Nutrition, Metabolism & Cardiovascular Diseases (2006) **16**, 466–470

ELSEVIER

Nutrition, Metabolism & Cardiovascular Diseases

www.elsevier.com/locate/nmcd

The mtDNA 15497 G/A polymorphism in cytochrome *b* in severe obese subjects from Southern Italy

Rosario Liguori [a,b], Cristina Mazzaccara [a], Fabrizio Pasanisi [c], Pasqualina Buono [d], Giovannangelo Oriani [b,e], Carmine Finelli [c], Franco Contaldo [c], Lucia Sacchetti [a,b,*]

[a] *Department of Biochemistry and Medical Biotechnology, University Naples Federico II, Via Pansini 5, 80131 Naples, Italy*
[b] *CEINGE Advanced Biotechnology S.C. a r. l., Via Comunale Margherita 482, 80145 Naples, Italy*
[c] *Department of Clinic and Experimental Medicine -Inter-University Center for Obesity and Eating Disorders (CISRO), University Naples Federico II, Via Pansini 5, 80131 Naples, Italy*
[d] *Faculty of Movement Sciences, University Parthenope, Naples, Via Acton 38, 80133 Napoli, Italy*
[e] *Department S.P.E.S., University Molise, Via De Sanctis, 86100 Campobasso, Italy*

Received 14 April 2005; received in revised form 14 June 2005; accepted 23 June 2005

KEYWORDS
Mitochondrial DNA;
Cytochrome;
Single nucleotide polymorphism;
Caucasians

Abstract *Background and aim:* A large number of mitochondrial DNA (mtDNA) mutations have been implicated in degenerative diseases and aging. The aim of this study was to evaluate whether the 15497 G/A mtDNA polymorphism (G251S) in the cytochrome *b* subunit of respiratory complex III, which has been associated with obesity-related variables and lipid metabolism in a Japanese population, is associated with severe obesity also in adult Caucasians from southern Italy.
Methods and results: Unrelated severely obese patients ($n = 317$; BMI $> 40\ kg/m^2$) and controls ($n = 217$; BMI $< 25\ kg/m^2$) from Southern Italy were genotyped by allelic discrimination TaqMan assay for the 15497 G/A mtDNA polymorphism. In obese patients fasting serum total cholesterol, triglycerides, HDL-cholesterol and glucose were measured enzymatically and sitting blood pressure and heart rate were also collected. Mean levels of total cholesterol, triglycerides and glucose were below the upper reference limit for healthy subjects. Female obese subjects showed lower levels of blood pressure and heart rate and higher levels of HDL cholesterol than male obese patients ($P < 0.001$). All the control subjects and

* Corresponding author. Department of Biochemistry and Medical Biotechnology, University Naples Federico II, Via Pansini 5, 80131 Naples, Italy. Tel.: +39 081 746 3532; fax: +39 081 746 2404.
E-mail address: sacchetti@dbbm.unina.it (L. Sacchetti).

0939-4753/$ - see front matter
doi:10.1016/j.numecd.2005.06.009

315/317 severely obese patients were homozygous for the G allele (wild type), whereas only 2/317, were females homozygous for the A allele.
Conclusions: The mtDNA 15497 G/A polymorphism in cytochrome b was present in 0.6% obese subjects, two females whose lipid parameters and BMI were similar to those of the overall group. Therefore, this mutation may appear to contribute in rare instances to severe obesity but does not explain the majority of cases in our population. A more extensive genetic haplogroup characterization is required to identify associations to obesity in Caucasians.

Introduction

Changes in mitochondrial DNA (mtDNA) influence human health. They may contribute to select individuals with advantageous metabolic phenotypes for longevity or individuals with enhanced susceptibility to degenerative diseases [1,2]. Mitochondrial DNA is maternally inherited; it has a very high mutation rate (10 fold that of nuclear DNA) and consists of a non-coding region (D-loop or control region) and a functional region that encodes two rRNAs (12S and 16S), 22 tRNAs and 13 polypeptides [3,4]. The latter are subunits of the 5-enzyme complexes (I—V) that are involved in the mitochondrial electron-transfer chain [4]. Cytochrome *b* is the only mtDNA-encoded subunit of respiratory complex III. The cytochrome *b* single nucleotide polymorphism (SNP) 15497 G/A, which leads to a glycine 251 serine (Gly251Ser) amino acid replacement, has been associated with obesity in a Japanese population. In fact, individuals bearing the A allele presented a waist-to-hip ratio and other obesity-related variables including triglycerides levels, greater than individuals bearing the G allele [5]. Because human mtDNA undergoes geographical variations, and because mtDNA variants are often functionally different [6], we determined whether the mtDNA 15497 G/A polymorphism is involved in obesity also in a Caucasian population. Thus, we studied this polymorphism in 317 severely obese and in 217 controls unrelated adult Italians from the same geographical area of Southern Italy.

Methods

Subjects were recruited from the Out-Patients Laboratory Service (217 control subjects: 98 males and 119 females, BMI $< 25\ kg/m^2$) and from the Out-Patients Obesity Clinic (317 patients with severe obesity: 126 males and 191 females with BMI $> 40\ kg/m^2$; no diabetes and no coronary heart disease) of the School of Medicine, University of Naples Federico II. The families of all subjects had lived in the same region of Southern Italy for at least two generations. Informed consent to participate in the study was obtained from each subject, and the study was approved by the Ethics Committee of our Medical School.

Serum total cholesterol (T Chol), triglycerides (Tg) and glucose (Glu) were measured enzymatically with standard techniques on an automated analyzer (Hitachi 747; Boehringer Mannheim). The HDL-cholesterol (HDL-Chol) concentration was determined enzymatically by measuring cholesterol in the supernatant after precipitation with phosphotungstate. The body mass index (BMI) was calculated as ratio weight (kg)/height (m^2). Systolic (SBP) and diastolic (DBP) blood pressure and heart rate (HR) were also collected by standard procedures.

Genomic DNA was extracted from peripheral blood samples with the Nucleon BACC2 kit (Amersham Life Science, England) and the mtDNA 15497 G/A SNP was assayed, in duplicate, by the Real Time Taqman method [7]. Briefly, two probes are used in a biallelic system; one probe is specific for the wild-type allele and the other is complementary to the mutant allele. The alleles are distinguished with fluorogenic probes, which consist of an oligonucleotide with a fluorescent reporter dye (VIC or FAM), a non-fluorescent quencher and a minor groove binder (MGB) [8]. The latter molecule forms a hyperstabilized duplex with complementary DNA thereby increasing the capacity of the hybridization probe to discriminate the SNP [9]. The Primer Express program (Applied Biosystems, Foster City, CA) was used to design the PCR primers (forward: 5′ ACT TCT CTT CCT TCT CTC CTT AAT GAC A 3′, and reverse: 5′GGG TTG GCT AGG GTA TAA TTG TCT3′) and the MGB TaqMan probes (FAM-CCT CCT A*A*G CGA CCC-Q-MGB and VIC-CCT CCT A*G*G CGA CCC-Q-MGB).

Reaction mixtures were assembled in a 384-well plate using a Biomek 2000 Workstation (Beckman Instruments, Inc. Fullerton, CA). Each mixture consisted of: 2 μL (40 ng) of genomic DNA, 36 nM of primer, 8 nM of probe and 2.5 μL TaqMan Universal Master mix (Applied Biosystems Foster City, CA) in a total reaction volume of 5 μL. Together with samples from patients, we tested negative (i.e., no DNA sample) and positive controls (i.e., G/G and

A/A homozygotes and a G/A heterozygote, obtained by mixing G/G and A/A DNA samples, for the 15497 G/A SNP). The positive controls had been previously typed by sequence analysis on an ABI 3100 Genetic Analyzer (Applied Biosystems Foster City, CA). Real time PCR was performed on an ABI Prism 7900-HT instrument with the Sequence Detection System (SDS 2.1) and the SDS Enterprise Database (Applied Biosystems Foster City, CA). The amplification protocol consisted of 95 °C for 10 min; 95 °C for 15 s and 60 °C for 1 min for 40 cycles with a final soak at 25 °C. During PCR, each TaqMan MGB probe anneals specifically to the complementary sequence. When the probe is intact, the proximity of the quencher to the reporter dye reduces the fluorescence signal, whereas when the fluorogenic probe is hybridized to the target sequence, it is cleaved by AmpliTaq Gold DNA polymerase. The cleavage results in the release of the reporter dye and the consequent increased fluorescence indicates that the specific probe target has been amplified. In each amplification step, the intensity of fluorescence increases in relation to the accumulation of the PCR product, which is automatically measured directly in the reaction well. At the end of the PCR, a laser collects the fluorescence spectrum of each dye and the SDS software elaborates the data to produce a scatter diagram of the mtDNA 15497 G/A polymorphism. Statistical analysis, Student's *t* test, was performed with SPSS for Windows software (Ver. 11.5). The distribution of genotypes in the severe obese population was tested (Hardy–Weinberg equilibrium) using Pearson's chi square test.

Results

Table 1 shows the general and biochemical characteristics of our severely obese Caucasian patients. Mean levels of total cholesterol, triglycerides and glucose were below the upper reference limit for healthy subjects. No statistically significant difference, evaluated by Student's *t* test, was observed among females and males obese patients except for mean levels of total cholesterol ($P < 0.02$) and HDL cholesterol, SBP, DBP ($P < 0.001$). MtDNA 15497 G/A genotypes in obese subjects were not in Hardy–Weinberg equilibrium ($P = 0.001$). Fig. 1 shows the TaqMan allelic discrimination plot of the cytochrome *b* 15497 G/A mtDNA polymorphism in both severely obese patients and control subjects of Southern Italy. Samples are grouped in distinct clusters. An increase in the fluorescent signal of VIC or FAM indicates homozygous genotypes for the G and A allele, respectively, whereas an increase in both signals indicates heterozygous genotypes.

Table 1 General and biochemical characteristics (mean ± S.D.) of severely obese Caucasian subjects of Southern Italy ($n = 317$)

Variables	Female ($n = 191$)	Male ($n = 126$)
Age (years)	34.1 ± 12.7	32.7 ± 10.5
BMI (kg/m^2)	48.5 ± 7.2	48.9 ± 8.5
Glucose (mmol/L)	5.20 ± 1.2	5.52 ± 1.7
Total cholesterol (mmol/L)[a]	4.93 ± 1.0	4.66 ± 0.9
HDL cholesterol (mmol/L)[b]	1.24 ± 0.2	1.02 ± 0.2
Triglycerides (mmol/L)	1.42 ± 0.6	1.59 ± 0.9
SBP (mmHg)[b]	124.5 ± 15.9	132.3 ± 15.4
DBP (mmHg)[b]	79.6 ± 10.1	84.2 ± 10.3
HR (bpm)	79.1 ± 10.6	80.4 ± 11.3

[a] $P < 0.02$.
[b] $P < 0.001$ indicate statistically significant difference (evaluated by the Student's *t*-test) among males and females.

The screening of the mtDNA 15497 G/A polymorphism revealed that all the control subjects and 315/317 severely obese patients were homozygous for the G allele (wild type), whereas only 2/317 (0.6%) were homozygous for the A allele. The general characteristics of these subjects (case1: a female with BMI 54.5 kg/m^2, Glu 6.16 mmol/L, T Chol 5.12 mmol/L, HDL-Chol 1.29 mmol/L, Tg 1.98 mmol/L; case 2: a female with BMI 56.2 kg/m^2, Glu 5.38 mmol/L, T Chol 5.74 mmol/L, HDL-Chol 1.65 mmol/L, Tg 0.97 mmol/L) were similar to those of the overall group.

Discussion

Although many of the mtDNA sequence variants found in geographically separated populations are evolutionarily interesting, caution must be exercised in attributing a pathological role to ethnic-specific variants [10]. In fact, many mtDNA base substitutions do not affect known genetic functions, whereas others alter conserved amino acids and can thus lead to mild or severe organ failure or disease [10]. In particular, the mtDNA 15497 G/A polymorphism produces a Gly251Ser amino acid replacement in the cytochrome *b* protein [1]. Glycine 251 is a relatively well conserved residue in the ubiquinone binding site of the cytochrome *b* protein. Therefore, the replacement of Gly251 by other amino acids could alter ubiquinone-binding and cause dysfunction of cytochrome *b* as occurs for the Gly251Asp substitution associated with heart failure [1].

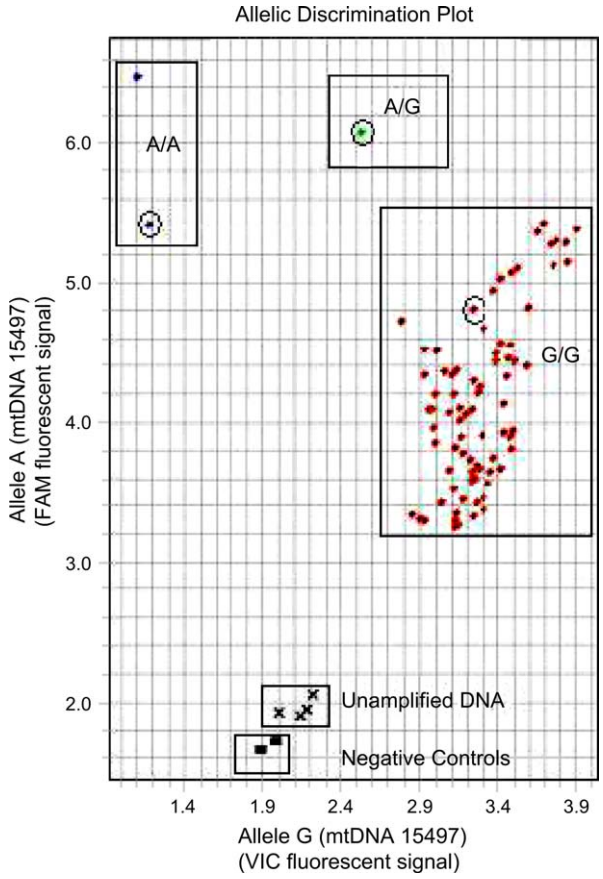

Figure 1 Allelic discrimination assay of mtDNA 15497 G/A polymorphism. Increases in the fluorescent signal VIC and/or FAM indicate the homozygous genotype for the G or A alleles, respectively, whereas an increase in both signals indicates heterozygous genotypes (A/G). Very low fluorescent signals (lower part of the figure) indicate negative controls (■) or unamplified samples (×), positive controls: G/G, A/G and A/A are circled.

In this study, which is the first investigation of the mtDNA 15497 G/A polymorphism in severely obese Caucasian subjects, we found the Gly251Ser replacement in 0.6% of cases. The two patients homozygous for the A allele, were females aged respectively 31 and 50 years, with obesity variables similar to those of the overall group of patients and triglyceride levels below the upper reference limit for healthy controls. This finding contrasts with data obtained in a Japanese population, where the A allele was detected in 3.5% of 1731 subjects (about 6 fold more than in our Italian Caucasian population) in association with obesity-related variables and increased triglyceride levels [5].

An explanation for this discrepancy could be that this polymorphism is associated with increased lipid levels rather than to obesity. In fact, in the Japanese population both cholesterol and triglyceride levels were higher than in our Caucasian obese subjects, whereas the mean BMI (<25 kg/m^2) was lower in the Japanese subjects than in our severely obese subjects (mean BMI $>$ 48 kg/m^2) with mean triglyceride levels in the reference range (<2.03 mmol/L).

Mitochondrial DNA genes are involved in energy production, through ATP synthesized via the oxidative phosphorylation system, both for work performance and to maintain body temperature, and differences in nucleotides may reflect adaptation to different climates [6]. Thus, the 15497 G/A SNP, representative of haplogroup G3, is present in people from central Asia and the Arctic as an advantageous adaptation to the cold climate, whereas the same SNP in westernized Japan could predispose to more fat accumulation and obesity [11]. Other haplogroups are present in European Caucasians, such as our Mediterranean population [12], where natural ancestral selection of different mtDNA variations probably occurred in relation to the temperate climate. We plan to investigate such haplogroups to look for specific associations with severe obesity. In conclusion, the 15497 G/A mutation appear to contribute only in rare cases to severe obesity but does not explain the majority of cases in our population.

Acknowledgment

Work supported by grants from CEINGE-Regione Campania (Convenzione — Del. G.R. 27/12/2002 N. 6276) and MIUR (PRIN 2002). We are grateful to Jean Gilder for editorial assistance and for revising the English text.

References

[1] Tanaka M, Fuku N, Takeyasu T, et al. Golden means to longevity: rareness of mitochondrial cytochrome *b* variants in centenarians but not in patients with Parkinson's disease. J Neurosci Res 2002;70:347—55.
[2] Autere J, Moilanen JS, Finnilä S, et al. Mitochondrial DNA polymorphisms as risk factors for Parkinson's disease and Parkinson's disease dementia. Hum Genet 2004;115:29—35.
[3] Anderson S, Bankier AT, Barrell BG, et al. Sequence and organization of the human mitochondrial genome. Nature 1981;290:457—65.
[4] Fuku N, Oshida Y, Takeyasu T, et al. Mitochondrial ATPase subunit 6 and cytochrome b gene polymorphisms in young adults. Biochem Biophys Res 2002;290:1199—205.
[5] Okura T, Koda M, Ando F, Niino N, Tanaka M, Shimokata H. Association of the mitochondrial DNA 15497 G/A polymorphism with obesity in a middle-aged and elderly Japanese population. Hum Genet 2003;113:432—6.
[6] Mishmar D, Ruiz-Pesini E, Golik P, et al. Natural selection shaped regional mtDNA variation in humans. Proc Natl Acad Sci USA 2003;100:171—6.

[7] Livak KJ. Allelic discrimination using fluorogenic probes and the 5′ nuclease assay. Genet Anal 1999;14:143—9.
[8] Kutyavin IV, Afonina IA, Mills A, et al. 3′-minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. Nucleic Acids Res 2000;28: 655—61.
[9] Afonina I, Zivarts M, Kutyavin I, Lukhtanov E, Gamper H, Meyer RB. Efficient priming of PCR with short oligonucleotides conjugated to a minor groove binder. Nucleic Acids Res 1997;25:2657—60.
[10] Wallace CW. Mitochondrial DNA sequence variation in human evolution and disease. Proc Natl Acad Sci USA 1994; 91:8739—46.
[11] Tanaka M, Takeyasu T, Fuku N, Guo LJ, Kurata M. Mitochondrial genome single nucleotide polymorphisms and their phenotypes in the Japanese. Ann N Y Acad Sci 2004;1011:7—20.
[12] Torroni A, Huoponen K, Francalacci P, et al. Classification of European mtDNAs from an analysis of three European populations. Genetics 1996;144:1835—50.