Polymorphisms of the *ENPP1* gene are not associated with type 2 diabetes or obesity in the Chinese Han population

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Running Title: *ENPP1*, diabetes and obesity in Chinese

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Abstract

Objective: Type 2 Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia and with a major feature of insulin resistance. Genetic association studies have suggested that *ENPP1* might play a potential role in susceptibility to type 2 diabetes and obesity. Our study aimed to examine the association between *ENPP1* and type 2 diabetes and obesity.

Design: Association study between two SNPs, rs1044498 (K121Q) and rs7754561 of ENPP1 and diabetes and obesity in the Chinese Han population.

Subjects: 1912 unrelated patients (785 male and 1127 female with a mean age 63.8 ± 9 years), 236 IFG/IGT subjects (83 male and 153 female with a mean age 64 ± 9 years) and 2041 controls (635 male and 1406 female with a mean age 58 ± 9 years).

Measurements: Subjects were genotyped for two SNPs using TaqMan technology on an ABI7900 system and tested by regression analysis.

Results: By logistic regression analysis, rs1044498 (K121Q) and rs7754561 showed no statistical association with type 2 diabetes, obesity under additive, dominant and recessive models either before or after adjusting for sex and age. Haplotype analysis found a marginal association of haplotype C-G (p=0.05) which was reported in the previous study.

Conclusion: Our investigation did not replicated the positive association found previously and suggested that the polymorphisms of *ENPP1* might not play a major role in the susceptibility to type 2 diabetes or obesity in the Chinese Han population.

Keywords: Type 2 diabetes, obesity, Chinese, single nucleotide polymorphism, *ENPP1*

Introduction

Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism. WHO estimates that more than 180 million people worldwide have diabetes and the number is likely to double by 2030. Meanwhile, the problem is considered to be much worse and more widespread in the developing countries, like China. 90% of diabetic cases are type 2 diabetes characterized by hyperglycemia due to a defect in insulin secretion usually with a contribution from insulin resistance. The role of genetic factors in the illness has been consistently supported by population, family and twin studies. The offspring of a diabetic parent has a 40% lifetime chance of developing diabetes in contrast to a population risk of 7%, and if both parents are affected, the risk rises to 70%(1). Twin studies have reported concordance rates of 0.20 to 0.91 in monozygotic twins and 0.10 to 0.43 in dizygotic twins(2). Genetic and genome-wide association studies in recent decades have identified many different candidate genes which are related to the susceptibility in a variety of populations(3, 4).

The ectonucleotide pyrophosphatase/phosphodiesterase 1 (*ENPP1*) gene, also known as plasma cell membrane glycoprotein 1 (*PC-1*) localizes on chromosome 6q23.2, a region which has been reported as being linked to obesity(5, 6), serum insulin levels(5) and type 2 diabetes(7) in different populations. *ENPP1* encodes for a type II transmembrane glycoprotein which inhibits insulin receptor (IR) tyrosine kinase activity(8) and subsequent insulin signaling probably by direct interaction with α subunit of IR(9). The *ENPP1* gene is up-regulated in peripheral insulin target tissues, such as fibroblasts, muscle and adipose tissue, in insulin resistance subjects with or without type 2 diabetes(10-12). The overexpression of *ENPP1* increases insulin resistance in mammals(13). The presence of insulin resistance is the earliest detectable defect in pre-diabetic individuals and a major feature of type 2 diabetes(14). *ENPP1* is considered as a candidate gene for the etiological and pharmacological study of type 2 diabetes.

A number of studies investigating the effects of *ENPP1* on insulin resistance and the pathogenesis of type 2 diabetes, obesity and other metabolic syndromes have focused on the association with genetic variants. Pizzuti and his colleagues first reported strong association between a nonsynonymous single nucleotide polymorphism (SNP) K121Q (rs1044498) on exon4 of *ENPP1* and insulin resistance(15). Subsequent cellular transfection experiments showed that the Q allele

was several times more effective than the K allele in reducing insulin stimulation of IR autophosphorylation, insulin receptor substrate-1 phosphorylation, phosphatidylinositol 3-kinase activity, glycogen synthesis, and cell proliferation(16). During the last decade, genetic association studies have found positive association between K121Q and type 2 diabetes in the Caucasian population(17), in the South Asians living in the US and India(17), in African-Americans(18), and in the Dominican population(19). The Q allele has also been observed to increase the risk of myocardial infarction(20) and obesity in the Caucasian population(21). Meyre et al. found positive association between a three-allele risk haplotype (K121Q (rs1044498), IVS20delT-11 (rs1799774) and A-->G+1044TGA (rs7754561); QdelTG) and childhood obesity, morbid or moderate obesity in adults and type 2 diabetes(22). Conversely, negative results were also reported between K121Q and type 2 diabetes in the Chinese, Japanese and Caucasian populations(23-26). Recently, Meyre and his colleagues found no association of the QdelTG risk haplotype with adult obesity and type 2 diabetes in the French population(27). In addition, recent genome-wide association studies have revealed a series of susceptibility SNPs of type 2 diabetes-related genes, which are mostly related to insulin secretion rather than insulin resistance(28) in large-scale samples and gene pools, but, with one exception(29), no evidence of association between *ENPP1* and type 2 diabetes have been reported(3, 4). Our own study in which we examined two representative SNPs in sequence, rs1044498 (K121Q) and rs7754561, in a Chinese case-control population used the largest sample investigated in China so far, with the aim of providing enhanced detection power and investigating possible association between polymorphisms of *ENPP1* and type 2 diabetes and obesity in the Chinese Han population.

Methods

Subjects and DNA preparation. For the case–control investigation, 1912 unrelated type 2 diabetes patients and 236 IFG/IGT subjects were recruited from Shanghai, China. The patients consisted of 785 male and 1127 female with a mean age 63.8 ± 9 years (HbA1c, 7.4 ± 1.6%; fasting plasma glucose, 7.9 ± 2.9 mmol/l; BMI, 25.2 ± 3.4 kg/m^2; waist/hip, 0.89 ± 0.06). The IFG/IGT subjects were made up of 83 male and 153 female with a mean age 64 ± 9 years (HbA1c, 6.2 ± 0.5%; fasting plasma glucose, 5.6 ± 0.68 mmol/l; BMI, 24.9 ± 3.2 kg/m^2; waist/hip, 0.88 ± 0.06). All patients and IFG/IGT were defined according to the World Health Organization criteria. Control subjects were enrolled from the same geographic region with fasting plasma glucose concentration < 6.1 mmol/l and consisted of 635 male and 1406 female with a mean age 58 ± 9 years (HbA1c, 5.8 ± 0.6%; fasting plasma glucose, 4.9 ± 0.7 mmol/l; BMI, 24.6 ± 3.2 kg/m^2; waist/hip, 0.86 ± 0.06). All values are expressed as mean ± SD as shown in Table 1.

High-molecular-weight genomic DNA was prepared from venous blood using the QuickGene 610L Automatic DNA/RNA Extraction System (Fijifilm, Tokyo, Japan).

Genotyping. In order to insure that the minor allele frequencies (MAF) of the two polymorphisms were not too low in the Chinese Han population to distinguish between cases and controls, we checked the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/) and the hapmap human SNP database (http://www.hapmap.org/). The MAF of rs1044498 and rs7754561 are 0.111 and 0.311 respectively in the Chinese Han population.

Two SNPs (rs1044498 and rs7754561) were genotyped using TaqMan technology on an ABI7900 system (Applied Biosystems, Foster City, California). All probes and primers were designed by the Assay-by-Design or Assay-on-Demand service of Applied Biosystems. The standard 5μL polymerase chain reaction (PCR) procedures were performed using TaqMan Universal PCR Master Mix reagent kits under the provided guidelines. Genotype data were obtained from more than 97% of the DNA samples and the duplicate quality control samples (32 samples) were included and genotyped with 100% concordance.

Statistical analysis. The association of SNPs with type 2 diabetes was assessed by logistic regression. All analysis procedures were adjusted for sex, age and BMI. For the additive model, homozygotes (1/1), heterozygotes (1/0) for the risk allele and homozygotes for the non-risk allele

(0/0) were encoded to an ordered categorical variable for genotypes (2, 1, and 0). The dominant model was defined as 1/1 + 1/0 versus 0/0 and the recessive model as 1/1 versus 1/0 + 0/0. Hardy-Weinberg equilibrium testing, allele, genotype and haplotype frequencies analysis were carried out online on a robust and user-friendly software platform (http://analysis.bio-x.cn/)(30) developed by our lab. It was used to estimate LD measured with standardized D' and to compare the discrepancies of allele, genotype and haplotype frequencies on single loci between cases and controls using a Monte Carlo simulation strategy(31), the normal chi-square test and the odds ratio test. The same association study was repeated for obesity in the patients and controls. Obesity and non-obesity were defined as BMI≥28 kg/m^2 and BMI <28 kg/m^2 respectively according to the Chinese criteria(32).

The difference in the quantitative traits including BMI, Fasting plasma glucose (FPG), Triglycerides (Trig), Hba1c and waist-to-hip ratio (w/h) was analyzed using a general linear model with BMI, FPG, Trig, Hba1c and w/h as the dependent variable, genotype as the independent variable and sex and age as covariates. The statistical analyses were performed using the SPSS (SPSS Inc., Chicago, IL, USA) program.

Power calculations were performed on the G*Power program(33). All p values reported were two-tailed. Statistical significance was defined at $p<0.05$.

Statement of Ethics

We certify that all applicable institutional and governmental regulations concerning the ethical use of human volunteers were followed during this research. A standard written informed consent, reviewed and approved by the ethics committee of the Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, was obtained from all participating subjects who were given a full explanation of the study.

Results

Genotypic distributions of the two SNPs in cases and controls were in Hardy-Weinberg equilibrium ($p>0.05$). In power calculations using the G*Power 3 program, we found that the sample size had >97% power for rs1044498 and >99% for rs7754561 for detecting a relatively weak gene effect (OR=1.2) with $\alpha \leq 0.05$.

However, we found no significant difference in either allele or genotype frequencies between the total 1912 type 2 diabetes patient group and 2041 normal controls at either rs1044498 or rs7754561 under additive, dominant and recessive models either before or after adjusting for sex and age (Table 2).

The estimation of linkage disequilibrium showed that rs1044498 and rs7754561 were not in a strong LD relationship with the standardized D'=0.782 and r2=0.049. We further analyzed the frequencies of the haplotypes of the 2 SNPs above the common lowest frequency threshold (at least 3% frequency in both case and control groups) and found a marginal association of haplotype C-G ($p=0.05$) which was reported in the previous study (Table 3).

Given the important role played by the *ENPP1* gene in insulin resistance and the association widely reported with obesity and metabolic syndromes, we carried out the same logistic regression model analysis for obesity and non-obesity as for type 2 diabetes subjects and normal controls (Table 4), and specifically examined the relationship between 2 SNPs and BMI, Fasting plasma glucose (FPG), Triglycerides (Trig), Hba1c, waist-to-hip ratio (w/h) in the control group using general linear model analysis (Table 5). No significant associations between polymorphisms of *ENPP1* and obesity or the quantitative traits were observed either before or after adjusting for sex and age.

Discussion

Diabetes mellitus has reached epidemic proportions and affects more than 180 million individuals worldwide. Global estimates for the year 2010 predict a further growth of almost 50%, with the greatest increases in the developing countries of Africa, Asia, and South America(34). The problem is becoming serious especially in China, a country with a 1.3 billion population, with predictions that the number of patients who suffer type 2 diabetes is going to be highly significant in a future aging society. According to the WHO criteria, Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism. This clinical phenomenon is likely to be partially attributable to impaired insulin secretion and insulin resistance in which some key genes play important roles. Research based on family, twin and adoption studies has established the genetic contribution to susceptibility to type 2 diabetes. Over the past decade, genetic and gnome-wide association studies on *TCF7L2*, *SLC30A8*, *HHEX*, *FTO*, *PPARG*, *KCNJ11*, as well as *ENPP1* have suggested possible relationship between allele and genotype frequencies and the pathogenesis of type 2 diabetes.

The ectonucleotide pyrophosphatase/phosphodiesterase 1 (*ENPP1*) gene, also known as plasma cell membrane glycoprotein 1 (*PC-1*), encodes for a type II transmembrane glycoprotein, which inhibits the insulin receptor and subsequent insulin signaling. When over-expressed in peripheral insulin target tissues, it is involved in human insulin resistance. The molecular mechanism responsible for the phenomenon was first reported to be associated with a nonsynonymous SNP K121Q(15) which produced the Q allele “gain-of-function” effect several times more effective than the K allele in reducing insulin stimulation of IR autophosphorylation, insulin receptor substrate-1 phosphorylation, phosphatidylinositol 3-kinase activity, glycogen synthesis, and cell proliferation(16). Further, genetic association analyses have supported these findings and have suggested that polymorphisms of *ENPP1* are associated with the risk of type 2 diabetes, obesity and a series of metabolic syndromes.

However, we failed to replicate positive association between rs1044498, rs7754561 and type 2 diabetes or obesity in the Chinese Han population, except a marginal association between the two risk allele haplotype C-G and type 2 diabetes ($p=0.05$) which was reported in the previous study(22). In comparable studies the positive associations have often been reported in white(17, 20)

and African(18) populations, whereas , negative results were found recently in Chinese(23) and Japanese(24) populations. Heterogeneity is still a factor, especially for a complicated metabolic disease. Moreover, unlike a single gene disease, type 2 diabetes and obesity are more attributable to multi-factorial pathogenesis caused by alterations in many gene products. Our study suggests that polymorphisms of *ENPP1* might not play a major role in susceptibility to type 2 diabetes or obesity, but we cannot completely rule out the possible potential effects of *ENPP1* allowing for the association evidence suggested by haplotype analysis and masking influence of different genetic backgrounds and environmental conditions. On the other hand, association studies are mainly concerned with the minor effect of genes or genotypes, so samples have to be large enough and have sufficient statistical power to deliver satisfactory results. Previous studies with positive results have been based on several relatively small samples, so further studies should have larger and more ethnically diverse samples. Our investigation on *ENPP1* provides with the largest sample to date relating to type 2 diabetes and obesity in the Chinese population.

The sample size of the IFG/IGT subject group was so small that we did not perform a case-control analysis between the IFG/IGT subject group and the control group, although it has been reported that both IFG and IGT are strong risk factors for the development of type 2 diabetes(35).

In addition, we examined the relationship between the two SNPs and BMI, FPG, Trig, Hba1c and w/h in the control group using general linear model analysis, given that the physiological conditions of the patient group might be altered by medicines and treatments. None showed significant association with any of these metabolic quantitative traits.

In conclusion, our case-control investigation in the Chinese Han population did not support positive association between two SNPs of *ENPP1*, rs1044498 and rs7754561, and type 2 diabetes or obesity. We hope the study may act as a reference point for further replication studies on the effects of *ENPP1* on type 2 diabetes and obesity, and for the comprehensive meta-analyses which are required for validation.

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Table 1. Clinical characteristics of the subject samples

	Type 2 diabetes	IFG/IGT	Control
Age (years)	63.8±9	64±9	58.1±9
BMI (kg/m^2)	25.3±3.4	24.9±3.2	24.5±3.2
Waist-to-hip ratio	0.90±0.06	0.88±0.06	0.86±0.06
Fasting blood glucose(mmol/l)	8.1±2.9	5.6±0.68	4.8±0.64
Triglycerides (mmol/l)	1.9±1.6	1.9±1.2	1.6±1.1
Cholesterol (mmol/l)	4.5±0.9	4.5±0.8	4.5±0.9
HDL (mmol/l)	1.2±0.3	1.1±0.3	1.3±0.3
LDL (mmol/l)	2.8±0.8	2.8±0.7	2.8±0.7
Systolic blood pressure(mmHg)	140.1±20.4	141.3±18.9	132±19.3
Diastolic blood pressure(mmHg)	82.0±10.2	82.1±10.6	81.1±10.1
HbA1C (%)	7.5±1.6	6.2±0.5	5.8±0.6

Data are means ± SD

Table 2. Allele and genotype distributions of the SNPs in cases and controls

	Risk-allele frequencies				Unadjusted	Genotype distribution				Adjusted by sex, age and BMI		
SNPs	Risk allele	Control n (freq)	IFG/IGT n (freq)	T2D n (freq)	OR (95% CI)	Genotype	Control n (freq)	IFG/IGT n (freq)	T2D n(freq)	Additive model OR (95% CI)	Dominant model OR (95% CI)	Recessive model OR (95% CI)
rs1044498	C	423(0.105)	50(0.108)	439(0.117)	0.89(0.77-1.02)	CC	19(0.009)	4(0.017)	23(0.012)	1.15(0.97-1.34)	1.15(0.98-1.36)	1.33(0.70-2.54)
					P_{freq} =0.097	CA	385(0.191)	42(0.181)	393(0.209)	P_{add} =0.076	P_{dom} =0.090	P_{rec} =0.387
						AA	1610(0.799)	186(0.802)	1463(0.779)			
rs7754561	G	2264(0.601)	224(0.526)	2063(0.596)	1.03(0.93-1.13)	GG	678(0.360)	62(0.291)	631(0.364)	0.94(0.86-1.04)	0.85(0.71-1.03)	0.98(0.84-1.13)
					P_{freq} =0.607	GA	908(0.482)	100(0.469)	801(0.462)	P_{add} =0.261	P_{dom} =0.094	P_{rec} =0.734
						AA	296(0.157)	51(0.239)	300(0.173)			

Table 3. Estimated haplotype analysis in the case-control samples

Haplotype	Type 2 diabetes n(freq)	Control n(freq)	Fisher's p	Odds Ratio (95%CI)
A-A	1353.25(0.395)	1434.82(0.386)	0.485481	1.035 (0.940-1.138)
A-G	1680.75(0.491)	1900.18(0.511)	0.063866	0.916 (0.834-1.005)
C-G	362.25(0.106)	340.82(0.092)	0.050313	1.168 (1.000-1.365)

Haplotype analysis excluded those with less than 3% estimated haplotype probability. The individual haplotype frequency differences between cases and controls were calculated using the SHEsis software platform. For each haplotype, alleles are arranged in order of rs1044498 - rs7754561.

Table 4. Allele and genotype distributions of the SNPs in the obese and non-obese subjects

SNPs	Risk allele	Risk-allele frequencies		Unadjusted	Genotype	Genotype distribution		Adjusted by sex and age		
		Obesity n (freq)	Non-obesity n (freq)	OR (95% CI)		Obesity n (freq)	Non-obesity n(freq)	Additive model OR (95% CI)	Dominant model OR (95% CI)	Recessive model OR (95% CI)
rs1044498	C	154(0.115)	697(0.1107)	0.96(0.85-1.09)	CC	5(0.007)	37(0.012)	1.05(0.87-1.27)	1.09(0.89-1.33)	0.63(0.25-1.62)
				P_{freq} =0.550	CA	144(0.216)	623(0.196)	P_{add} =0.597	P_{dom} =0.411	P_{rec} =0.337
					AA	519(0.777)	2518(0.792)			
rs7754561	G	749(0.607)	3526(0.597)	0.95(0.79-1.14)	GG	678(0.360)	1065(0.361)	1.03(0.91-1.16)	1.02(0.83-1.24)	1.03(0.86-1.23)
				P_{freq} =0.552	GA	908(0.482)	1396(0.473)	P_{add} =0.678	P_{dom} =0.878	P_{rec} =0.789
					AA	296(0.157)	492(0.167)			

Table 5. Associations between the SNPs and quantitative traits of BMI, Fasting plasma glucose (FPG), Triglycerides (Trig), Hba1c and waist-to-hip ratio (w/h)

SNPs	Genotype (1/2)	Trait	11	12	22	P
rs1044498	A/C	BMI (kg/m^2)	24.3(22.3-26.5)	24.5(22.6-26.7)	24.8(22.9-26.6)	0.454
		FPG (mmol/l)	4.90(4.51-5.26)	4.86(4.50-5.23)	4.74(4.44-5.00)	0.062
		Trig	1.34(0.95-1.91)	1.32(0.93-1.96)	1.35(1.03-1.92)	0.846
		Hba1c	5.81(5.47-6.10)	5.77(5.40-6.12)	5.89(5.41-6.13)	0.279
		w/h	0.86(0.82-0.90)	0.86(0.82-0.91)	0.86(0.82-0.90)	0.186
rs7754561	A/G	BMI (kg/m^2)	24.1(22.0-26.2)	24.3(22.3-26.5)	24.5(22.3-26.7)	0.054
		FPG (mmol/l)	4.88(4.49-5.26)	4.89(4.539-5.27)	4.90(4.46-5.26)	0.123
		Trig	1.34(0.94-1.88)	1.33(0.93-1.90)	1.36(0.94-1.96)	0.955
		Hba1c	5.82(5.44-6.13)	5.82(5.47-6.13)	5.81(5.47-6.09)	0.938
		w/h	0.86(0.82-0.89)	0.86(0.82-0.90)	0.86(0.82-0.90)	0.342

Data are median (1st–3rd quartile). P values were derived using the additive model adjusted for sex and age.