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胰島素抗阻、糖尿病與肥胖相關基因之研究

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計畫主持人：莊立民

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(計畫名稱)

胰島素抗阻、糖尿病與肥胖相關基因之研究

Study on the genes related to insulin resistance, type 2 diabetes and obesity

計畫類別：☒ 個別型計畫 ☐ 整合型計畫

計畫編號：NSC 91－3112－B－002－019

執行期間：91 年 5 月 1 日至 92 年 4 月 30 日

計畫主持人：莊立民

共同主持人：

計畫參與人員：楊偉勛、唐存凱

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執行單位：台大醫學院、內科

中 華 民 國 92 年 5 月 30 日

中文摘要

糖尿病是一種常見的新陳代謝失調，估計至少佔世界人口的 5%。在台灣糖尿病的盛行率約為 4%，其中 95%以上之患者為第二型糖尿病；第二型糖尿病經常與肥胖、高血壓、血脂不良症及動脈硬化症同時存在，據世界衛生組織估計，下個十年 75%的醫療花費將用在非傳染性之慢性疾病上。這些異常含有第二型糖尿病、肥胖、高血壓、血脂不良症、冠狀動脈疾病，以上全部統稱為代謝症候群，今年我們計畫分析引發這些疾病的致病路徑主要缺陷，即胰島素阻抗性，經過修正著重目標在：

在此提案第一年的具體的研究：

尋找候補基因之 SNPs，並確定其與胰島素阻抗性及相關臨床第二型糖尿病、肥胖及冠狀動脈疾病之關係。

我們在臨床結合基因學之研究，顯示與 APM1 基因的 SNP 與胰島素阻抗性有關連，並與 PPAR 基因型產生交互作用，影響肥胖與胰島素阻抗性(APM1 與肥胖關係之研究，已被 JMM 接受刊登，而 APM1 與 PPAR 基因之交互作用，也已被 Diabetologia 接受刊登)。

更重要的是，我們研究 APM1 基因之對偶基因之表達，和 SORBS1 基因在脂肪組織的表達，與肥胖或胰島素阻抗性有密切之關連(前者在 JMM 中刊登，而後者則在 Ob Res 刊登)。

經由本年的研究發現有更多的有興趣的基因標記，並與臨床複雜的疾病有關，如代謝症候群。此經驗可提供未來大規模的基因型分析，得出更多的數據資料，以達到對糖尿病與代謝症候群更深入之瞭解。

關鍵詞：代謝症候群、mRNA 表現, 單核苷酸多態性, *APM1*, *SORBS1*, *PPAR*

英文摘要

Type 2 diabetes is one of the most common metabolic disorders affecting at least 5% of the whole population in the world. In Taiwan, the prevalence of diabetes mellitus was about 4% and more than 95% of the cases were type 2 diabetes mellitus. Since type 2 diabetes is frequently associated with obesity, hypertension, dyslipidemia and atherosclerosis, it has been projected by the WHO that more than 75% of the health cost will be consumed for the non-communicable disease (NCD) in the next decade.

The underlying cause of these abnormalities including type 2 diabetes, obesity, hypertension, dyslipidemia and coronary artery disease, collectively termed metabolic syndrome, is not well understood. However, it has been clearly shown that both environmental and genetic factors are important in the pathogenesis of metabolic syndrome.

In this year (of the originally proposed 3-year project), we plan to dissect pathogenic pathways leading to the major defect underlying the metabolic syndrome, i.e. insulin resistance. With the revision, we focus only on some the targets:

Specific research of this proposal in the first year granted:

Isolation of the SNPs of the candidate genes and confirm the association with insulin resistance and the related clinical disorders such as type 2 diabetes, obesity and coronary artery disease in Chinese population. We have confirmed by clinical association studies to show a positive correlation of SNP of the APM1 gene and its interaction with PPAR gene in subjects with obesity and insulin resistance (A study on APM1 locus with obesity and different allelic expressions in the adipose tissue has been accepted by JMM; A study on gene-to-gene interaction between APM1 and PPAR has been accepted by Diabetologia). More importantly, we showed the allele-specific expression of APM1 gene and expression of SORBS1 gene in the adipose tissue to correlate clinical obesity and insulin sensitivity (A study on tissue expression of SORBS1 gene correlated with clinical phenotype has been accepted by Ob Res; A study on APM1 locus with obesity and different allelic expressions in the adipose tissue has been accepted by JMM).

Currently, we have identified more interesting genetic markers that might be related to clinical complex disorder such as the metabolic syndrome. Future large-scale genotyping might yield more remarkable data via high throughput genome center.

keywords: metabolic syndrome, mRNA expression, SNP, APM1, SORBS1, PPAR

Progress Report

Background.

Type 2 diabetes is a multifactorial disorder involving both genetic and environmental factors (7). Detailed studies reveal two interacting basic defects are established in pathophysiology for human type 2 diabetes, i.e., β cell dysfunction (8,9), and insulin resistance (10-12). Understanding the mechanisms of insulin resistance and β cell defect is the key to elucidating the pathogenesis of type 2 diabetes and obesity. However, the genetic susceptibility factors for this disorder remains to be identified, due to a limited success in gene finding for the multifactorial diseases in general (14,15).

From our and others' observations, obesity is the major determinant for the development of type 2 diabetes (13). In addition to the environmental contribution to the development of obesity, obesity is now can also be considered as a genetic disease of adipose tissue (16). The molecular mechanisms of programming of adipocytes differentiation have been extensively studied and theses studies also provide a basic understanding of obesity in animals and humans (for review, see 17). It is therefore possible to isolate the genes involved in syndrome of obesity and diabetes from the understanding of adipocyte gene expression (18). Interestingly, the induction of adipocyte differentiation is associated with an increase in insulin sensitivity (19).

Aims of the study.

1. To isolate and characterize the genes related to insulin resistance, obesity and type 2 diabetes.
2. Study of the functions of the candidate genes relating to insulin resistance

Literature review.

Current studies using differential display and other techniques to isolate genes differentially expressed during adipogenesis have advance our understanding of the genetic factors involved in obesity/type 2 diabetes. Others and we have isolated several of the candidate genes for obesity and type 2 diabetes that were isolated during adipocytes differentiation. Others and we have isolated several of the candidate genes for obesity and type 2 diabetes that were isolated during adipocytes differentiation. For examples, *adipoQ* and *HMGIC* have been found in our differential display study (*Chuang, unpublished*). These two genes have been also found by others and found to be important in obesity and type 2 diabetes (20-22). We have been able to show the association between genetic variants of the adiponectin, human

homologue of adipoQ, and subjects with obesity and coronary artery disease (*Chuang, in submission*). In addition, we have focused on a signaling molecule, SORBS1/CAP, in insulin signaling pathway. We have cloned the human gene for SORBS1, identified 14 SNP on this gene, and demonstrated a positive association of a variant, T228A, with a reduced risk to obesity and type 2 diabetes in our laboratory (23,24). Thus, isolating a candidate with differential expression during a disease pathway or the changes in insulin sensitivity might be a good way for gene hunting.

Study design and methods.

1. Differential display, subtraction cloning, and microarray to isolate the candidate genes that involve insulin signaling/insulin action and adipocytes differentiation.

In the first year, we focused on 3 genes isolated from adipogenesis: i.e. ***PPAR*** , ***APM1***, ***SORBS1***.

2. SNP typing and association studies.

a. Nucleotide G/T polymorphism of *APM1* gene. Total genomic DNA was purified from peripheral blood leukocytes using a DNA extraction kit (Puregene, Minneapolis, MN). Exon 2 of human adiponectin gene was amplified using primers, 5'-TAG AAG TAG ACT CTG CTG AGA TG-3' and 5'-CTC CCT GTG TCT AGG CCT TAG-3', with the annealing temperatures at 68 to 60°C stepping down 2°C for every 5 cycles. The amplified DNA was digested with *Bsp*HI enzyme (New England BioLabs, Inc., Beverly, MA) prior to electrophoresis on a 2% agarose gel. The resulting fragment of PCR was 422 base pairs (bp). Because the T94G substitution abolished a *Bsp*HI restriction site, two fragments of 265 and 157 bp after digestion indicate the presence of T allele.

b. Pro12Ala polymorphism of the *PPAR* γ 2 gene. Genotyping was performed on genomic DNA. A 270 base-pair (bp) region encompassing the site of the Pro12Ala polymorphism in the *PPAR* γ 2 gene was detected by PCR-based restriction fragment length polymorphism (PCR/RFLP) analysis. PCR amplification was performed using upstream primer (5' GCC AAT TCA AGC CCA GTC 3') and mutagenic downstream primer (5' GAT ATG TTT GCA GAC AGT GTA TCA GTG AAG GAA TCG CTT TCC G 3'). The mutagenic primer introduces a *Bst* UI restriction site only when the C→G substitution at nucleotide 34 is present. PCR conditions were: denaturation at 94° C for 5 minutes, followed by 30 cycles of denaturation at 94° C for 30

seconds, annealing at 65° C for 45 seconds, and extension at 72° C for 1 minute with a final extension at 72° C for 10 minutes.

3. Tissue mRNA expression studies.

a. Quantitation of relative mRNA levels of G and T alleles of the APM1 gene.

The adipose tissues were extracted for RNA by Trizol reagent and 1 µg of the total RNA was reverse transcribed into cDNA following manufacturer's recommendation (Life technologies, Rockville, MD). PCR with primers: 5'-ATG CTG TTG CTG GGA GCT GT-3' and 5'-CCA TCC AAC CTG TGC AGG C-3') was performed with an initial denaturation at 95°C for 10 min., followed by denaturing at 95°C for 10 sec, annealing at 60°C for 1 min for a total of 31-37 cycles; then by a final extension at 72°C for 10 min. The PCR products were digested with *SmaI* followed by electrophoresis on 2% agarose gels stained with ethidium bromide. Because the T→G change creates a *SmaI* site, the uncut 134-bp band was the T allele, whereas the presence of 79- and 55-bp bands was indicative of the G allele. The band intensity was determined by densitometric analyses (1-D Gel Analysis Software, One-Dscan, Scanalytics Inc., Fairfax, VA). Because the T/G alleles were amplified in the same reaction, and the relative levels between them were used in the analysis, a control gene such as β-actin or GAPDH in the ordinary quantitative PCR was not required.

b. Quantitation of mRNA of SOBBS1 by real-time PCR. Two µl of diluted cDNA was added to a 12.5 µl 2x SYBR Green PCR Master Mix (Perkin-Elmer Applied Biosystems, Foster City, CA, USA), and variable amounts of the respective primers for the human SORBS1 and β-actin, and water to a final volume of 25µl. The primers were: *SORBS1-F493*: 5'-TAG CCT TTC AGA TCC TGT CTC AGA-3', *SORBS1-R602*: 5'-CGC CTA CTG TCA TCC TTT GCT-3', *β-actin-F*: 5'-CCT CAT GAA GAT CCT CAC CGA GC-3' and *β-actin-R*: 5'-GCC AAT GGT GAT GAC CTG GC-3'. The PCR conditions were: 10 minutes at 95°C and 40 cycles of 15 seconds at 95°C plus 1 minute at 60°C. Briefly, the fluorescent signal from each PCR reaction is collected as the peak-normalized values plotted versus the cycle numbers. Reactions are characterized by comparing the threshold cycle (Ct) values. The Ct is a unit-less value defined as the fractional cycle number at which the normalized sample fluorescence signal passes a fixed threshold above baseline, when it is always located within the linear phase of amplification. Samples with a high starting copy number of cDNA show an increase in fluorescence earlier in the PCR process, therefore resulting in a low Ct number. The comparative Ct method, which eliminates the need for

standard curves, was used. The Ct number of β -actin minus that of SORBS1 (Δ Ct) represents as a $\log_2$ transform of the SORBS1 mRNA expression corrected with β -actin mRNA expression.

4. Cloning of human adiponectin gene and transfection of 3T3-L1.

A PCR reaction using human adipose tissue cDNA as template and primers (5'-ATG CTG TTG CTG GGA GCT GT-3' and 5'-TCA GTT GGT GTC ATG GTA GAG-3') was performed to generate the coding sequences of human adiponectin gene. The PCR product was cloned into pGEM-Teasy (Promega, Madison, WI), subsequently subcloned into the EcoRI site of pCDNA3.1+ (Invitrogen BV, Gronigen, The Netherlands) in either sense or anti-sense orientation driven by a CMV promoter. Two μ g of each plasmid was transfected into 3T3-L1 cells by lipofectamine method (Life Technologies, Rockville, MD). Transfected cells were selected 2 days after transfection in a medium containing 500 μ g/mL G418 for two weeks, thereafter maintained in 200 μ g/mL G418 as pools. Each pool had at least 200 colonies from each stable transfection. Because the differentiated 3T3-L1 adipocytes are quite heterogeneous in phenotypes, the experimental results from a few selected clones could be misleading. Therefore we decided to use the pools from a large number of colonies to avoid the bias from selection.

5. Glucose transport assays.

3T3-L1 cells stably transfected with the plasmids containing vector-alone, sense, or anti-sense of adiponectin gene were induced to differentiation. Glucose transport assayed by the uptake of 2-deoxy-D-[3 H]-glucose, was performed in triplicate as described in the differentiated adipocytes after 24-hour incubation in serum-free medium. The count of uptake in the presence of 10 μ M cytochalasin-B was used as the background, therefore subtracted from all the raw values for subsequent analysis. A final insulin concentration of 100 nM was used to stimulate glucose uptake. The data on basal and insulin-stimulated glucose uptake for the cells with sense or anti-sense adiponectin gene were adjusted using the basal glucose uptake of the cells harboring vector-alone as 100%.

Results and Discussion.

- a. **T/G polymorphism of the APM1 gene is correlated with BMI.** We found that BMI were inversely correlated with the dose of G allele ($p=0.023$, $\gamma=-0.15$). Multivariate linear regression analyses showed that the adiponectin genotypes were significantly related to BMI ($p=0.022$, $\beta=-0.05\pm0.02$), while adjusting for age and gender. The dose of the G allele was associated with a reduction of approximately 1.12 kg/m² in BMI. (See appendix 1).

- b. Interaction of the T/G polymorphism of the APM1 gene with PPAR Pro12Ala polymorphism is correlated with insulin sensitivity.** We found that the T allele of adiponectin gene was associated with a higher insulin level at 1-hour (Ins60) in oral glucose tolerance tests (OGTT) utilizing sib pairs discordant for the adiponectin genotypes; and with a higher Ins60 and higher area under curve of insulin (AUC_i) in OGTT utilizing all subjects in a mixed model that corrected for family effects. Significant interactions between adiponectin and PPAR γ 2 genotypes were found in fasting insulin levels (Ins0), insulin levels at 2-hour (Ins120) in OGTT and insulin resistance index (HOMA_{IR}). The subjects carrying the adiponectin G allele and the PPAR γ 2 Ala12 allele appeared to be more insulin sensitive. Our results showed that adiponectin is a genetic factor associated with insulin sensitivity. Interactions with PPAR γ 2 genotypes significantly modified this association. (See appendix 2).
- c. Differential expression of the T- or G- alleles of the APM1 Gene might explain the biological consequence of the silent SNP.** The T94G polymorphism in exon 2 of the human adiponectin gene is a synonymous mutation (GGT→GGG, Gly→Gly), which has no biological effects on its own. It is plausible that the polymorphism is in linkage disequilibrium with some other functional genetic alterations. Omental adipose tissue from 21 subjects was assayed by semi-quantitative PCR (Fig. 1A). The mean level of G allele was significantly higher than that of T allele ($p < 10^{-10}$, Fig. 1B). We concluded that the biological effects of the T94G polymorphism might be at least in part attributable to the difference in allele-specific expression levels. (See appendix 1).
- d. Adipose tissue mRNA expression of the SORBS1 correlates with clinical obesity.** The relative SORBS1 mRNA levels from the omental depots correlated with BMI ($\gamma = -0.41$, $p = 0.0008$) and total cholesterol ($\gamma = -0.30$, $p = 0.017$). With adjustment for age, only the correlation with BMI remained significant ($\gamma = -0.30$, $p = 0.017$). Using multivariate linear regression analysis, the relative SORBS1 mRNA levels from the omental depots were only related to BMI significantly ($\beta = -0.20 \pm 0.09$, $p = 0.031$), but not to the other variables. From the linear regression model, every 5 kg/m² change in BMI would be associated with approximately 2-fold difference ($= 2^{5 \times -0.2}$) in the omental SORBS1 mRNA levels. (See appendix 3).

In this year's approach, we demonstrated the possibility of finding genes, from the differentially expressed genes during a pathway of adipogenesis, which are involved in pathogenesis of clinical disorders such as obesity, type 2 diabetes and insulin resistance. This would provide us a better understanding of pathogenesis of insulin

sensitivity and the clinical related diseases.

Papers published:

1. W-S Yang, P-L Tsou, W-J Lee, D-L Tseng, C-L Chen, C-C Peng, K-C Lee, M-J Chen, C-J Huang, T-Y Tai, LM Chuang (2003) Allele-specific Differential Expression of a Common Adiponectin Gene Polymorphism Related to Obesity. JMM (in press)
2. WS Yang, CA Hsiung, LT Ho, YT Chen, CT He, JD Curb, J Grove, T Quertermous, YDI Chen, SS Kuo, LM Chuang, for the SAPPHIRE Study Group (2003) Genetic Epistasis of Adiponectin and PPAR γ 2 Genotypes in Modulation of Insulin Sensitivity: a Family-based Association Study. Diabetologia (in press).
3. W-S Yang, W-J Lee, K-C Huang, K-C Lee, C-L Chao, C-L Chen, T-Y Tai, LM Chuang (2003) mRNA Levels of the Insulin-Signaling Molecule SORBS1 in the Adipose Depots of Nondiabetic Women. Ob Res 11:586-90.

Self evaluation. Based on the first year's experience, we demonstrated the feasibility of finding genes from the differentially expressed genes during a pathway, which is involved in pathogenesis of clinical disorders for a complex disorder. This would provide us a ground base for a plan in the next two years for a larger set of the genes that are isolated during adipogenesis for better understanding of the pathogenetic mechanism of insulin resistance and the related clinical disorders. In addition, we have 3 papers accepted for publications in the prestigious journals.

附件 1

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Original Article

Allele-specific differential expression of a common adiponectin gene polymorphism related to obesity

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Abstract Adiponectin gene polymorphisms have recently been reported to be associated with obesity, insulin sensitivity, and the risk of type 2 diabetes. We examined a T94G polymorphism of the adiponectin gene in 245 ostensibly normal nondiabetic subjects. The G allele frequency was lower among subjects with higher BMI (≥ 27) than in those with lower BMI. BMI was inversely correlated with the dose of G allele. Multivariate linear regression analyses showed that the adiponectin genotypes were significantly related to BMI after adjusting for age and gender. The dose of the G allele was associated with a reduction of approximately 1.12 kg/m² in BMI. We further found that the relative mRNA levels of G allele were consistently

higher than those of T allele in the omental adipose tissue from 21 heterozygous subjects. Finally, we observed that the expression levels of adiponectin affected insulin-stimulated glucose uptake in differentiated 3T3-L1 adipocytes. In conclusion, the allele-specific differential expression of this common polymorphism could be responsible for its biological effects observed in this and the other studies.

Keywords Obesity - Insulin resistance - Adiponectin - Polymorphism - mRNA

Abbreviations

BMI Body mass index

SNP Single nucleotide polymorphism

Introduction

Adipose tissue secretes many adipocytokines to modulate a wide array of biological functions [1]. Along with the well-known examples, such as leptin and resistin [2, 3], a relatively novel adipocytokine, adiponectin has been identified in mouse (adipoQ or ACRP30) [4, 5] as well as in human (apM1 or GBP-28) [6, 7]. Recombinant adiponectin proteins were recently shown to decrease tumor necrosis factor α induced monocyte adhesion, nuclear transcription factor κ B signaling, and the expression of adhesion molecules in endothelial cells in vitro [8, 9, 10]. They also attenuated cholesterol ester accumulation and the expression of class A scavenger receptor in human monocyte-derived macrophages [11]. Lower plasma adiponectin levels are reported in patients with coronary heart disease [8]. Furthermore, immunoreactive adiponectin is detectable in the walls of catheter-injured vessels but not in the intact vascular walls [11, 12]. Taken together these reports indicate that adiponectin could be a protective adipocytokine against atherosclerosis.

A longitudinal observation of rhesus monkeys found a progressive decline in adiponectin paralleled the decrease in insulin sensitivity during the development of diabetes [13]. In mice injection of the recombinant adiponectin decreased plasma glucose and fatty acid levels and improved insulin sensitivity [14, 15, 16]. Long-term injection of adiponectin in mice led to significant weight reduction [14]. Hypoadiponectinemia is also documented in subjects with obesity or diabetes [17, 18].

An inverse correlation between plasma adiponectin levels and body mass index (BMI), fasting insulin, and glucose and triglyceride levels but a positive relationship with high-density lipoprotein cholesterol and measurement of insulin sensitivity in clamp studies has been reported [[17](#), [18](#), [19](#)]. These findings suggest a role of adiponectin in the pathophysiology of insulin resistance syndrome.

Genetic variations in the human adiponectin gene have recently been reported to be associated with the risk of obesity, insulin resistance, type 2 diabetes, and high levels of low-density lipoprotein cholesterol [[20](#), [21](#), [22](#), [23](#), [24](#), [25](#)]. In contrast, others have found no association with obesity or type 2 diabetes [[25](#), [26](#), [27](#)]. Almost all of the studies used a T/G polymorphism in the exon 2 of the human adiponectin gene as the sole genetic marker. One study performed extensive SNP genotyping, ending up with 14 single nucleotide polymorphisms (SNPs) across the whole gene [[20](#)]. However, only the T/G polymorphism in exon 2 along with an SNP276 was strongly associated with the risk of type 2 diabetes [[21](#)]. Since the T/G polymorphism at exon 2 is a synonymous mutation (GGT→GGG, Gly15Gly) and SNP276 is in intron 2, the exact molecular mechanisms responsible for the biological effects of these variations are not known at present. On the other hand, it was recently reported that SNPs in the adiponectin promoter region are associated with the risk of type 2 diabetes in the French population [[24](#)].

In this study we surveyed the frequency of the T94G polymorphism in the exon 2 among 245 ostensibly normal nondiabetic subjects with a wide range of BMI. We also estimated the relative mRNA levels of T/G alleles in the adipose tissue of heterozygous subjects. Moreover, we measured insulin-stimulated glucose uptake in 3T3-L1 adipocytes stably transfected with either sense or antisense gene of adiponectin. Our data suggest that adiponectin may be a genetic contributor to body weight and the risk of obesity. The allele-specific differential expression of adiponectin is at least one of the causes responsible for the biological effects associated with this polymorphism.

Materials and methods

Subjects

We recruited 245 ostensibly normal nondiabetic subjects (WHO criteria) aged 30 years or over and with a BMI less than 45 (Table [1](#)) during a routine health examination at National Taiwan University Hospital (NTUH). Adipose tissue

specimens were obtained from 10 severely obese heterozygous subjects (seven women, three men; aged 32.2 ± 9.6 years; BMI 41.76 ± 4.00) during gastric partition surgery [28] and from 11 lean heterozygous women (age 48.4 ± 4.8 years; BMI 21.56 ± 2.22) during uterine myomectomy. BMI of at least 27.0 was defined as obesity according to the guideline for obesity management issued by the Ministry of Health of Taiwan. Informed consent was obtained. The study was approved by the Medical Ethics Committee of National Taiwan University Hospital.

Table 1. Characteristics of 245 ostensibly normal nondiabetic subjects in this study

Age (years)	47.7 ± 10.9
Body mass index	26.87 ± 6.59
Gender: F/M	124/121
Hypertension	76 (31%)
Hyperlipidemia	47 (19%)
Diabetes mellitus	0 (0%)

Laboratory and phenotypic characterization of the subjects

A 75 g oral glucose tolerance test was given to all subjects in health examination. Determination of the concentrations of plasma glucose, total cholesterol, triglyceride, and serum insulin levels and the estimation of insulin resistance index and beta cell function by homeostasis model assessment (HOMA) were carried out as previously reported [28].

Extraction of genomic DNA and genotyping

Total genomic DNA was purified from peripheral blood leukocytes using a DNA extraction kit (Puregene, Minneapolis, Minn., USA). Exon 2 of the human adiponectin gene was amplified using primers, 5'-TAGAAGTAGACTCTGCTGAGATG-3' and 5'-CTCCCTGTGTCTAGGCCTTAG-3', with the annealing temperatures at 68–60°C stepping down 2°C for every five cycles. The amplified DNA was digested with *Bsp*HI enzyme (New England BioLabs, Beverly, Mass., USA) prior to electrophoresis on a 2% agarose gel. The resulting fragment of PCR was 422 bp. Because the T94G substitution abolished a *Bsp*HI restriction site, two fragments of 265 and 157 bp after

digestion indicate the presence of T allele.

RNA extraction and quantitation of relative mRNA

levels of G and T alleles

The adipose tissues were extracted for RNA by Trizol reagent and 1 g of the total RNA was reverse transcribed into cDNA following manufacturer's recommendation (Life Technologies, Rockville, Md., USA). PCR (primers: 5'-ATGCTGTTGCTGGGAGCTGT-3' and 5'-CCATCCAACCTGTGCAGGC-3') was performed with an initial denaturation at 95°C for 10 min, followed by denaturing at 95°C for 10 s, annealing at 60°C for 1 min for a total of 31–37 cycles, then by a final extension at 72°C for 10 min. The amplification of 33 cycles was chosen because the amount of PCR product was located within the linear range of amplification (data not shown). The PCR products were digested with *Sma*I followed by electrophoresis on 2% agarose gels stained with ethidium bromide. Because the T→G change creates a *Sma*I site, the uncut 134-bp band was the T allele, whereas the presence of 79- and 55-bp bands was indicative of the G allele. The band intensity was determined by densitometric analyses (1-D Gel Analysis Software, One-Dscan, Scanalytics, Fairfax, Va., USA). Because the T/G alleles were amplified in the same reaction, and the relative levels between them were used in the analysis, a control gene such as β -actin or GAPDH in the ordinary quantitative PCR was not required.

Cloning of human adiponectin gene and transfection

of 3T3-L1

A PCR reaction using human adipose tissue cDNA as template and primers (5'-ATG CTG TTG CTG GGA GCT GT-3' and 5'-TCA GTT GGT GTC ATG GTA GAG-3') was performed to generate the coding sequences of human adiponectin gene. The PCR product was cloned into pGEM-Teasy (Promega, Madison, Wis., USA), subsequently subcloned into the *Eco*RI site of pCDNA3.1+ (Invitrogen, Groningen, The Netherlands) in either sense or antisense orientation driven by a cytomegalovirus promoter. Of each plasmid 2 μ g was transfected into 3T3-L1 cells by lipofectamine method (Life Technologies). Transfected cells were selected 2 days after transfection in a medium containing 500 μ g/ml G418 for 2 weeks, thereafter maintained in 200 μ g/ml G418 as pools. Each pool had at least 200 colonies from each stable

transfection. Because the differentiated 3T3-L1 adipocytes are quite heterogeneous in phenotypes, the experimental results from a few selected clones could be misleading. Therefore we decided to use the pools from a large number of colonies to avoid the bias from selection.

Generation of antibody against adiponectin and western blot analysis

A polyclonal antibody against peptide residues 15–29 (GHDQETTTQGPGVLL) of adiponectin was produced in rabbits. Crude cellular extracts of stably transfected cells were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by western blot analyses with the antiadiponectin antibody. Mouse β -actin monoclonal antibody (Chemicon International, Calif., USA) was used as a standard control antibody. The protein of interest was detected by enhanced chemiluminescence (Amersham Pharmacia, Piscataway, N.J., USA).

Glucose transport assays

3T3-L1 cells stably transfected with the plasmids containing vector-alone, sense, or antisense of adiponectin gene were induced to differentiation as previously described [29]. Glucose transport assayed by the uptake of 2-deoxy-D- ^3H glucose, was performed in triplicate as described in the differentiated adipocytes after 24-h incubation in serum-free medium [29]. The count of uptake in the presence of 10 μM cytochalasin-B was used as the background, therefore subtracted from all the raw values for subsequent analysis. A final insulin concentration of 100 nM was used to stimulate glucose uptake. The data on basal and insulin-stimulated glucose uptake for the cells with sense or antisense adiponectin gene were adjusted using the basal glucose uptake of the cells harboring vector-alone as 100%.

Statistical analyses

Data are presented as mean $\pm$ SD. Statistical analyses by analysis of variance, χ^2 tests, Mann-Whitney rank sum test, and univariate and multivariate linear regression analyses were performed by using the PC version of the Statistical Analysis System (SAS, 6.12 edition, SAS Institute, Cary, N.C., USA) where appropriate. Logistic regression analyses were performed with Stata (College Station, Tex., USA). The

original data were log transformed before used for statistical analyses. A *P* value less than 0.05 was considered significant statistically.

Results

We sequenced the protein-coding regions in exons 2 and 3 of the human adiponectin gene using the genomic DNA from 20 unrelated randomly selected Taiwanese subjects. Only one T/G polymorphism at nucleotide 94 in exon 2 was identified. Although it does not alter the amino acid (Gly15Gly), it seemed to be quite common in our population and therefore could be useful as a marker for genetic association studies. We genotyped 245 ostensibly normal nondiabetic subjects. The frequencies of T and G alleles were 0.637 and 0.363, respectively. Comparison of selected characteristics of these subjects categorized by genotypes revealed that only the BMI differed between them, with a borderline significance (Table 2). Further, we found that only BMI was inversely correlated with the dose of G allele ($P=0.023$, $\gamma=-0.15$). Multivariate linear regression analyses showed that the adiponectin genotypes were significantly related to BMI ($P=0.022$, $\beta=-0.05\pm0.02$), while adjusting for age and gender. The dose of the G allele was associated with a reduction of approximately 1.12 in BMI.

Table 2. Means $\pm$ SD of selected characteristics among 245 normal subjects by adiponectin genotypes (*HOMA* homeostasis model assessment)

	T/T		T/G		G/G		<i>P</i> ^b
	<i>n</i>	Mean $\pm$ SD ^a	<i>n</i>	Mean $\pm$ SD	<i>n</i>	Mean $\pm$ SD	
Age (years)	91	48.35 $\pm$ 10.60	130	46.65 $\pm$ 10.69	24	51.13 $\pm$ 12.78	NS*
Triglyceride (mmol/l)	91	1.44 $\pm$ 0.86	130	1.42 $\pm$ 0.10	24	1.32 $\pm$ 0.08	NS
Cholesterol (mmol/l)	91	4.66 $\pm$ 0.85	130	4.66 $\pm$ 1.03	24	4.40 $\pm$ 1.04	NS
Body mass index	91	27.94 $\pm$ 7.45	130	26.53 $\pm$ 6.04	24	24.59 $\pm$ 5.42	0.067
Fasting plasma glucose (mmol/l)	91	5.27 $\pm$ 0.57	129	5.18 $\pm$ 0.65	24	5.10 $\pm$ 0.64	NS
Fasting plasma insulin (pmol/l)	91	69.17 $\pm$ 65.44	126	58.26 $\pm$ 52.16	24	48.28 $\pm$ 26.12	NS
Insulin resistance (HOMA)	91	2.34 $\pm$ 2.37	126	1.94 $\pm$ 1.75	24	1.51 $\pm$ 0.92	NS

^aThe original mean $\pm$ SD is presented without transformation

^bAnalysis of variance after log transformation

* $P>0.1$

To further illustrate the effect of the adiponectin genotypes on BMI, BMI values of 27 or higher (based on the guideline issued by the Ministry of Health of Taiwan) was used to categorize subjects (Table 3). Significantly lower frequency of G allele was found in the higher BMI group ($P=0.043$). The odds ratio of higher BMI for the G allele relative to the T allele was 0.65. In multivariate logistic regression analyses the odds ratio of higher BMI for the subjects carrying G/T genotype relative to the subjects carrying T/T genotype was 0.54, adjusting for age and gender ($P=0.028$ for linear trend, Table 3). Using 30 as the cutoff point instead of 27 produced similar results. The G allele frequency in subjects with BMI of 30 or higher was less than that with BMI less than 30 (28% vs. 39%; $P=0.044$, χ^2). The odds ratio for obesity for G/T genotype relative to T/T genotype was 0.48 (95% CI 0.24–0.95, P for linear trend=0.019) with adjustment for age and gender in logistic regression. Therefore the G allele of the adiponectin gene may be associated with lower BMI and the reduced risk to obesity, defined by BMI value of either 27 or more or one of 30 or more.

Table 3. Adiponectin genotype and allele frequencies according to BMI of the total among 245 normal subjects and odds ratios (OR) of obesity by adiponectin genotypes and alleles

	BMI $\geq$ 27.0		BMI < 27.0			
Genotype	<i>n</i>	%	<i>n</i>	%	OR	95% CI
G/G	5	6.3	19	11.5	0.42	0.13–1.28
G/T	38	47.5	92	55.8	0.54	0.30–0.97 ^a
T/T	37	46.3	54	32.7	1	—
Allele						
G	48	30.0	130	39.4	0.65*	0.42–0.99*
T	112	70.0	200	60.6	1	—

^aOdds ratios after adjusting for age and gender and treating BMI $\geq$ 27.0 as 1 and BMI < 27 as 0.

P for trend=0.028 for genotypes using T/T as reference in multiple logistic regression model

* χ^2 , $P=0.043$

The T94G polymorphism in exon 2 of the human adiponectin gene is a synonymous mutation (GGT→GGG, Gly→Gly), which has no biological effects on its own. It is plausible that the polymorphism is in linkage disequilibrium with some other functional genetic alterations, resulting in the difference in mRNA expression levels of the T/G alleles. Therefore we assayed the relative T/G allele-specific expression within the same heterozygous individuals. The relative mRNA levels of T/G alleles should faithfully reflect the effects caused by the genomic changes linked with the T94G polymorphism, since both alleles were in exactly the same "environmental"

conditions. Omental adipose tissue from 21 subjects was assayed by semiquantitative PCR (Fig. 1A). The mean level of G allele was significantly higher than that of T allele ($P < 10^{-10}$, Fig. 1B). We conclude that the biological effects of the T94G polymorphism might be at least in part attributable to the difference in allele-specific expression levels.

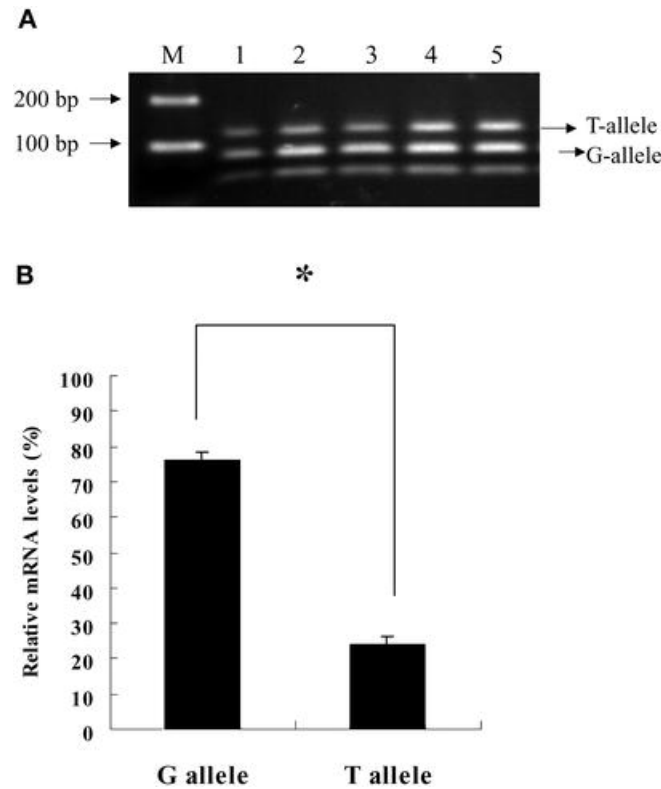


Fig. 1. The allele-specific mRNA expression of adiponectin gene in the omental adipose tissue from 21 subjects with heterozygous T/G genotype assayed by PCR. **A** A representative agarose gel showing the PCR products from five subjects (*lanes 1–5*) at the 33rd cycle digested with *Sma*I; *M* 100-bp marker; *T allele* 134 bp; *G allele* 79 and 55 bp; The higher intensity of the G allele bands relative to that of the T allele bands indicates higher expression levels. **B** The relative mRNA levels of adiponectin G and T alleles in the omental adipose tissue from 21 heterozygous subjects estimated from the assays shown in **A**. In each individual the total intensity of G/T alleles calculated from densitometric analyses was set to 100% to adjust the relative amount of G/T alleles. *Bars* means $\pm$ SEM of the data; $*P < 1 \times 10^{-10}$

We then examined whether the difference in adiponectin gene expression has a direct biological effect on insulin sensitivity in adipocytes themselves. The plasmids containing sense, antisense, or no adiponectin gene were stably introduced into 3T3-L1 cells. Pools of more than 200 colonies of the stable transfectants were differentiated into adipocytes and assayed for glucose uptake. The basal glucose

uptake of the sense-expressing pools was increased by 40%, whereas the basal glucose uptake from the pools expressing antisense was exactly the same as that from the pools with vector-alone. However, they did not differ statistically (Fig. 2A). Insulin-stimulated glucose uptake in the pools with vector alone by an average of sixfold. The insulin-stimulated glucose uptake in the pools expressing sense gene was 135% of that from the insulin-stimulated vector alone. In contrast, the insulin-stimulated glucose uptake in the pools expressing antisense gene was decreased to 75% of that from the insulin-stimulated vector-alone. The difference in mean insulin-stimulated glucose uptake between the sense- and antisense-expressing pools was significant ($P=0.035$, Fig. 2A). The difference in adiponectin gene expression in the differentiated 3T3-L1 adipocytes (Fig. 2B) might be the cause of the difference in insulin-stimulated glucose uptake.

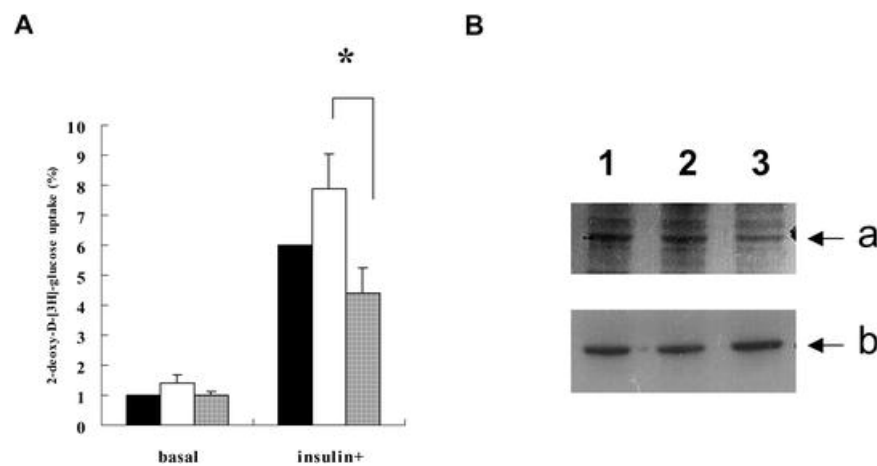


Fig. 2. A The basal and insulin-stimulated glucose uptake and adiponectin protein levels in the differentiated 3T3-L1 adipocyte pools harboring sense, antisense adiponectin gene, or vector alone, The basal glucose uptake of the cells transfected with vector alone (*black bar* in basal group) was set to 1 to adjust the values from the others. The insulin stimulated glucose uptake for the vector-alone (*black bar* in insulin+) was enhanced by approximately sixfold. *White bars* results using sense adiponectin-expression clones; *shaded bars* results using antisense adiponectin-expression clones (mean and SEM. of seven separate experiments); * $P=0.035$. B Western blot gel showing the relative amount of adiponectin (*a*) and β -actin (*b*) proteins in the differentiated 3T3-L1 cells harboring the plasmids with vector alone (*lane 1*), sense (*lane 2*), or antisense adiponectin gene (*lane 3*). The adiponectin protein level of the antisense pool was lower adjusted for the level of the control gene β -actin

Discussion

This study provides the genetic evidence that adiponectin influences BMI, which is consistent with the findings in several very recent genetic reports [[20](#), [21](#), [22](#), [23](#)]. Most importantly, we found the differential expression of the alleles associated with the polymorphism. We also demonstrate that the difference in expression levels of adiponectin can lead to different glucose uptake in differentiated 3T3-L1 adipocytes.

Since the T94G polymorphism is a synonymous mutation without a biological effect on its own, all these genetic association studies in a sense led to the same conclusions that the adiponectin gene is a genetic contributor to insulin sensitivity, BMI, and risk of type 2 diabetes [[20](#), [21](#), [22](#), [23](#), [24](#)]. Exactly what genetic alterations are in linkage disequilibrium with this polymorphism is not clear at present. A difference in the allele-specific expression of an SNP of the gene has been suggested recently to correlate functionally with disease phenotype [[30](#)]. Our study showed for the first time that the steady-state mRNA levels transcribed by the G allele are higher than those by the T allele in the adipose tissue of heterozygous subjects, suggesting that the promoter, the introns, and/or the other potential regulatory elements such as upstream enhancers or locus control regions of the adiponectin gene should be carefully examined. Recently the SNPs in the adiponectin promoter region were shown to be associated with type 2 diabetes [[24](#)]. Whether these SNPs are functional and are responsible for the allele-specific expression observed in our study has not been explored. Alternatively, this synonymous mutation may affect steady-state mRNA levels by altering RNA splicing or stability. The T/G or linked polymorphism nearby may affect mRNA splicing or stability [[31](#)]. The allele-specific differential expression of adiponectin in the adipose tissue is also consistent with the observations that higher plasma adiponectin was associated with reduced risk of obesity, diabetes, insulin resistance and coronary heart disease in humans [[8](#), [17](#), [18](#)]. Our data provide an explanation for the indirect genetic association studies at the molecular levels. However, the exact genetic mechanisms responsible for the allele-specific expression await further exploration.

Our transfection experiments further demonstrated that the expression level of adiponectin affects insulin-stimulated glucose uptake in adipocytes. Previous studies have shown the insulin-sensitizing effects of adiponectin in liver and skeletal muscle [[15](#), [16](#)]. This is the first to show that adiponectin also affects insulin sensitivity on adipocytes themselves. Therefore adiponectin may act in the adipose tissue via an autocrine or paracrine manner. However, our experiments were too preliminary to address the exact mechanism of actions. In conclusion, our study suggests that the human adiponectin gene is an important genetic factor linking insulin resistance syndrome and related disorders such as obesity. The allele-specific differential

expression of adiponectin at least partially explains the biological effects observed in many genetic association studies.

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附件 2

Genetic Epistasis of Adiponectin and PPAR γ 2 Genotypes in Modulation of Insulin Sensitivity: a Family-based Association Study

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Key words: *adiponectin, PPAR γ , polymorphisms, gene interaction, insulin sensitivity*

Abstract.

Aims/hypothesis. Genetic interactions in modulating the phenotypes of a complex trait, such as insulin sensitivity, were usually taken for granted. However, this has not been commonly demonstrated. Previous studies have suggested that both PPAR γ 2 and adiponectin genes may influence insulin sensitivity. Therefore it is likely that they could modulate insulin sensitivity through gene-gene interactions.

Methods. We genotyped 1793 subjects of Chinese and Japanese descendents from 601 hypertensive families recruited in SAPPHIRE study for a T94G in the adiponectin gene exon 2 and the PPAR γ 2 Pro12Ala polymorphisms. **Serum insulin levels and insulin resistance index (HOMA_{IR}) were used as the markers of insulin sensitivity.**

Results. We found that the T allele of adiponectin gene was associated with a higher Ins60 and higher area under curve of insulin (AUCi) in oral glucose tolerance tests (OGTT) utilizing all subjects in a mixed model that corrected for family effects. Significant interactions between adiponectin and PPAR γ 2 genotypes were found in fasting insulin levels (Ins0), insulin levels at 2-hour (Ins120) in OGTT and insulin resistance index (HOMA_{IR}). The main effects of the PPAR γ 2 genotypes were in the plasma glucose levels in OGTT. In contrast, the main effects of adiponectin genotypes were in every insulin variables, including Ins0, Ins60, Ins120, AUCi and HOMA_{IR}. The subjects carrying the adiponectin G allele and the PPAR γ 2 Ala12 allele appeared to be more insulin sensitive.

Conclusion/interpretation. These results showed that adiponectin is a genetic factor regulating-associated with insulin sensitivity. Interactions with PPAR γ 2 genotypes significantly modulate defined this association ~~its contributions~~.

INTRODUCTION

Type 2 diabetes mellitus (T2DM) has daunted many countries in the developed or developing world [1]. Insensitivity to insulin stimulation is generally accepted to be the fundamental pathophysiology of T2DM [2]. As a classical example of poly-genic (also called multi-factorial or complex) diseases, the pathogenesis of T2DM is attributed to the collective actions of many genetic and environmental factors and the complex interactions among them [3]. The contribution of gene-gene interactions in shaping the phenotypes of T2DM, such as insulin sensitivity, is usually taken for granted. However, it is only occasionally demonstrated [4, 5].

Among many potential thrifty genes, the Pro12Ala variants of the human PPAR γ 2 gene have been quietly extensively surveyed in many genetic association studies [by other investigators](#) [6-8]. Previously we have also reported the association of PPAR γ 2 Ala12 variant with better glucose tolerance and insulin sensitivity among subjects from Japanese and Chinese hypertension families in Stanford Asia Pacific Program in Hypertension and Insulin Resistance (SAPPHIRE) study [9]. Because of its family-based study design utilizing the comparisons between siblings, the genetic association study in SAPPHIRE is expected to have better control in early childhood environmental factors and genetic background than a population-based genetic association study [9-11].

The other sensible genetic candidates for T2DM are adipose-derived secreted molecules regulated by PPAR γ 2, such as leptin, resistin and adiponectin. Among them, the recombinant adiponectin protein has been recently demonstrated in mice to be capable of lowering plasma glucose and fatty acids, improving insulin sensitivity and reducing body weight [12-14]. It has also been reported that adiponectin genotypes in humans influenced insulin sensitivity and the risk for T2DM [15-19]. Recent studies using knockout mice in general confirmed these biological functions of adiponectin [20, 21].

We have recently demonstrated that treating T2DM patients with rosiglitazone, a PPAR γ 2 agonist raised their plasma adiponectin levels by more than 2-fold [22]. The other investigators have also shown that the administration of glitazones increased the expression of adiponectin in humans as well in animals and in cultured adipocytes [23-26]. These results, all together, suggest a possibility of genetic interactions between PPAR γ 2 and adiponectin, and its attribution to insulin sensitivity.

In this study, we investigated the association of adiponectin T94G polymorphism with metabolic phenotypes and the effects of its interaction with PPAR γ 2 Pro12Ala polymorphism on these phenotypes among the large cohort from the SAPPHIRE [9, 10]. We found that the T allele of adiponectin was associated with post-glucose load

hyperinsulinemia. The main effects of the adiponectin and PPAR γ 2 genotypes and the genetic interactions between them on the metabolic variables were revealed in further analyses. The subjects having both the G allele of adiponectin and the Ala12 allele of PPAR γ 2 appeared to be more insulin sensitive.

RESEARCH DESIGNS AND METHODS

Subjects and Phenotypic Characterization

The characteristics of study population, the inclusion and exclusion criteria of SAPPHiRe study were detailed in several previous publications [9, 10]. Notably, diabetic subjects diagnosed based on the WHO criteria were excluded [27]. This study incorporated both the concordant (both hypertension) and the discordant sib-pairs (one hypertension, the other hypotension) in design. A total of 2525 subjects of Japanese or Chinese descendents were recruited from 6 centers at San Francisco, Hawaii and Taiwan. In this report, 1793 subjects including parents and sibs from 601 families were genotyped. Written informed consent was obtained from all participants. The study was approved by the ethics board of each participating institute. Characterization of the subjects by fasting plasma glucose, insulin, triglyceride, total cholesterol, lipoprotein profiles, OGTT and anthropometric measurements, including height, weight, waist and hip circumferences were previously described in details previously [9, 10]. A 75-g oral glucose tolerance test (OGTT) was performed and plasma glucose and insulin levels at 1-hour and 2-hour post-glucose load were measured [9, 10]. The fasting plasma sample was obtained right before the OGTT was conducted.

Extraction of genomic DNA and genotyping

Total genomic DNA was purified from peripheral blood leukocytes using DNA extraction kit of Puregene (Minneapolis, MN, USA), following the manufacturer's protocol. The primers used for PCR amplification for the exon 2 of human adiponectin gene were: 5'-TAG AAG TAG ACT CTG CTG AGA TG-3' and 5'-CTC CCT GTG TCT AGG CCT TAG-3'. The PCR reactions were performed in a total volume of 15 μ l containing 20 ng of genomic DNA with an initial denaturation at 94°C for 5 min, followed by denaturing at 94°C for 30 sec, annealing at 68 to 60°C for 1 min with the annealing temperature stepping down 2°C for every 5 cycles, and polymerization at 72°C for 40 sec; then by a final extension at 72°C for 10 min. Four μ l of the amplified DNA was digested with *Bsp*HI enzyme (New England BioLabs, Inc., Beverly, MA, USA), and then electrophoresed on a 2% agarose gel. The resulting fragment of PCR was 422 base pairs (bp). Because the T94G substitution abolished a *Bsp*HI restriction site, two fragments of 265 and 157 bp after digestion indicate the presence of T allele. This polymorphism is equivalent to the SNP45 described by Hara et al. [16]. The genotyping of PPAR γ 2 Pro12Ala was published previously [9].

Statistical Analyses

Association analysis for adiponectin genotypes- All variables were in S.I. units except that in HOMA-IR. The data were given in means and S.E. In order to compare each outcome variable between the G variant-carrying individuals (including the genotypes T/G and G/G) and their sibs with the genotype T/T, only the siblings with discordant genotypes in T94G polymorphism of the adiponectin gene were included in the analysis. Paired analysis was used to test whether there was difference in variables of interest between the sibs discordant for genotypes after adjustment for age, gender, BMI, ethnicity and area of enrollment BMI by employing analysis of covariance with these variables as covariates. The residuals were then used to test the null hypothesis that there is no difference in the adjusted variables between genotype-discordant sibs among all families (Table 2). The analyses were performed using the SAS version 8.0 PROC GLM. We also performed analysis of covariance using a mixed model to assess whether the adiponectin genotypes affect the outcome variables controlling for covariates age, gender, BMI, ethnicity and area of enrollment BMI as fixed effects, and controlling for clustering among families as a random effect (Table 23). The analyses were performed using the SAS 8.0 PROC MIXED. All statistical tests were two-tailed. The p-values less than 0.05 were considered statistically significant. These statistical analyses were previously described [9, 10].

Analyses on adiponectin gene and PPAR γ 2 gene interactions- In order to assess the interactions between the adiponectin and PPAR γ 2 genotypes, we performed analysis of covariance using a mixed model to evaluate the contribution of the main effects of adiponectin T49G, PPAR γ 2 Pro12Ala respectively, and their interactions for each variable of interest in the same model controlling for covariates age, gender, BMI, ethnicity, and BMI area of enrollment as fixed effects and entering family as a random effect (Table 34). The analyses were performed using the SAS 8.0 PROC MIXED.

The differences of several variables between the subjects with different adiponectin genotypes within fixed PPAR γ 2 genotypes after adjusting for age, gender, ethnicity, BMI, area of enrollment and family effects were compared (Table 45, Figure 1). The values/bars reported are the least square means for each subgroup. The p values were obtained using the SAS PROC MIXED.

RESULTS

The genotype and allele frequencies of adiponectin T94G polymorphism in Japanese and Chinese subjects were comparable (Table 1A). The genotype and allele frequencies in the hypertensive probands were similar and did not deviate from Hardy-Weinberg equilibrium (Table 1B). The genotype and allele frequencies, and the basic characteristics of these subjects categorized by the PPAR γ 2 genotypes were reported previously [9]. Using a sibling-based comparison for phenotypic variables, we found that the subjects with the T/T genotype (n=282) had significantly higher mean plasma insulin level at 1 hour (Ins60) —during oral glucose tolerance tests (OGTT)—than that of the sibs with the discordant genotypes (T/G and G/G, n=312) after adjustment for age, gender, BMI, ethnicity and the area of enrollment BMI (562 $\pm$ 27 vs. 483 $\pm$ 18 pmol/L, p=Table 20.0067). The differences in three other variables including—in the mean area under curve of plasma insulin (AUCi) in OGTT, total cholesterol and LDL cholesterol—were of borderline statistical significance (Table—2828 $\pm$ 37 vs. 743 $\pm$ 27 pmol.h/L, p=0.0578). The differences between discordant adiponectin genotypes in the other variables did not reach statistical significance (data not shown).

To confirm the above findings, we also used a mixed model to correct for familial effects. This method enabled us to include more subjects for analyses. The basic characteristics of the subjects included in this analysis were shown in Table 1(C). After adjusting for age, gender, BMI, ethnicity and area of enrollment BMI, only the means in Ins60 and AUCi in OGTT were significantly different between subjects with the T/T genotype and those otherwise (Table 32). Post-glucose load hyperinsulinemia among subjects with the T/T genotypes in order to normalize plasma glucose levels shown in these two analyses suggests that they may be more insulin resistant. We also performed the analyses by categorize the subjects into three adiponectin genotypes (n=844 of T/T, n=701 of T/G and n=168 of G/G). The results were quite similar. Only the mean Ins60 (525 $\pm$ 18 vs. 455 $\pm$ 20 vs. 461 $\pm$ 35 pmol/L, p=0.0035) and AUCi (785 $\pm$ 26 vs. 702 $\pm$ 28 vs. 719 $\pm$ 50 pmol.h/L, p=0.0204) were significantly different among the three genotypes. The differences were primarily between the T/T and the other two genotypes, suggesting a dominant effect of the G allele in association with insulin sensitivity. Thus this allowed us to pool G/T and G/G genotypes together in all the analyses. No significant difference among the three genotypes was observed in the other variables (data not shown).

Next we investigated the effects of genetic interactions between the adiponectin T94G and the PPAR γ 2 Pro12Ala polymorphisms. Previously, we found in the same study population that the PPAR γ 2 Ala12 variant was significantly associated with better glucose tolerance at 0 (Glu0) and 1 hour (Glu60) in OGTT and with lower

insulin resistant index by HOMA (HOMA_{IR}) [9]. Using two-way ANOVA analysis, we found that there were significant interactions between the adiponectin and PPAR γ 2 genotypes in insulin levels at 0 (Ins0) and 2-hour (Ins120) in OGTT and HOMA_{IR}, with adjustment for age, gender, BMI, ethnicity and area of enrollment-BMI, whereas their interactions in Glu0 and AUCi were lessof borderline significance (Table 43). Interestingly, the main effects of adiponectin genotypes were all significant on every parameter (Ins0, Ins60, Ins120 and AUCi) of insulin in OGTT and HOMA_{IR}. The main effect of the adiponectin genotypes on Glu0 was of borderline significance (Table 3). In contrast, the main effects of PPAR γ 2 genotypes were only significant on Glu0-Glu60 and area under curve of glucose (AUCg) in OGTT (Table 43).

To more clearly illustrate the effects of interactions, these subjects were grouped by their genotypes. It was apparent that the subjects carrying both the adiponectin G and PPAR γ 2 Ala12 alleles were more insulin sensitive. They tended to have lower fasting plasma glucose and insulin, plasma insulin levels in OGTT, and HOMA_{IR} (Table 54, Figure 1). Subjects with the adiponectin G allele even when coupled with PPAR γ 2 Pro/Pro still had lower insulin levels at 1-hour and AUCi in OGTT than subjects with the T/T genotype (Table 54 and Figure 1A).

DISCUSSION

In this report, we demonstrated that the adiponectin T94G genotypes indeed ~~influenced—~~were associated with the variation in insulin sensitivity, ~~indicated indirectly by the serum insulin levels and insulin resistance index by HOMA (HOMA_{IR})~~, in a cohort of Chinese and Japanese hypertension families recruited in the SAPPHIRe study. The subjects with the T/T genotype lower post-glucose load insulin levels but similar glucose levels than those with the other genotypes, indicating insulin resistance associated with T/T genotypes. It should be noted that diabetic subjects were excluded in the recruitment of SAPPHIRe study [10]. Compared with four recent population-based genetic association studies [15-18], the advantage of the current study is that this is a family-based association study utilizing siblings as controls. Because they have relatively higher sharing in both genetic and environmental backgrounds than the population controls, the observed phenotypic differences between them are more likely the results of genetic differences in interest [11].

There has been a great interest in tackling the genetic make-up of human complex traits or diseases, such as diabetes, obesity and hypertension. Although a huge amount of genetic data of such has accumulated in the past, still not very many have addressed the issue of genetic interactions [4, 5]. To our knowledge, our study is one of the earliest to investigate the genetic interactions in connection with insulin resistance. Significant interactions between the genotypes of adiponectin and PPAR γ 2 in relation to fasting insulin and post-glucose load insulin level at 2-h and HOMA_{IR} were noted, providing a piece of genetic evidence in humans that these two genes may participate either in the same or in two interdependent pathways ~~regulating-related to~~ the biological processes of insulin sensitivity. Consistent with this, previous studies in cell cultures, in animals and in humans have shown that adiponectin expression either at mRNA or at protein levels could be up-regulated by PPAR γ 2 activation [21-25]. This study also demonstrated that a genetic association study is capable of detecting genetic epistasis.

The molecular mechanisms behind the genetic interactions between adiponectin and PPAR γ 2 genotypes are unclear at present, but would be fascinating to elucidate. The T94G polymorphism of adiponectin is a silent mutation for Gly15 (GGT to GGG). We speculated that it might be in linkage dis-equilibrium with the other genetic alterations, probably a regulatory mutation. On the other hand, the PPAR γ 2 Ala12 variant was previously shown to have reduced transactivating ability [8]. Although only a half-site of PPAR γ response element (PPRE) was recognized in the proximal promoter of adiponectin by sequence analysis, synthetic PPAR γ 2 agonists were

demonstrated to enhance transcription of luciferase driven by the proximal 2 kb of adiponectin promoter [22]. How PPAR γ 2 variants may activate adiponectin gene in different promoter contexts is an important question to address in the future.

In conclusion, we found that the T94G polymorphism of the adiponectin gene was associated with insulin sensitivity, **represented by serum insulin levels and HOMA_{IR}**, in the subjects from a large hypertensive family cohort. More importantly, the effect of adiponectin was modified by the PPAR γ 2 Pro12Ala genotypes, indicating a genetic interaction in modulating the association with insulin sensitivity. These observations suggest that both the adiponectin and PPAR γ 2 genes and the interactions between them may play a role in the etiopathogenesis of insulin resistance.

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Legends to the figures:

Figure 1 A. Comparisons of AUC_i between the subjects with different combinations of the adiponectin and PPAR γ 2 genotypes, the bars showed means $\pm$ S.E., *-p=0.0357, **-p=0.0118. **B.** Comparisons of AUC_g between the subjects with different combinations of the adiponectin and PPAR γ 2 genotypes, the bars showed means $\pm$ S.E.
Comparisons of HOMA_{IR} between the subjects with different combinations of the adiponectin and PPAR γ 2 genotypes, the bars showed means $\pm$ S.E.M., *** p=0.0192.
C. Comparisons of HOMA_{IR} between the subjects with different combinations of the adiponectin and PPAR γ 2 genotypes, the bars showed means $\pm$ S.E., ***p=0.0201.

Table 1. The proportions (%) and the numbers of subjects with specific adiponectin T94G genotypes and alleles among Chinese or Japanese subjects (A) and hypertensive probands (B). Characteristics of the subjects discordant for the adiponectin genotypes. ~~Data~~ are compared in subjects with the T/T vs. those with the T/G or G/G (C).

A.

	Genotypes			Alleles	
	TT	TG	GG	T	G
Chinese	50.15%	39.77%	10.08%	70.04%	29.96%
	667	529	134	1863	797
Japanese	50.28%	41.25%	8.47%	70.90%	29.10%
	273	224	46	770	316

B.

	Genotypes			Alleles	
	TT	TG	GG	T	G
All	47.83%	41.52%	10.65%	68.59%	31.41%
	265	230	59	760	348
Chinese	47.90%	41.48%	10.62%	68.64%	31.36%
	194	168	43	556	254
Japanese	47.65%	41.61%	10.74%	68.46%	31.54%
	71	62	16	204	94

C.

<u>Variables</u>	<u>T/T</u> <u>(N=844)</u> <u>Mean±S.E.</u>	<u>T/G or G/G</u> <u>(N=869)</u> <u>Mean±S.E.</u>	<u>P=**</u>
<u>Gender (male/female)</u>	<u>372/472</u>	<u>389/480</u>	<u>NS</u>
<u>Ethnicity (Chinese/Japanese)</u>	<u>619/225</u>	<u>631/238</u>	<u>NS</u>
<u>Area</u> <u>(Taiwan/San Francisco/Hawaii)</u>	<u>513/96/235</u>	<u>509/92/268</u>	<u>NS</u>
<u>Age (years)</u>	<u>50.78±0.38</u>	<u>50.88±0.38</u>	<u>NS</u>
<u>BMI* (kg/m²)</u>	<u>25.65±0.16</u>	<u>25.96±0.16</u>	<u>NS</u>
<u>Waist (cm)</u>	<u>85.71±0.47</u>	<u>86.64±0.47</u>	<u>NS</u>
<u>Waist/hip ratio</u>	<u>0.88±0.00</u>	<u>0.89±0.00</u>	<u>NS</u>

*Abbreviation: BMI, body mass index

** NS for $p > 0.1$

|

Table 2. Pair comparisons of metabolic variables between siblings discordant for the adiponectin genotypes after adjustment for age, gender, ethnicity and BMI

Variables ¹	T/T (N=282)	T/G or G/G (N=312)	P= ³
	Mean±S.D.	Mean±S.D.	
Glu0 (mmol/L)	5.17±0.89	5.22±1.11	NS
Glu60 (mmol/L)	9.72±2.44	9.72±2.67	NS
Glu120 (mmol/L)	7.83±2.44	7.83±2.72	NS
AUCg	16.15±3.59	16.19±3.98	NS
Ins0 (pmol/L)	52±37	56±40	NS
Ins60 (pmol/L)	562±453	483±318	0.0064
Ins120 (pmol/L)	491±470	461±408	NS
AUCi (pmol.h/L)	828±623	743±482	0.0565
HOMA _{IR} ²	1.73±1.42	1.89±1.92	NS

¹Abbreviations: Glu, plasma glucose at the specific time point in OGTT; Ins, plasma insulin at the specific time point in OGTT; AUCg, area under curve of plasma glucose in OGTT, AUCi, area under curve of plasma insulin in OGTT. ²HOMA: homeostasis model assessment calculated by $[\text{Insulin}/22.5e^{-\ln(\text{Glucose})}]$

³P-values greater than or equal to 0.1 are marked NS (not significant), P-values less than 0.05 are in bold.

Table 32. Comparisons of metabolic variables between subjects with discordant adiponectin genotypes after adjustment for age, gender, BMI, ethnicity and area of enrollment BMI, using a mixed model corrected for family effects.

	<u>Mean±S.E.</u>	<u>Mean±S.E.</u>	<u>P=</u>
<u>Glu0</u>	5.39±0.05	5.33±0.05	NS
<u>Glu60</u>	9.67±0.13	9.50±0.13	NS
<u>Glu120</u>	7.89±0.13	7.78±0.13	NS
<u>AUCg</u>	16.30±0.20	16.06±0.19	NS
<u>Ins0</u>	54±1	53±1	NS
<u>Ins60</u>	529±17	496±17	0.0008
<u>Ins120</u>	476±18	448±18	NS
<u>AUCi</u>	790±24	711±24	0.0058
<u>HOMA_{IR}</u>	1.85±0.06	1.85±0.06	NS

<u>Variables*</u>	<u>T/T</u> (N=844)	<u>T/G or G/G</u> (N=869)	<u>P=***</u>
	<u>Mean±S.E.</u>	<u>Mean±S.E.</u>	
<u>Glu0</u>	<u>96.325.35±0.900.05</u>	<u>95.545.31±0.890.05</u>	<u>NS</u>
<u>Glu60</u>	<u>171.319.52±2.440.14</u>	<u>168.369.35±2.440.14</u>	<u>NS</u>
<u>Glu120</u>	<u>140.667.81±2.480.14</u>	<u>138.817.71±2.490.14</u>	<u>NS</u>
<u>AUCg</u>	<u>289.9316.11±3.760.21</u>	<u>285.515.86±3.760.21</u>	<u>NS</u>
<u>Ins0</u>	<u>7.654.5±0.211.5</u>	<u>7.4453.4±0.211.5</u>	<u>NS</u>
<u>Ins60</u>	<u>73.17525.0±2.5718.4</u>	<u>63.54455.9±2.5718.4</u>	<u>0.0008</u>
<u>Ins120</u>	<u>66.25475.3±2.7920.0</u>	<u>62.31447.1±2.820.0</u>	<u>NS</u>
<u>AUCi</u>	<u>109.36784.7±3.6626.3</u>	<u>98.28705.2±3.6526.3</u>	<u>0.0056</u>

HOMA_{IR}**

1.84±0.06

1.84±0.06

NS

1*Abbreviations: Glu, plasma glucose at the specific time point in OGTT; Ins, plasma insulin at the specific time point in OGTT; AUCg, area under curve of plasma glucose in OGTT, AUCi, area under curve of plasma insulin in OGTT.

2** HOMA: homeostasis model assessment calculated by $[\text{Insulin}/22.5e^{-\ln(\text{Glucose})}]$

3*** P-values greater than or equal to 0.1 are marked NS (not significant), P-values less than 0.05 are in bold.* All abbreviations, units, and P-values are as in Table 2.

Table 43. Table 3. Significance levels of the adiponectin or PPAR γ 2 genotypes on the main effects and their interactions after adjustment for age, gender, ethnicity, BMI, and area of enrollment using a mixed model corrected for family effects.

<u>Variables*</u>	<u>Adiponectin T94G</u>		<u>PPARγ2 Pro12Ala</u>		<u>Interactions</u>	
	<u>Main effects</u>		<u>Main effects</u>			
	<u>F(d.f.s)=**</u>	<u>P=</u>	<u>F(d.f.s)=</u>	<u>P=</u>	<u>F(d.f.s)=</u>	<u>P=</u>
<u>Glu0</u>	<u>3.61(1,811)</u>	<u>0.0671</u>	<u>0.36(1,811)</u>	<u>0.5491</u>	<u>2.96(1,811)</u>	<u>0.0859</u>
	)		)		)	
<u>Glu60</u>	<u>0.47(1,687)</u>	<u>0.4951</u>	<u>5.76(1,687)</u>	<u>0.0167</u>	<u>0.01(1,687)</u>	<u>0.9271</u>
	)		)		)	
<u>Glu120</u>	<u>0.37(1,715)</u>	<u>0.5459</u>	<u>2.47(1,715)</u>	<u>0.1167</u>	<u>0.09(1,715)</u>	<u>0.7640</u>
	)		)		)	
<u>AUCg</u>	<u>0.69(1,678)</u>	<u>0.4063</u>	<u>5.35(1,678)</u>	<u>0.0210</u>	<u>0.08(1,678)</u>	<u>0.7751</u>
	)		)		)	
<u>Ins0</u>	<u>4.72(1,808)</u>	<u>0.0301</u>	<u>0.41(1,808)</u>	<u>0.5243</u>	<u>4.81(1,808)</u>	<u>0.0285</u>
	)		)		)	
<u>Ins60</u>	<u>8.64(1,696)</u>	<u>0.0034</u>	<u>0.58(1,691)</u>	<u>0.4468</u>	<u>1.81(1,696)</u>	<u>0.1795</u>
	)		)		)	
<u>Ins120</u>	<u>5.54(1,722)</u>	<u>0.0189</u>	<u>0.68(1,722)</u>	<u>0.4083</u>	<u>4.09(1,722)</u>	<u>0.0435</u>
	)		)		)	
<u>AUCi</u>	<u>9.07(1,682)</u>	<u>0.0027</u>	<u>0.73(1,682)</u>	<u>0.3937</u>	<u>3.32(1,682)</u>	<u>0.0691</u>
	)		)		)	
<u>HOMA_{IR}</u>	<u>4.20(1,807)</u>	<u>0.0408</u>	<u>0.64(1,807)</u>	<u>0.4255</u>	<u>5.90(1,807)</u>	<u>0.0154</u>
	)		)		)	

*All abbreviations, units, and P-values are as in Table 2.

** F(d.f.s): F-value (degrees of freedom for the numerator and denominator of the relevant F statistics)

~~Table 3. Significance levels of the adiponectin or PPAR γ 2 genotypes on the main effects and their interactions after adjustment for age, gender, BMI, ethnicity and area of enrollment using a mixed model corrected for family effects.~~

<u>Variables*</u>	<u>Adiponectin</u>	<u>PPARγ2</u>	<u>Interactions</u>
	<u>T94G</u>	<u>Pro12Ala</u>	
	<u>Main effects</u>	<u>Main effects</u>	
	<u><u>p=**</u></u>	<u><u>p=</u></u>	<u><u>p=</u></u>
<u>Glu0</u>	<u>0.0671$\ddagger$</u>	<u>0.5491</u>	<u>0.0859$\ddagger$</u>
<u>Glu60</u>	<u>0.4951</u>	<u>0.0167</u>	<u>0.9271</u>
<u>Glu120</u>	<u>0.5459</u>	<u>0.1167</u>	<u>0.7640</u>
<u>AUCg</u>	<u>0.4063</u>	<u>0.0210</u>	<u>0.7751</u>
<u>Ins0</u>	<u>0.0301</u>	<u>0.5243</u>	<u>0.0285</u>
<u>Ins60</u>	<u>0.0034</u>	<u>0.4468</u>	<u>0.1795</u>
<u>Ins120</u>	<u>0.0189</u>	<u>0.4083</u>	<u>0.0435</u>
<u>AUCi</u>	<u>0.0027</u>	<u>0.3937</u>	<u>0.0691$\ddagger$</u>
<u>HOMA_{IR}</u>	<u>0.0408</u>	<u>0.4255</u>	<u>0.0154</u>

* All abbreviations, units, and P-values are as in Table 2.

** P-values greater than or equal to 0.1 are marked NS (not significant), P-values less than 0.05 are in bold.

Table 4. Comparisons between the subjects with different combinations of adiponectin and PPAR γ 2 genotypes. The p values in the 1st column from the right indicate the comparisons between the different adiponectin genotypes within a group with the same PPAR γ 2 genotypes. The p-values at the bottom indicate the comparisons between the different PPAR γ 2 genotypes within a group with the same adiponectin genotypes. All the abbreviations, units and p-values used are as in Table 2.

<u>Variables*</u>	<u>PPARγ2 genotypes</u>	<u>Adiponectin genotypes</u>		<u>P=**</u>
		<u>T/T</u>	<u>G/-</u>	
<u>N</u>	<u>Pro/Pro</u>	<u>779</u>	<u>804</u>	
	<u>A/a/-</u>	<u>63</u>	<u>62</u>	
<u>Glu0</u>	<u>Pro/Pro</u>	<u>96.165.34$\pm$0.92</u>	<u>95.925.33$\pm$0.910</u>	<u>NS</u>
		<u>0.05</u>	<u>.05</u>	
<u>P=</u>	<u>Ala/-</u>	<u>98.205.46$\pm$2.61</u>	<u>91.465.08$\pm$2.820</u>	<u>0.0649</u>
		<u>0.15</u>	<u>.16</u>	
<u>Glu60</u>	<u>Pro/Pro</u>	<u>172.299.57$\pm$2.5</u>	<u>169.439.41$\pm$2.50</u>	<u>NS</u>
		<u>10.14</u>	<u>0.14</u>	
<u>P=</u>	<u>Ala/-</u>	<u>160.208.90$\pm$6.8</u>	<u>156.468.69$\pm$7.17</u>	<u>NS</u>
		<u>60.38</u>	<u>0.40</u>	
<u>Glu120</u>	<u>Pro/Pro</u>	<u>141.267.85$\pm$2.5</u>	<u>139.717.76$\pm$2.55</u>	<u>NS</u>
		<u>50.14</u>	<u>0.14</u>	
<u>P=</u>	<u>Ala/-</u>	<u>134.257.46$\pm$7.0</u>	<u>129.707.21$\pm$7.49</u>	<u>NS</u>
		<u>90.39</u>	<u>0.42</u>	
<u>AUCg</u>	<u>Pro/Pro</u>	<u>291.2816.18$\pm$3.</u>	<u>287.2615.95$\pm$3.8</u>	<u>NS</u>
		<u>870.22</u>	<u>40.21</u>	
<u>P=</u>	<u>Ala/-</u>	<u>274.9415.27$\pm$1</u>	<u>266.7714.82$\pm$10.</u>	<u>NS</u>
		<u>0.440.58</u>	<u>930.61</u>	
<u>Ins0</u>	<u>Pro/Pro</u>	<u>7.5554.2$\pm$0.221</u>	<u>7.5654.2$\pm$0.211.</u>	<u>NS</u>
		<u>.6</u>	<u>5</u>	
<u>*P=</u>	<u>Ala/-</u>	<u>8.2359.1$\pm$0.614</u>	<u>6.2945.1$\pm$0.664.</u>	<u>0.0234</u>
		<u>.4</u>	<u>7</u>	
<u>Ins60</u>	<u>Pro/Pro</u>	<u>72.37519.3$\pm$2.6</u>	<u>63.98459.1$\pm$2.63</u>	<u>0.0050</u>
		<u>519.0</u>	<u>18.9</u>	
	<u>Ala/-</u>	<u>83.60599.8$\pm$7.3</u>	<u>61.25439.5$\pm$7.70</u>	<u>0.0256</u>

		<u>752.9</u>	<u>55.3</u>	
		<u>NS</u>	<u>NS</u>	
<u>P=</u>				
<u>Ins120</u>	<u>Pro/Pro</u>	<u>65.09467.0±2.8</u>	<u>63.17453.2±2.86</u>	<u>NS</u>
		<u>720.5</u>	<u>20.5</u>	
	<u>Ala/-</u>	<u>80.77479.5±7.6</u>	<u>57.26410.8±8.16</u>	<u>0.0225</u>
		<u>354.8</u>	<u>58.6</u>	
<u>P=</u>		<u>0.0430</u>	<u>NS</u>	
<u>AUCi</u>	<u>Pro/Pro</u>	<u>107.95774.5±3.</u>	<u>99.20711.8±3.74</u>	<u>0.0357</u>
		<u>7727.1</u>	<u>26.8</u>	
	<u>Ala/-</u>	<u>127.72916.4±1</u>	<u>92.80665.8±10.7</u>	<u>0.0118</u>
		<u>0.3574.3</u>	<u>176.8</u>	
<u>P=</u>		<u>0.0605</u>	<u>NS</u>	
<u>HOMA_{IR}</u>	<u>Pro/Pro</u>	<u>1.83±0.07</u>	<u>1.88±0.07</u>	<u>NS</u>
	<u>Ala/-</u>	<u>2.04±0.19</u>	<u>1.44±0.20</u>	<u>0.0201</u>
<u>PP=</u>		<u>NS</u>	<u>0.0294</u>	

~~* All the abbreviations and units used are as in Table 2. Significance levels of the adiponectin or PPAR γ 2 genotypes on the main effects and their interactions after adjustment for age, gender, ethnicity, and BMI using a mixed model corrected for family effects.~~

Variables*	Adiponectin-T94G	PPAR γ 2-Pro12Ala	Interactions
	Main-effects	Main-effects	
	P=	P=	P=
<u>Glu0</u>	0.0695	NS	0.0820
<u>Glu60</u>	NS	0.0075	NS
<u>Glu120</u>	NS	0.0723	NS
<u>AUCg</u>	NS	0.0106	NS
<u>Ins0</u>	0.0291	NS	0.0272
<u>Ins60</u>	0.0035	NS	NS
<u>Ins120</u>	0.0191	NS	0.0449
<u>AUCi</u>	0.0028	NS	0.0699
<u>HOMA_{IR}</u>	0.0409	NS	0.0140

~~** P-values greater than or equal to 0.1 are marked NS (not significant), P-values less than 0.05 are in bold. * All abbreviations, units, and P-values are as in Table 2.~~

Table 54. Comparisons of the means of each variable between the subjects with different combinations of adiponectin and PPAR γ 2 genotypes, (including N=779 of Pro/Pro+T/T, N=804 of Pro/Pro+G/-, N=63 in of Ala/-+T/T and N=62 of Ala/-+G/- genotypes). The p-values in the 1st column from the right indicate the comparisons between the different adiponectin genotypes within a group with the same PPAR γ 2 genotypes. The p-values at the bottom indicate the comparisons between the different PPAR γ 2 genotypes within a group with the same adiponectin genotypes.

Variables	PPAR γ 2 genotypes	Adiponectin genotypes		P=
		T/T	G/-	
Glu0	Pro/Pro	5.33 $\pm$ 0.05	5.33 $\pm$ 0.05	NS
	Ala/-	5.50 $\pm$ 0.15	5.11 $\pm$ 0.16	0.0644
P=		NS	NS	
Glu60	Pro/Pro	9.71 $\pm$ 0.13	9.55 $\pm$ 0.13	NS
	Ala/-	8.93 $\pm$ 0.38	8.77 $\pm$ 0.40	NS
P=		0.0435	0.0535	
Glu120	Pro/Pro	7.94 $\pm$ 0.13	7.84 $\pm$ 0.13	NS
	Ala/-	7.44 $\pm$ 0.39	7.25 $\pm$ 0.41	NS
P=		NS	NS	
AUCg	Pro/Pro	16.38 $\pm$ 0.20	16.15 $\pm$ 0.20	NS

	Ala/-	15.31±0.58	14.93±0.60	NS
P=		0.0728	0.0473	
Ins0	Pro/Pro	54±1	54±1	NS
	Ala/-	60±4	45±5	0.0224
P=		NS	0.0587	
Ins60	Pro/Pro	522±18	463±17	0.0051
	Ala/-	604±52	444±54	0.0259
P=		NS	NS	
Ins120	Pro/Pro	468±19	454±19	NS
	Ala/-	578±54	410±58	0.0231
P=		0.0472	NS	
AUCi	Pro/Pro	780±25	717±25	0.0362
	Ala/-	922±74	672±76	0.0120
P=		0.0591	NS	
IR-HOMA	Pro/Pro	1.83±0.06	1.89±0.06	NS
	Ala/-	2.06±0.19	1.46±0.20	0.0192
P=		NS	0.0326	

mRNA Levels of the Insulin-Signaling Molecule *SORBS1* in the Adipose Depots of Nondiabetic Women

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Abstract

YANG, WEI-SHIUNG, WEI-JEI LEE, KUO-CHIN HUANG, KUAN-CHING LEE, CHIA-LING CHAO, CHI-LING CHEN, TONG-YUAN TAI, AND LEE-MING CHUANG. mRNA levels of the insulin-signaling molecule *SORBS1* in the adipose depots of nondiabetic women. *Obes Res.* 2003;11:586-590.

Objectives: The *SORBS1* gene has been shown to be an important adaptor protein in the insulin-signaling pathway in many molecular and cellular biology studies. However, its roles in humans either in health or disease are rarely explored. In this report, we measured the *SORBS1* mRNA levels in human adipose tissues.

Research Methods and Procedures: Adipose tissues of both the abdominal subcutaneous and omental depots were obtained from 62 nondiabetic women. The relative *SORBS1* mRNA levels were quantified using real-time polymerase chain reaction.

Results: The relative *SORBS1* mRNA levels from these two depots significantly correlated with each other ($\gamma = 0.85$, $p = 0.0000$). The relative *SORBS1* mRNA levels in the omental depots were lower than those in the subcutaneous depots ($p = 0.053$ by two-tailed test, $p = 0.026$ by one-tailed paired Student's *t* test). The mean *SORBS1* expression level in the omental depots was $\sim 70\%$ that in the subcutaneous depots. Moreover, the relative *SORBS1* mRNA levels in the omental depots were significantly related to BMI using either correlation analysis ($\gamma = -0.41$, $p = 0.0008$) or multivariate linear regression analysis ($\beta = -0.20 \pm 0.09$,

$p = 0.031$) with adjustment for age, plasma glucose, serum insulin, triglyceride, and total cholesterol levels.

Discussion: Our preliminary results indicate the depot-specific differential expression of *SORBS1* in relation to BMI. Further investigation of the functional significance of this phenomenon in human obesity is warranted.

Key words: *SORBS1*, mRNA, adipose, depot

Introduction

The concept of adipose tissue as an endocrine organ having influences on the whole-body physiology has been well taken recently (1,2). Therefore, many genes expressed in the adipose tissue, such as leptin, adiponectin, tumor necrosis factor- α , and peroxisome proliferator-activated receptor γ (PPAR γ),¹ may have significant systemic effects, including those on insulin sensitivity, glucose disposal, and fatty acid metabolism (for review, see 2 and references therein).

The human *SORBS1* gene (Sorbin and SH3 domain-containing-1; also called *CAP*, *SH3P12*, or *Ponsin*; see 3–5) encodes an insulin-signaling molecule mainly expressed in the insulin-sensitive tissues, such as adipose tissue, liver, and skeletal muscle (for review, see 6). The *SORBS1* protein forms a complex with the oncoprotein Cbl. On insulin stimulation in adipocytes, another Cbl-associated protein, APS, is phosphorylated (7). The phospho-APS facilitates the phosphorylation of Cbl (7). ("APS" in "phospho-APS" represents an adapter protein containing a PH and an SH₂ domain.) The phospho-Cbl-CAP complex is then sequestered to the lipid raft domains of plasma membrane. Subsequent interactions of this phospho-complex with the other proteins, including flotillin, CrkII, C3G, and TC10, eventu-

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¹Nonstandard abbreviations: PPAR γ , peroxisome proliferator-activated receptor γ ; RT, reverse transcription; PCR, polymerase chain reaction; Ct, threshold cycle; Δ Ct, threshold cycle number of β -actin minus that of *SORBS1*.

Table 1. The characteristics of 62 nondiabetic women

Variables	Mean $\pm$ SD
Age (years)	39.4 $\pm$ 10.3
BMI (kg/m ²)	30.4 $\pm$ 9.5
Glucose (mM)	5.29 $\pm$ 0.72
Insulin (pM)	133 $\pm$ 108
Triglyceride (mM)	1.3 $\pm$ 0.8
Total cholesterol (mM)	4.4 $\pm$ 0.9

ally lead to the translocation of glucose transporter Glut-4 vesicles from cytoplasm to plasma membrane (6,7). This pathway is now considered to be the second insulin-signaling pathway for insulin-stimulated glucose transport in parallel with the well-recognized phosphoinositide 3'-kinase pathway (6). Furthermore, the expression of *SORBS1* was shown to increase in the cultured adipocyte cell line treated with the new antidiabetic agent, PPAR γ 2 agonist thiazolidinedione, whose major mechanism of action is to improve insulin sensitivity (8).

Although the participation of *SORBS1* in the insulin-signaling pathway has been beautifully demonstrated using tools of molecular and cellular biology (3,5–11), suggesting its potential roles in the pathogenesis of certain human diseases clustered in the metabolic syndrome, the number of human studies regarding *SORBS1* has been surprisingly limited. We were the first, to our knowledge, to suggest that a Thr228Ala polymorphism of the human *SORBS1* gene is associated with the risk of obesity and type 2 diabetes (12). In this report, we investigated the mRNA levels of *SORBS1* in the adipose tissues of 62 nondiabetic females to correlate the degree of obesity and metabolic phenotypes with its expression levels. We found that the expression levels of *SORBS1* in the abdominal visceral adipose depots were lower than those from the abdominal subcutaneous depots and that the *SORBS1* mRNA levels in the visceral adipose depots correlated negatively with BMI.

Research Methods and Procedures

Subjects

Sixty-two nondiabetic (by American Diabetes Association criteria) Taiwanese female subjects 19 to 59 years old undergoing elective abdominal surgery were included (Table 1). Both the abdominal subcutaneous and omental adipose tissues were biopsied during a scheduled operation either for benign uterine myoma or for a gastric partition surgery at a fasting state with intravenous supplement of glucose water. The fasting plasma glucose, serum insulin, total cholesterol, triglyceride, and the derived insulin resistance index by homeostasis model assessment were sur-

veyed as described previously (13). Informed consent was obtained, and the study was reviewed and approved by the institutions.

Adipose Tissue RNA Extraction and Reverse Transcription (RT)

The adipose tissue was immediately dipped into liquid nitrogen after removal and then stored at -80°C freezer until use. Total RNA was extracted using RNeasy (Promega, Madison, WI) following the manufacturer's protocol. RT was performed using an RT kit (Promega) with 1 μg of total RNA and 0.5 μg of random hexamers in a final volume of 25 μL containing 200 U of Moloney murine leukemia virus reverse transcriptase, 20 mM deoxynucleoside triphosphate, and 25 U of rRNasin for 1 hour at 37° . The reaction products were diluted to 100 μL with distilled, deionized water before polymerase chain reaction (PCR) amplification.

Quantitation of mRNA by Real-Time PCR

Two microliters of diluted cDNA were added to a 12.5- μL 2 $\times$ SYBR Green PCR Master Mix (Perkin-Elmer Applied Biosystems, Foster City, CA), variable amounts of the respective primers for the human *SORBS1* and β -actin, and water to a final volume of 25 μL . The primers were: *SORBS1*-F493, 5'-TAG CCT TTC AGA TCC TGT CTC AGA-3', *SORBS1*-R602, 5'-CGC CTA CTG TCA TCC TTT GCT-3'; β -actin-F, 5'-CCT CAT GAA GAT CCT CAC CGA GC-3'; and β -actin-R, 5'-GCC AAT GGT GAT GAC CTG GC-3'. The sequences of these primers were designed by Primer Express software (Perkin-Elmer Applied Biosystems). The primers *SORBS1*-F493 and *SORBS1*-R602 were designed to amplify the exon 9 that existed in all the isoforms of *SORBS1* in various tissues, including the adipose tissue (3). The PCR conditions were: 10 minutes at 95° and 40 cycles of 15 seconds at 95° plus 1 minute at 60° . For the detailed principle and procedures for the real-time quantitative PCR analyzed by the ABI PRISM 5700 Sequence Detection System (Perkin-Elmer Applied Biosystems), please see the users' bulletin or manual of ABI PRISM 5700 Sequence Detection System. In brief, the fluorescent signal from each PCR reaction is collected as the peak-normalized values plotted vs. the cycle numbers. Reactions are characterized by comparing the threshold cycle (C_t) values. The C_t is a unitless value defined as the fractional cycle number at which the normalized sample fluorescence signal passes a fixed threshold above baseline, when it is always located within the linear phase of amplification. Samples with a high starting copy number of cDNA show an increase in fluorescence earlier in the PCR process, therefore resulting in a low C_t number. The comparative C_t method, which eliminates the need for standard curves, was used. The C_t number of β -actin minus

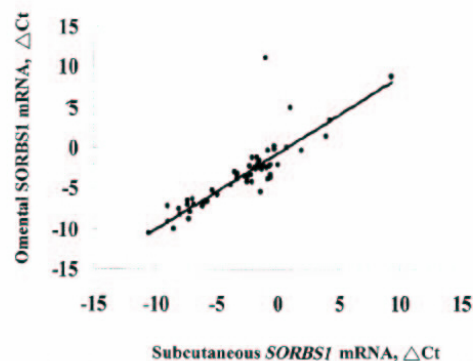


Figure 1: The correlation between the relative *SORBS1* mRNA levels in the subcutaneous and the omental adipose depots represented by the ΔCt among 62 nondiabetic women. The equation of the regression line: $y = 0.95x - 0.65$.

that of *SORBS1* (ΔCt) represents as a $\log_2$ transform of the *SORBS1* mRNA expression corrected with β -actin mRNA expression.

Statistical Analysis

Results were expressed as means $\pm$ SD. The paired Student's *t* test was used to test the difference between two depots from the same subjects. Pearson's correlation and partial correlation were used to examine the correlation between the mRNA levels and the other variables. Multivariate linear regression analyses using the relative mRNA levels of *SORBS1* as the dependent variables and age, BMI, plasma glucose, serum insulin, triglycerides, and total cholesterol levels as the independent variables were also performed. The statistical analyses were performed using STATA (release 6.0, Stata Corp., College Station, TX).

Results

We surveyed the relative mRNA levels of the human *SORBS1* gene in adipose tissues from both the abdominal omental and subcutaneous depots obtained from 62 nondiabetic females. The relative mRNA levels of *SORBS1* in the abdominal omental adipose tissues were lower than those in the abdominal subcutaneous depots (ΔCt , -3.24 ± 0.49 vs. -2.73 ± 0.44 ; paired Student's *t* test $p = 0.053$ by two-tailed, $p = 0.026$ by one-tailed). The mean *SORBS1* mRNA level in the omental depots was $\sim 70\%$ ($100\% \times 2^{-0.51}$) that of the subcutaneous depots. The relative mRNA levels of *SORBS1* from these two depots were significantly correlated ($\gamma = 0.85$, $p = 0.0000$, Figure 1). Because the subjects with higher BMI recruited from the groups for weight reduction surgery tended to be younger, age could be a confounding factor in our analyses. With the adjustment for age, the correlation between the relative *SORBS1* mRNA from these two depots remained significant ($\gamma = 0.84$, $p = 0.000$). The correlation stayed almost the same after further adjustment for age and BMI ($\gamma = 0.83$, $p = 0.000$).

The relative *SORBS1* mRNA from the subcutaneous depots correlated significantly with age ($\gamma = 0.40$, $p = 0.0012$), BMI ($\gamma = -0.42$, $p = 0.0006$), and total cholesterol ($\gamma = -0.27$, $p = 0.031$) (Table 2). With adjustment for age or for both age and BMI, none of these remained significantly related to the relative *SORBS1* mRNA levels in the subcutaneous depots. With adjustment for BMI, age also was not significantly related to the relative *SORBS1* mRNA levels in the subcutaneous depots (data not shown). Using multivariate linear regression analysis, the relative *SORBS1* mRNA levels from the subcutaneous depots were not related to any of these variables (Table 3).

The relative *SORBS1* mRNA levels from the omental depots correlated with BMI ($\gamma = -0.41$, $p = 0.0008$, Figure 2) and total cholesterol ($\gamma = -0.30$, $p = 0.017$) (Table 4). With adjustment for age, only the correlation with BMI remained significant ($\gamma = -0.30$, $p = 0.017$) (Table 4).

Table 2. Correlation analyses of the subcutaneous relative *SORBS1* mRNA (by the ΔCt) with the listed variables with or without adjustment

	Variables					
	Age	BMI	Glucose	Insulin	TG	TC
Adjustment						
No	0.40 (0.0012)	-0.42 (0.0006)	-0.02 (0.88)	-0.08 (0.52)	-0.21 (0.11)	-0.27 (0.031)
Age	—	-0.21 (0.11)	0.05 (0.69)	-0.01 (0.94)	-0.01 (0.93)	-0.12 (0.37)
Age + BMI	—	—	0.06 (0.65)	-0.004 (0.98)	0.11 (0.38)	-0.01 (0.94)

TG, triglycerides; TC, total cholesterol.

The correlation coefficients are listed. The corresponding *p* values are listed in the parentheses.

Table 3. Multivariate linear regression of the relative *SORBS1* mRNA (by ΔCt) from the subcutaneous or omental depots as the dependent variables, and age, BMI, plasma glucose, serum insulin, triglyceride and total cholesterol levels as the independent variables

Independent variables	Dependent variables (<i>SORBS1</i> mRNA levels by ΔCt)			
	Subcutaneous		Omental	
	$\beta \pm \text{S.E.}$	<i>p</i>	$\beta \pm \text{S.E.}$	<i>p</i>
Age	0.07 ± 0.06	0.27	-0.006 ± 0.068	0.93
BMI	-0.13 ± 0.08	0.13	-0.20 ± 0.09	0.031
Glucose	0.24 ± 0.59	0.69	0.23 ± 0.66	0.73
Insulin	-0.0003 ± 0.0040	0.95	-0.0009 ± 0.0045	0.83
TG	0.62 ± 0.69	0.38	1.03 ± 0.78	0.19
TC	-0.24 ± 0.66	0.72	-0.56 ± 0.74	0.46

Using multivariate linear regression analysis, the relative *SORBS1* mRNA levels from the omental depots were related significantly only to BMI ($\beta = -0.20 \pm 0.09$, $p = 0.031$) but not to the other variables (Table 3). From the linear regression model, every 5 kg/m² change in BMI would be associated with ~ 2 -fold difference ($= 2^{5 \times -0.2}$) in the omental *SORBS1* mRNA levels. In any of our analyses, the insulin resistance index derived by the homeostasis model assessment was never found to be significantly related to the *SORBS1* expression levels in the adipose tissues (data not show).

Discussion

The major findings of this study are that the expression levels of *SORBS1* gene are lower in the adipose tissues from

the abdominal visceral depots than those from the abdominal subcutaneous depots and that its expression levels in the visceral depots are negatively related to the BMI among the nondiabetic women. Because of the limitations on the collections of our subjects and tissue samples, our clinical investigation was quite preliminary and did not delineate the functional significance of our findings. We could not address the issue of protein levels. Nevertheless, this report is the first human study to our knowledge investigating the mRNA levels of the *SORBS1* gene, a molecule that has been strongly implicated, in many elegant molecular and cellular biology experiments (3,5–11), to be a key component in insulin signaling. Whether this depot-specific differential expression of *SORBS1* in relation to BMI plays any part in the pathophysiology of human obesity requires further investigation.

The expression of the *SORBS1* gene was induced during adipocyte differentiation and could be further enhanced by treatment with insulin sensitizer thiazolidinediones (8), suggesting an association of higher *SORBS1* gene expression with better insulin sensitivity. The inverse relationship demonstrated in our study between the *SORBS1* mRNA levels in the visceral adipose depots and BMI suggests that the lower *SORBS1* gene expression probably has a part in the pathogenesis of insulin resistance in obesity.

Although the expression levels of *SORBS1* gene in the visceral adipose depots were lower than those in the subcutaneous depots and the expression levels from both depots were highly correlated, it is the expression levels in the visceral depots rather than the subcutaneous ones that were significantly related to BMI. Whether this observation is related to the fact that, in many epidemiological and clinical studies, visceral fat is consistently found to be more strongly related to insulin resistance than subcutaneous fat remains to be explored (2).

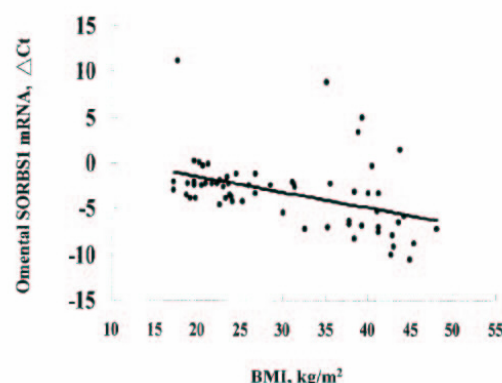


Figure 2: The correlation between the relative *SORBS1* mRNA (by ΔCt) in the omental adipose depots and BMI among 62 nondiabetic women. The equation of the regression line: $y = -0.17x + 1.90$.

Table 4. Correlation analyses of the omental relative *SORBS1* mRNA (by the ΔC_t) with the listed variables with or without adjustment

	Variables					
	Age	BMI	Glucose	Insulin	TG	TC
Adjustment						
No	0.29 (0.02)	-0.41 (0.0008)	-0.01 (0.91)	-0.08 (0.51)	-0.17 (0.18)	-0.30 (0.017)
Age	—	-0.30 (0.017)	0.04 (0.78)	-0.03 (0.81)	-0.03 (0.81)	-0.19 (0.13)
Age + BMI	—	—	0.05 (0.71)	-0.02 (0.85)	0.16 (0.23)	-0.051 (0.71)

The correlation coefficients are listed. The corresponding *p* values were listed in the parentheses.

In this study, we found a significant relation of *SORBS1* gene expression only with BMI but not with the other metabolic variables. This is probably caused by the relatively small sample size of this study. However, the alternative explanation for this could be that we surveyed the *SORBS1* gene expression only in the adipose tissue but not in other insulin-sensitive tissues such as the liver and muscle. The expression of *SORBS1* in the adipose tissue may affect only adiposity, thereby influencing BMI more strongly. But the effects on the whole-body insulin sensitivity and glucose and lipid metabolism may also involve the *SORBS1* expression in the other insulin-sensitive tissues. The *SORBS1* gene is highly expressed in the liver and skeletal muscle (3). How the expression of *SORBS1* in these tissues is related to insulin sensitivity and glucose and lipid metabolism merits further investigation.

The insulin sensitizer thiazolidinedione was shown to increase the mRNA levels of *SORBS1* in cultured adipocyte cell line and the treated Zucker (fa/fa) rats, a model of human type 2 diabetes (8). This may be one of the mechanisms by which the PPAR γ synthetic ligand increases insulin sensitivity. Therefore, it is plausible to speculate that treating type 2 diabetic patients with thiazolidinediones may enhance the *SORBS1* expression in the adipose tissues, while improving their glycemic control. Whether this is an important pathway for this new class of antidiabetic agents to improve insulin sensitivity in vivo remains to be investigated.

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