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# Single nucleotide polymorphisms in the mitochondrial control region are associated with metabolic phenotypes and oxidative stress

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ABSTRACT

*Objective:* To identify the mitochondrial DNA (mtDNA) single nucleotide polymorphisms (SNPs) in the control region and elucidate their role in metabolic phenotypes and oxidative stress.
*Methods:* A total of 861 nondiabetic subjects were enrolled, including 250 impaired fasting glucose (IFG) and 370 obese subjects (body mass index [BMI] > 25 kg/m$^2$). Antioxidant status presented as total free thiol level was determined from serum samples. DNA was extracted from peripheral blood leucocytes, and the sequences were analyzed using the DNASTAR software. SNPs were identified by comparison with the Cambridge Reference Sequence.
*Results:* After adjusting odds ratios for age, sex, and BMI, the selected independently significant SNPs indicated 4 susceptible SNPs: SNP-16126C and SNP-16261T, which were related to abdominal obesity ($P = 0.009$; 0.06); SNP-16390A, related to hypertension (HTN) ($P = 0.007$); and SNP-16092C, related to decreased antioxidant capacity ($P = 0.015$). In the obese subgroup, 3 susceptible SNPs included SNP-16189C and SNP-16260T, which showed significantly higher IFG prevalence ($P = 0.016$ and 0.024, respectively), and SNP-16519C, which was significantly higher in the HTN group ($P = 0.036$). As to protective SNPs, 5 protective SNPs were identified in all subjects but only one SNP-16093C is consistent in obese group, which showed a significantly lower prevalence in patients with abdominal obesity and was associated with a higher antioxidant status ($P < 0.001$).
*Conclusion:* SNPs in the mtDNA control region are associated with metabolic phenotypes and oxidative stress markers. Some SNPs are relating to the interaction between obesity and genetic factors. The beneficial effects of these protective SNPs were insignificant and some susceptible SNPs became dominant within the obese subgroup. Subjects harboring these SNPs should avoid excessive weight gain.


## 1. Introduction

The mitochondrial genome is highly compacted on the double-stranded circular mitochondrial DNA (mtDNA), which is 16569 bp in length. MtDNA contains a noncoding control region of approximately 1.1 kb (between positions 16024 and 576). The initiation of mtDNA replication in cells occurs within the control region (Arnberg et al., 1971; Yasukawa et al., 2005). Human mtDNA is more susceptible to oxidative damage and consequently acquires mutations at a higher rate than nuclear DNA. This is because of the increased exposure to high reactive oxygen species (ROS) levels generated during respiration, lack of protective histones, and limited capacity for repair of damaged mtDNA (Croteau et al., 1999; Yakes and Van Houten, 1997). Moreover, the production of superoxide anions by mitochondria is increased by defects in the respiratory chain in the affected tissue of patients with mitochondrial disease or of aged individuals (Turrens, 2003; Wei et al., 1998). Most variations in mtDNA occur in the control region. The control region functions as a promoter for both heavy and light strands of mtDNA. Hence, presumably, the effectiveness of oxidative stress and mitochondrial biogenesis may be altered by polymorphisms in the control region of mtDNA.

Both qualitative and quantitative changes in mtDNA have been involved in the pathogenesis of type 2 diabetes. Reduction of the mitochondrial function in the insulin-resistant offspring of parents with type 2 diabetes can be mostly attributed to reductions in mitochondrial density (Morino et al., 2006). An altered mitochondrial function such as defective oxidative metabolism in fat and skeletal muscle seems to be involved in visceral fat gain and in the development of insulin resistance (IR) (Nisoli et al., 2007). A common variant in mtDNA at bp 16,189 (T → C transition) has been suggested to be related to both thinness at birth and impaired glucose tolerance/type 2 diabetes mellitus (DM) at adulthood in men born in Hertfordshire between 1920 and 1930 (Marchington et al., 1996; Poulton et al., 2002). We demonstrated that

*Abbreviations:* mtDNA, mitochondrial DNA; SNPs, single nucleotide polymorphisms; BMI, body mass index; TBARS, thiobarbituric acid reactive substance; IFG, impaired fasting glucose; HTN, hypertension; AO, abdominal obesity.

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the prevalence of the position 16189 variant is higher in type 2 DM patients than in the age- and sex-matched non-DM subjects in a larger group of ethnic Chinese subjects in Taiwan. The higher levels of fasting insulin, insulin resistance, and ß-cell function in our subjects, who harbored the position 16189 variant, were indicative of a pre-diabetic state (Liou et al., 2007). In spite of the suggestion that the mtDNA status might be a hereditary factor that could indicate for IR and fat deposition (Song et al., 2001), evidence about the role of control region variations in metabolic phenotypes is rare and should be studied in nondiabetic groups.

In this study, the mtDNA sequence variation of a segment of the control region was determined to elucidate its role in metabolic phenotypes and its effect on oxidative stress and mtDNA copy number in nondiabetic subjects in Taiwan.

## 2. Materials and methods

### 2.1. Ethics statement

The protocol for the present study was approved by the Committee on Human Research at Kaohsiung Chang Gung Memorial Hospital (CMRPG891081, IRB 92-673 and 94-1092B) and conducted in accordance with the Declaration of Helsinki. All participants signed the written informed consent form to obtain approval to take part in the study.

### 2.2. Subjects

A total of 861 nondiabetic, unrelated Taiwanese subjects of Han ethnic backgrounds were enrolled in this study, including 250 IFG and 370 obese subjects (body mass index [BMI] > 25 kg/m$^2$). The study participants were randomly selected from the health-screening center or outpatient service. The nondiabetic status was determined on the basis of the patient's history and a fasting plasma glucose (FPG) < 100 mg/dL. Impaired fasting glucose (IFG) was defined by an FPG between 100 and 125 mg/dL. Hypertension was defined by systolic blood pressure > 140 mm Hg and/or diastolic blood pressure > 90 mm Hg and/or anti-hypertensive drug therapy. A waist circumference (WC) measurement, as recommended by the World Health Organization (WHO), was measured from midway between the crest of the ileum and the inferior margin of the last rib in a horizontal plane. For Asians, abdominal obesity was defined as male subjects with WC of at least 90 cm and female subjects with WC of at least 80 cm (Tan et al., 2004).

### 2.3. Methods for determining the mtDNA control region sequence

The mtDNA control region segment (relative to region 15911–602 in the Cambridge Reference Sequence) was amplified using the forward primer L15911 (5′-ACCAGTCTTGTAAACCGGAG-3′) and the reverse primer H602 (5′-GCTTTGAGGAGGTAAGCTAC-3′). The products were purified using gel extraction kits (Watson BioMedicals Inc.) and sequenced using primer L15911 and primer L29 (5′-CTCACGGGAGCT CTCCATGC-3′) on an ABI 377XL DNA Sequencer (Applied Biosystems). However, because of the frequent conversion of thymine to cytosine and the presence of a homopolymeric cytosine tract at np 16184–16193 and np 303–315 within the control region, the sequencing procedure ceased each time with samples harboring these variants. Because of this, reverse sequencing using the following additional set of primers was required: H81 (5′-CAGCGTCTCGCAATGCTATC-3′) and H602 (5′-GCTTTGAGGAGGTAAGCTAC-3′). The DNA sequences were analyzed using the DNASTAR and BioEdit Sequencing Analysis Software.

### 2.4. Oxidative stress

The methods to detect free thiols and thiobarbituric acid reactive substance (TBARS) in plasma were as described previously (Lin et al., 2005). Free thiols in the plasma were determined by directly reacting thiols with 5,5-dithiobis-2-nitrobenzoic acid (DTNB) to form 5-thio-2-nitrobenzoic acid (TNB). The amount of thiols was calculated from the absorbance, which was determined using the extinction coefficient of TNB ($A_{412}$ = 13,600 $M^{-1}$ $cm^{-1}$) (Ellman and Lysko, 1979). The plasma TBARS concentration was assessed using the method of Ohkawa et al. (1979). After centrifugation, the plasma samples were stored at −80 °C for future analysis. The results are expressed as micromoles of TBARS per liter. A standard curve of TBARS was obtained by hydrolysis of 1,1,3,3-tetraethoxypropane (TEPP).

### 2.5. Statistical analysis

Chi-square tests were used to compare basic characteristics between patients and controls. A sequence of analyses was adopted for single nucleotide polymorphism (SNP) selection. Chi-square tests were first used to compare distributions of SNPs between patients and controls. SNPs showing significant differences between the patients and the controls were chosen for further analysis. SNPs were included in a logistic regression model with backward selection. Only independently significant SNPs were selected by logistic regression. In addition, the same logistic regression selection process was also conducted for the subgroup with obesity. Finally, the adjusted odds ratios (*AORs*) from the selected SNPs were computed based on the logistic regression with additional covariates of metabolic phenotype-related risk factors. The statistical data were expressed as mean ± SD. A $P$ value < 0.05 was considered to be statistically significant.

## 3. Results

### 3.1. Basic demographic characteristics

The study participants were 861 nondiabetic subjects, including 370 obese subjects (BMI > 25 kg/m$^2$), and their basic characteristics are shown in Table 1. All of these characteristics were found to be significantly different between the obese and non-obese groups. The obese subjects were older than the control subjects and had higher values of BMI, blood TBARS, FPG, and WC. The mean thiol level was lower in the obese group. Larger number of obese patients had comorbidities of hypertension (HTN), IFG, and abdominal obesity than controls.

### 3.2. Identification of SNPs and significance analysis for individual SNPs

Single nucleotide substitution polymorphisms showed a prevalence that was >3% in the control region between the nucleotide positions

**Table 1**
Basic demographic characteristics in the obesity and non-obesity groups.

| | Obesity (BMI > 25 kg/m$^2$) (n = 370) | Non-obesity (BMI < 25 kg/m$^2$) (n = 491) | $P$ value |
|---|---|---|---|
| Age (years) | 55.95 ± 12.0 | 52.43 ± 13.9 | <0.001 |
| Sex (M%) | 65.9 | 53.6 | <0.001 |
| BMI | 27.8 ± 2.5 | 22.2 ± 1.9 | <0.001 |
| TBARS (μmol/L) | 1.527 ± 0.835 | 1.383 ± 0.814 | 0.011 |
| Thiol (μmol/L) | 1.952 ± 0.384 | 2.025 ± 0.394 | 0.006 |
| Glucose (mg/dL) | 97.90 ± 10.09 | 93.16 ± 9.43 | <0.001 |
| Waist (cm) | 92.22 ± 7.93 | 79.11 ± 7.92 | <0.001 |
| IFG (%) | 38.1 | 22.2 | <0.001 |
| HTN (%) | 53.8 | 30.8 | <0.001 |
| Abdominal obesity (%) | 73.5 | 20.6 | <0.001 |

BMI: body mass index, TBARS: thiobarbituric acid reactive substance, IFG: impaired fasting glucose, HTN: hypertension.