行政院國家科學委員會專題研究計畫　成果報告

胰島素抗阻、糖尿病與肥胖相關基因之研究（3/3）

計畫類別：個別型計畫
計畫編號：NSC93-3112-B-002-005-
執行期間：93 年 05 月 01 日至 94 年 04 月 30 日
執行單位：國立臺灣大學醫學院內科

計畫主持人：莊立民
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報告類型：完整報告

處理方式：本計畫可公開查詢

中　華　民　國 94 年 10 月 28 日

行政院國家科學委員會補助專題研究計畫 □成果報告
□期中進度報告

（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
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成果報告類型(依經費核定清單規定繳交)：□精簡報告　□完整報告

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執行單位：台大醫學院、內科

中　華　民　國　94 年 10 月 27 日

中文摘要

胰島素阻抗常伴隨肥胖、第二型糖尿病、高血壓、脂質代謝異常、冠狀動脈疾病等臨床表現，臨床上統稱為代謝症候群，是現今全世界健康課題上最常見之重要的慢性病；吾等提出三年之計劃，旨在剖析胰島素阻抗之致病機轉，並採多種不同之策略來達成目標。首先我們會採用分子遺傳學之研究，來了解基因之單一核甘酸變異與臨床表現型之關聯，而候選基因主要是來自我們實驗室自己找出在脂肪細胞與前脂肪細胞有差異表現之基因，這些基因極可能與肥胖之發生有關，並與胰島素之敏感度有關；吾等將尋找這些基因之單一核甘酸變異並研究在正常人、肥胖、第二型糖尿病、冠狀動脈疾病病人之間的差異。而了解這些基因之重要性之後，與這些基因位在同一個路徑之基因（如位在同一個胰島素信息傳遞路徑）都可能為新的候選基因，我們預期將可完成數個分子遺傳學之研究報告。

吾等過去之研究發現有些基因之變異與肥胖、第二型糖尿病有關聯，為要證實這個發現，如能做出此基因之剔除小鼠來研究其表現型，將是最重要之證明；本計劃擬要剔除之基因為 SH3P12/SORBS1 與本計劃命名為 X 之基因。 前者在別人與吾等之研究中發現與胰島素促進細胞對葡萄糖之攝取作用有關，且此一基因之變異與國人之肥胖和第二型糖尿病有關；而後者則尚未在人類找出相對應之基因，然而從這個基因之表現來判斷是極為有趣之基因，它在脂肪細胞分化時會被誘發表現出來，它在肥胖與糖尿病鼠之脂肪組織表現量也比正常對照鼠為高，它在不同之組織表現也具特異性, 更重要的是以胰島素增敏劑處理細胞時，它的表現會被抑制下來，暗示它與胰島素阻抗有極密切之關聯。

經過修正著重目標在：

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 PPARγ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 PPARγ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, 單核甘酸多樣型, *APM1, SORBS1, PPAR*

英文摘要

Insulin resistance associates with multiple clinical manifestations such as obesity, type 2 diabetes, hypertension, dyslipidemia and coronary artery disease (CAD), collectively termed metabolic syndrome, and is a major health issue in our society and in the whole world. In this limited 3-years project, we propose to dissect pathogenic pathways leading to the major defect underlying the metabolic syndrome, i.e. insulin resistance. We applied several approaches to accomplish the goals. The first is molecular genetic studies of the association between candidate SNPs and clinical phenotypes including insulin resistance, obesity and type 2 diabetes. The candidate genes that we choose to study are mostly identified from a differential display between adipocytes vs. preadipocytes in our own laboratory. Based on our large collection of the samples, we can analyze and correlate the genetic variants with clinical phenotypes in the control, obese, type 2 diabetes and CAD subjects. Once confirmed, the other molecules involved in the same pathway might serve as other candidates for testing. We expected to accomplish several molecular genetic studies for publication in 3 years.

Since we have characterized some of the candidate genes previously, we will confirm the biological function of the gene(s) that are shown to associated with the diseases. For this purpose, the best way is to knockout the genes and to see if phenotypes occur. Others and we have shown the role of SH3P12/SORBS1 in the insulin-stimulated glucose uptake. In addition, we have found SNP of this gene correlated with obesity and type 2 diabetes in our population. We will create knockout mice of the SH3P12/SORBS1 gene and establish an animal model for studying insulin resistance, obesity, and diabetes mellitus. Another new gene designated X in this proposal is also very interesting in that the expression of this gene is increased in the adipose tissues of heterozygous and homozygous db/db mice as compared to the wild type mice. Moreover, an insulin sensitizer down regulated the expression of this gene in the 3T3-L1 adipocytes, suggesting that this gene might involve insulin sensitivity. Sequence study indicated this gene was a secretory protein that contained a signal peptide. It is therefore speculated that overproduction will cause insulin resistance and the related phenotypes such as obesity and type 2 diabetes.

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 PPARγ gene in subjects with obesity and insulin resistance. 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.

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, PPARγ

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., βcell dysfunction (8,9), and insulin resistance (10-12). Understanding the mechanisms of insulin resistance and βcell 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

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. ***PPARγ, APM1, SORBS1.***

Designs and Methods

Subjects. Two independent samples, one of normal non-diabetic and normotensive individuals serving as control and the other of type 2 diabetes mellitus as cases.

Genotyping. (i) Genomic DNA. Total genomic DNA from peripheral blood leukocytes of each individual was extracted using Puregene DNA extraction kit, in accordance with the provider's instruction (Minneapolis, MN). For the genotyping analysis which need more high quality DNA , the DNA concentration of each sample will be measured more exactly by fluorometric quantitation method using PicoGreen dsDNA Quantitation Reagent (Molecular Probes, Eugene, Oregon) and gel electrophoresis. The genomic DNA was stored at –20 degree C until SNP analysis wsa performed.

(ii) SNP analysis.

a) Candidate genes selection: In the previous study, we isolated 56 candidate genes for diabetes/obesity development in mouse adipogenesis system. Two genes such as Sorbs1/Sh3d5/Cap and Acrp30 had been demonstrated which play some role in the pathogenesis of human disorder with insulin resistance. After human homology searching, we

identified 45 genes for further SNP allele frequencies determination.

b) SNPs selection: In principle, we will select 4 SNPs from public database such as dbSNP in NCBI (http://www.ncbi.nlm.nih.gov/SNP/), JSNPs in Japanese JSNP Database (http://snp.ims.u-tokyo.ac.jp/) and SNPper database (http://bio.chip.org:8080/bio/) for each gene to perform genotyping. Each SNP will been identified the validation and heterozygosity with 96 case and control subjects first then additional subjects genotyping been performed as its real and has more than 10 % polymorphism. The sequence surrounding a SNP site will be obtained from above mentioned SNP databases. PCR primers for evaluating polymorphic sites will be designed by employing SpectroDESIGNER™ software (MassARRAY™, Sequenom). We will try to perform three or four SNPs variant analysis in one genotyping analysis. In this purpose, we will use SpectroDESIGNER to design optimal multiple primer sets for SNPping reaction.

c) MALDI-TOF Mass Spectrometry: After PCR reaction, the terminal phosphate of non-incorporated dNTPs will be removed from the amplification products by shrimp alkaline phosphatase (SAP) treatment. Homogeneous MassExtend (hME) reaction can detect polymorphisms in amplified DNA by specifically extending a specific primer annealing near the SNP site. Then, the analyte DNA of 1 μl will be added to the 384-spectroCHIP by using SpectroPOINT and dried at room temperature. The DNA on the matrix spot will be introduced into the source region of a mass spectrometer (Sequenom). SNP genotyping from a 384-well target will be performed automatically.

Results and Discussion.

a. **Selected candidate genes and clinical metabolic diseases:** We have demonstrated 3 major genes isolated from differential display, i.e. the *APM1* gene, the *SORBS1* gene and *PPARγ*, that showed significant association of metabolic phenotypes in humans. In the 3 papers that we published, we did show significant SNPs of the three genes either alone or interact with each others affect insulin sensitivity, glucose/insulin metabolisms, and clinical diseases such as obesity and type 2 diabetes (See publications #1~3).

b. **Mechanism of SNP and clinical disorders.** We identified certain genetic interaction of the T/G polymorphism of the *APM1* gene with *PPARγ* Pro12Ala polymorphism in determination of insulin sensitivity, serving as a good model of gene-gene interaction for such a complex disorder or a complex trait. Furthermore, in correlation of the tissue expressions of the genes with clinical phenotypes, we found that tissue mRNA expressions of the *APM1* and *SORBS1* genes are indeed correlated with clinical phenotypes (See publications #1,3). More interestingly, we found a specific allelic expression of the silent SNP of *APM1* gene might be the underlying molecular mechanism that leads to clinical phenotypes (See publication #1). More recently, we found a significant correlation of *APM1* genetic polymorphism with coronary artery disease (manuscript 1 in submission). Due to our clinical human association of adiponectin gene, *APM1*, our manuscript on reviewing the genetic association of APM1 gene has been accepted as a review article in the Journal of Molecular Medicine (See publication #4).

c. **Two additional candidate genes, *GLUT10* and *APOA5* that contribute to clinical phenotypes.** We have tested for the contribution of *GLUT10* and *APOA5* to clinical phenotypes such as type 2 diabetes and hypertriglyceridemia. We found that certain uncommon SNP haplotypes of the *GLUT10* gene were associated with type 2 diabetes although with a modest significance level (manuscript 2 in submission). On the other hand, the SNP of the *APOA5* gene, which interacts with fasting plasma glucose, was associated with hypertriglyceridemia, indicating another example of complex disease caused by gene-environment interaction (See publication #5).

d. **Large-scale SNP genotyping for the genes involved in adipogenesis in human type 2 diabetes.** We demonstrated the feasibility of finding genes from the differentially expressed genes during a pathway of adipogenesis, which is involved in pathogenesis of clinical disorders such as insulin resistance, obesity and type 2 diabetes. For now, we have chosen 4 SNP sites for each of the genes isolated from differential display that resulted in 106 SNP which were informative and successfully genotyped. In the first set of 380 normal and 380 diabetic samples, total genotyping of 60,040, we found 7 SNPs that were significantly correlated with type 2 diabetes. For replication, we genotype another set of 380 normal and 380 diabetic samples and found two of the SNPs that were significant associated with type 2 diabetes. The following summarizes the processes that we conducted for this association study:

   · Overall success rate for the SNP genotyping : 97.02% for the control samples; 90.78% for the diabetic samples

   · Testing allelic and genotypic distribution difference (association) between control and patient groups;

   · Testing HWE for all SNPs in the control group (*http://www.biostat-resources.com/stata/*);

   · Significant level determined by Monte Carlo simulation (with CLUMP from *http://www.gene.ucl.ac.uk/~dcurtis*). SNPs with $p<0.05$ after correction for multiple testing are labeled significant.

   · The final two SNPs that were significant in association with type 2 diabetes are now under more SNP typing and for further haplotype analyses.

   Some statistical results are shown in the following:

   1. Firstly, we can analyzed the different SNP/gene on the quantitative traits, such as fasting plasma glucose, HbA1c, HOMA-IR or –beta, and triglyceride levels (Table 1~4) in the normal non-diabetic and normotensive individuals. These examples illustrate a multigenetic nature for the complex disorder.

   2. There are good examples to show a diverse effect of one single gene on metabolic phenotypes, genetic pleiotropism- a common feature of complex disorder (Table 5,6).

   3. Among the two genes that had associated with type 2 diabetes, we further used STRUCTURE and STRAT software to adjust for population structure in association study.

HMGA2 remained highly significant after adjustment for population structure. Interestingly, this encoded protein contains structural DNA-binding domains and may act as a transcriptional regulating factor. Identification of the deletion, amplification, and rearrangement of this gene that are associated with myxoid liposarcoma suggests a role in adipogenesis and mesenchymal differentiation. A gene knock out study of the mouse counterpart demonstrated that this gene is involved in diet-induced obesity, making this finding highly sensible. The other GM2A, though showed significant association with insulin resistance with HOMA as shown in the table 3 and association with type 2 diabetes, the explanation of its functional significance remains to be further explored (manuscript 3 in preparation).

Table 1. The SNPs that are associated with fasting plasma glucose in the normal individuals with quantitative analyses. The SNP numbering (the first two rows), gene names, and p-values are shown.

s48	6150	rs3205421	CHPT1	0.0013
s28	6115	rs340141	TRIP10	0.0113
s39	6133	rs2256923	IGFALS	0.0255
s50	6153	rs2695281	CHPT1	0.029
s94	7546	rs2303873	SORT1	0.0427
s48	6150	rs3205421	CHPT1	0.0006
s39	6133	rs2256923	IGFALS	0.02
s3	6065	rs3740297	GDF2	0.0331
s28	6115	rs340141	TRIP10	0.0362
s50	6153	rs2695281	CHPT1	0.0479

Table 2. The SNPs that are associated with HbA1c in the normal individuals with quantitative analyses. The SNP numbering (the first two rows), gene names, and p-values are shown.

s78	6223	rs1250259	FN1	0
s19	6096	rs2273181	ARHJ	0.0003
s71	6195	rs4260185	SDPR	0.0013
s77	6220	rs1530380	CDH2	0.002
s24	6106	rs340140	TRIP10	0.0065
s102	808	21843		0.0312
s101	807	18500		0.032

Table 3. The SNPs that are associated with HOMA-IR or HOMA-beta in the normal individuals with quantitative analyses. The SNP numbering (the first two rows), gene names, and p-values are shown.

s58	6167	rs1048719	GM2A	0.0458
s58	6167	rs1048719	GM2A	0.0355
s66	6184	rs153477	GM2A	0.0259
s66	6184	rs153477	GM2A	0.0284

Table 4. The SNPs that are associated with triglyceride levels in the normal individuals with quantitative analyses. The SNP numbering (the first two rows), gene names, and p-values are shown.

s15	6091	rs2293810	ABCD2	0.0115
s65	6182	rs10358	TK1	0.0155
s25	6112	rs2215448	CAV1	0.02
s72	6196	rs903147	MAD2L1	0.0251
s23	6101	rs1410563	GRF2	0.0264
s32	6123	rs2291865	ARL10C	0.0325
s73	6200	rs1251077	ACADM	0.0429
s85	7533	-3338		0.0445
s18	6095	rs1868844	ARHQ	0.0481
s15	6091	rs2293810	ABCD2	0.0063

Table 5. Correlation of the SNPs of caveolin 1 with various metabolic phenotypes.

ht	s26	6113	rs6867	CAV1	0.0103
ht_r	s26	6113	rs6867	CAV1	0.007
ltg	s25	6112	rs2215448	CAV1	0.02
ltg_r	s25	6112	rs2215448	CAV1	0.039
ua	s25	6112	rs2215448	CAV1	0.0143
ua_r	s25	6112	rs2215448	CAV1	0.0068

Ht: height; tg: triglyceride, ua: uric acid; _r: data transformed

Table 6. Correlation of the SNPs of CCRN4L with various metabolic phenotypes.

bmi	s8	6081	rs2292839	CCRN4L	0.0427
pc	s11	6085	rs2271777	CCRN4L	0.0296
ua	s8	6081	rs2292839	CCRN4L	0.0379

bmi: body mass index; pc: postprandial plasma glucose; ua: uric acid

Papers published:

1. Yang WS, Tsou PL, Lee WJ, Tseng DL, Chen CL, Peng CC, Lee KC, Chen MJ, Huang CJ, Tai TY, Chuang LM* (2003) Allele-specific differential expression of a common adiponectin gene polymorphism related to obesity. J Mol Med 81:428-434
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Manuscript in submission/preparation:

1. JY Jiang, YD Jiang, FT Chiang, JJ Hwang, WP Lien, LM Chuang*. Interaction of the Genetic Variant of the *APM1/ACDC* Gene and serum cholesterol level in Angiography-Proven Coronary Artery Disease. (submitted)
2. WH Lin, LM Chuang (equal to first author), CH Chen, JI Yeh, PS Hsieh, CH Cheng, YT Chen. The genetic variation of human *SLC2A10* is associated with type 2 diabetes mellitus in Taiwanese population. (submitted)
3. Yeh JI, Lin WS, Chen YT, Chuang LM*. SNP-based association study of the genes involved in adipogenesis in human type 2 diabetes. (in preparation)

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 many original papers and one review paper accepted for publications in the prestigious journals.