Rapid communications

Mutational analysis of the coding region of the uncoupling protein 2 gene in obese NIDDM patients: Impact of a common amino acid polymorphism on juvenile and maturity onset forms of obesity and insulin resistance

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Summary Recently, a gene encoding a novel human uncoupling protein, designated UCP2, was discovered. The murine UCP2 was mapped to a region on mouse chromosome 7 which in several models has been shown to be linked to obesity and hyperinsulinaemia. Single strand conformation polymorphism (SSCP) analysis and direct sequencing of the coding region of the UCP2 gene in 35 obese Caucasian NIDDM patients of Danish ancestry revealed one nucleotide substitution, replacing an alanine with a valine at codon 55. The amino acid polymorphism was present in 24 of the 35 (69%) examined subjects. The allelic frequency of the A/V55 variant was 48.3% (95% CI: 42.5–54.1%) among 144 subjects with juvenile onset obesity, 45.6% (40.5–50.7%) among 182 subjects randomly selected at the draft board examination, and 45.5% (37.1–53.9%) among lean control subjects selected from the same study cohort. Within these cohorts there were no differences in BMI

values at different ages among wild-type carriers and A/V55 carriers. In a population-based sample of 369 young healthy Caucasians the variant showed no association with alterations in BMI, waist-to-hip ratio, fat mass or weight gain during childhood or adolescence. The A/V55 polymorphism was not related to alterations in fasting values of serum insulin and C-peptide or to an impaired insulin sensitivity index. We conclude that genetic variability in the human UCP2 gene is not a common factor contributing to NIDDM in obese Danish Caucasian subjects and the common A/V55 amino acid polymorphism of the gene is not implicated in the pathogenesis of juvenile or maturity onset obesity or insulin resistance in Caucasians. [Diabetologia (1997) 40: 1227–1230]

Keywords Uncoupling protein 2, obesity, genetics, insulin resistance, amino acid polymorphism.

Obesity, which is a major risk factor for non-insulin-dependent diabetes mellitus (NIDDM) is, with rare exceptions, a multifactorial disorder with both environmental and genetic influences. Twin studies [1] as well as studies of adopted children [2] show that

most familial aggregation of obesity is due to genetic influence rather than to shared family environment. Recently, a gene encoding a novel human uncoupling protein, designated UCP2 was discovered [3]. Interestingly, the murine UCP2 gene was localized to a region on the distal mouse chromosome 7, which in several independent models has been shown to be linked to quantitative traits of obesity [4, 5]. Furthermore, quantitative trait loci analysis in mice has shown significant positive correlations between markers at this locus on chromosome 7 and hyperglycaemia as well as hyperinsulinaemia [6]. It was also demonstrated that UCP2 affects the mitochondrial activity and is capable, to a certain extent, of uncoupling the

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Abbreviations: UCP2, Uncoupling protein 2; NIDDM, non-insulin-dependent diabetes mellitus; SSCP, single strand conformation polymorphism.

respiration [3]. Unlike UCP1 which is activated by cold and $\beta 3$ agonists, UCP2 is induced by diet indicating that UCP2 may influence thermogenesis as well as energy expenditure [3]. Moreover, in contrast to UCP1, which is characterized by a unique expression in brown adipose tissue, UCP2 has a wide tissue distribution. UCP2 is expressed in skeletal muscle, heart, liver, kidney and the immune system [3]. Although the biochemical and physiological functions of UCP2 are still poorly understood, the gene is an attractive candidate for obesity and insulin resistance. Thus, the major objectives of the present study were: 1) to examine for genetic variability in the coding region of the UCP2 gene in obese NIDDM patients; 2) to evaluate whether variants of the gene were associated with juvenile onset or maturity onset obesity; and 3) to evaluate whether genetic variability of UCP2 was related to an impaired insulin sensitivity index or alterations in fasting levels of plasma glucose, serum insulin or serum C-peptide in young healthy subjects.

Subjects and methods

Subjects. In the primary mutation screening, single strand conformation polymorphism (SSCP) and heteroduplex analysis was performed on cDNA from 35 (20 men/15 women) obese Danish Caucasian NIDDM patients with an age of 50.5 (13.3)(median (interquartile range)) years. BMI was 32.4 (7.0) kg/m², HbA_{1c} 8.0 (3.2)% and duration of known diabetes 3.0 (3.2) years.

The subsequent association studies were performed in study groups selected from a population of young Caucasian men of Danish ancestry, who at the age of 18–26 years were examined at the draft board, and who, in addition, had attended school in the municipality of Copenhagen, where height and weight had been measured as part of the school health examinations [7]. The population was further restricted to those who were examined at the Copenhagen City Heart Study Program in 1981–1983 [8] and again in 1992–1994. The cohort of men with juvenile onset obesity included 156 subjects who had a BMI 31.0 kg/m² or more at the draft board examination. As a control cohort 205 draftees were selected at random as every hundredth from the same population. Weight and height at different ages were recorded. From this control cohort, 79 subjects who at the draft board examination and at the examination in 1992–1994 had a BMI less than 25 kg/m² were selected to comprise a lean control group. Genomic DNA was obtained from blood samples, drawn at the last examination at the Copenhagen City Heart Study.

Genotype-phenotype interaction studies were performed in 380 subjects randomly recruited from a population of young healthy individuals aged 18–32 years, who in 1979–1980 and again in 1984–1985 as children had participated in an epidemiological blood pressure survey in a specified part of Copenhagen [9]. All were Danish Caucasians by self-identification. The present examination took place in 1992–1993 where blood samples for DNA isolation were also drawn. Data on birth weight were obtained from the midwife records. Physiological and anthropometric characteristics of this population sample have been presented previously [9].

Prior to participation informed consent was obtained from all subjects. The study was approved by the ethical committee of Copenhagen and was in accordance with the principles of the Declaration of Helsinki II.

Anthropometric, biochemical and physiological studies in the cohort of 380 young healthy subjects. Waist-to-hip ratio, fat mass, fasting serum concentrations of triglyceride, total cholesterol and high density lipoprotein (HDL)-cholesterol and fasting plasma glucose were analysed as previously described [9]. The concentration of specific insulin (excluding des(31,32)- and intact proinsulin) in serum was measured by ELISA and the concentration of serum C-peptide was determined by radioimmunoassay RIA using Steno Diabetes Center routine intravenous methods. Insulin sensitivity index was estimated from an intravenous glucose tolerance test in combination with intravenous injection of tolbutamide as previously described [9].

Preparation of total RNA from human skeletal muscle in obese NIDDM patients and cDNA synthesis. A percutaneous biopsy (~0.5 g) of the vastus lateralis muscle was taken under local anaesthesia and total RNA was isolated. cDNA synthesis was performed on 1.0 µg of total muscle RNA in a volume of 25 µl as described previously [10].

Identification of mutations in cDNA of the UCP2 gene. SSCP-heteroduplex analysis was performed on cDNA generated from skeletal muscle. One primary PCR amplification of the total cDNA sequence (Genbank accession number: u82819) was carried out using 5 µl cDNA, 2 mmol/l MgCl₂ and 0.626 u of Amplitaq Gold DNA Polymerase (Perkin Elmer, Foster City, Calif., USA) and 0.2 µmol/l of primers designed from the non-coding regions: 5'-tgtaaacgacggccagtgttctccatctctgggacg-3' (forward) and 5'-caggaaacagctatgacccaaagccagaggtgatcagg-3' (reverse). A secondary PCR was carried out with 0.5 µl of the primary PCR as template and including 0.125 µl of a 37-MBq/ml solution (α -³²P)dCTP (Amersham, Buckinghamshire, UK). Three segments were amplified using the following primer pairs, annealing temperature and MgCl₂ concentrations: Segment 1: forward primer as above and reverse: 5'-caggaaacagctatgacctgtgcatgctcagagccc-3' (60°C/1.5 mmol/l); segment 2: 5'-tgtaaacgacggccagtctctgtccgcatcggtc-3' (forward) and 5'-caggaaacagctatgaccggtcatctgtcatgaggtg-3' (reverse)(65°C/1.5 mmol/l), segment 3: 5'-tgtaaacgacggccagtgtgtgacatgatcctac-3' (forward) and reverse primer as above (65°C/2 mmol/l). Cycles (denaturation at 94°C for 9 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing for 30 s and extension at 72°C for 1 min, with a final extension at 72°C for 9 min) were performed on a GeneAmp 9600 Thermocycler (Perkin-Elmer/Cetus, Norwalk, Conn., USA). PCR products were mixed with loading buffer, denatured and allowed to partially re-anneal in order to generate heteroduplexes and were finally analysed by non-denaturing gel electrophoresis under two different conditions as previously described [10]. Segments showing variation in migration were re-amplified by PCR, purified using Microcon 100 microconcentrators and sequenced directly using ABI PRISM Dye Primer Cycle Sequencing Kit with Amplitaq DNA Polymerase FS and ABI prism 377 (Perkin Elmer, Foster City, Calif., USA).

Screening for the A/V55 amino acid polymorphism in the UCP2 gene. Genomic DNA was obtained from human leukocyte nuclei using standard methods. The polymorphism was detected by restriction fragment length polymorphism-generating PCR. The genomic fragment containing codon 55 of the UCP2 gene was PCR amplified using primers: 5'-gggcccagtcgcgctacag-3' and 5'-catttggcgtcaggccgg-3' designed from the cDNA sequence. The PCR product was digested with 1 u of *Bbv*I for 1 h. Fragments were resolved on a 3% agarose gel and visualized by staining with ethidium bromide.

Table 1. Body mass index (BMI) at different ages according to genotype of the codon 55 polymorphism of the UCP2 gene in 144 (of 156) males with juvenile onset obesity selected at the

draft board examination and in 182 (of 205) male subjects randomly recruited at the draft board examination

	Genotype				
	AA-55	AV-55	<i>p</i> value	VV-55	<i>p</i> value
<i>Obese subjects</i>					
<i>n</i>	41	67		36	
BMI (age 20 years) (kg/m ²)	33.0 (3.4)	32.1 (2.0)	0.04	32.9 (2.3)	0.61
BMI (age 48 years) (kg/m ²)	33.6 (8.9)	36.4 (6.4)	0.07	34.2 (4.5)	0.98
ΔBMI (kg/m ²)	0.2 (7.7)	2.8 (8.1)	0.53	0.6 (4.4)	0.70
<i>Control subjects</i>					
<i>n</i>	56	86		40	
BMI (age 20 years) (kg/m ²)	21.1 (2.6)	21.3 (2.9)	0.91	21.8 (2.8)	0.31
BMI (age 48 years) (kg/m ²)	27.0 (4.8)	25.3 (5.2)	0.59	26.4 (4.1)	0.94
ΔBMI (kg/m ²)	5.3 (4.3)	4.0 (3.6)	0.41	4.3 (3.2)	0.46

Values are medians (interquartile range). The *p* value compares subjects heterozygous (AV-55) or homozygous (VV-55) for the A/V55 polymorphism of the UCP2 gene with AA homozygotes

Statistical analysis. Chi-square analysis was applied to test for significance of differences in allele frequencies. Differences in continuous variables between groups of subjects were tested with Student's *t*-test when the distribution of the variable or the logarithmically transformed variable approached a normal distribution and when the variances of the variables were equal in the groups compared. Otherwise the Mann-Whitney rank sum test was used. Data are expressed as medians (interquartile ranges). A *p*-value less than 0.05 (two-tailed) was considered significant. Statistical Package of Social Science (SPSS) for Windows, version 7.0 was used for statistical analysis.

Results

SSCP-heteroduplex scanning of the coding region of the UCP2 gene in 35 obese subjects with NIDDM revealed only one nucleotide substitution which was located at codon 55, replacing an alanine (gcc) with a valine (gtc) (A/V55). The amino acid polymorphism was present in 24 of the 35 (69%) examined subjects. No silent variants were identified. The allelic frequency of this amino acid polymorphism among 144 (of 156) subjects with juvenile onset obesity was 48.3% (95% confidence interval: 42.5–54.1%) compared to 45.6% (40.5–50.7%) (NS) in the cohort of 182 (of 205) subjects randomly selected at the draft board examination. The allelic frequency was 45.5% (37.1–53.9%) among 67 (of 79) lean subjects (BMI at the draft board examination and BMI at the age of 48 years less than 25 kg/m²) selected from the cohort of 205 draftees (NS). In the cohort of 369 (of 380) young healthy subjects the allelic frequency of the A/V55 polymorphism was 39.6% (36.1–43.1%). All the observed genotype frequencies were in Hardy-Weinberg equilibrium. Among the 182 draftees, BMI at the draft board examination and at the last examination at age 48 years did not differ between carriers of the A/V55 polymorphism and wild-type carriers (Table 1). Within the cohort of subjects with juvenile onset obesity heterozygous carriers of the A/V55 variant had a lower BMI compared to AA homozygotes (Table 1). However,

after Bonferroni correction for multiple testing the statistical significance disappeared. In the cohort of 369 young healthy subjects the A/V55 polymorphism showed no association with alterations in anthropometric or biochemical variables related to obesity or weight gain during childhood and adolescence (Table 2). Likewise, there were no significant differences between carriers of the variant and wild-type carriers with regard to fasting values of plasma glucose, serum insulin or C-peptide or to the insulin sensitivity index (Table 2).

Discussion

The present study, which to our knowledge is the first reported mutational analysis of the coding sequence of the human UCP2 gene, revealed one sequence variant resulting in an amino acid replacement, A/V55, with a prevalence which was not significantly different between males with juvenile onset obesity, randomly selected control males and lean control males from the same population. In addition, the variant allele was not associated with obesity related estimates like increased values of fat mass, waist-to-hip ratio or BMI or with hyperglycaemia, hyperinsulinaemia or impaired insulin sensitivity.

The tertiary structure of the UCP2 protein has not been defined and the biological functions are not known in detail. It is therefore difficult to conceive which protein changes may cause defects in the function of UCP2. Human UCP1 and UCP2 have 59% amino acid homology and it is known that UCP1 has three mitochondrial carrier protein motifs and a nucleotide binding site [3]. When extrapolated from the topology of UCP1, the A/V55 variant of UCP2 is not predicted to be located within any of these functional domains. Moreover, the Ala→Val change is a conservative amino acid substitution and therefore this amino acid replacement is not expected to cause major changes in the tertiary structure of the protein.

Table 2. Clinical and biochemical data of 369 (of 380) young healthy Caucasians when classified in accordance to their genotype of the A/V55 polymorphism of the UCP2 gene

	Genotype		<i>p</i> value	VV-55	<i>p</i> value
	AA-55	AV-55			
<i>n</i>	136	174		59	
Sex (men/women)	74/62	77/97		32/27	
Age (years)	25.0 (5.0)	26.0 (5.0)	0.34	26.0 (7.0)	0.73
BMI (kg/m ²)	22.4 (5.1)	23.4 (4.7)	0.17	23.2 (4.7)	0.31
Waist-to hip ratio	0.82 (0.09)	0.82 (0.12)	0.82	0.81 (0.11)	0.67
Fat mass (kg)	14.8 (10.1)	16.9 (10.3)	0.07	16.0 (10.1)	0.49
Weight gain (birth–1979) (kg)	44.3 (21.6)	43.4 (21.9)	0.99	46.3 (27.7)	0.88
Weight gain (1979–1992) (kg)	23.2 (21.4)	24.8 (22.0)	0.91	26.5 (26.3)	0.21
F-s-cholesterol (mmol/l)	4.3 (1.2)	4.4 (1.1)	0.55	4.4 (1.2)	0.63
F-s-HDL-cholesterol (mmol/l)	1.13 (0.95)	1.15 (0.39)	0.82	1.11 (0.37)	0.53
F-s-triglyceride (mmol/l)	0.82 (0.50)	0.92 (0.63)	0.23	0.96 (0.73)	0.06
F-p-glucose (mmol/l)	5.0 (0.6)	4.9 (0.7)	0.08	5.0 (0.7)	0.47
F-s-insulin (pmol/l)	30 (19)	30 (24)	0.39	31 (23)	0.43
F-s-C-peptide (pmol/l)	436 (169)	455 (184)	0.15	430 (498)	0.52
Si (10 ⁻⁵ × (min × pmol/l) ⁻¹)	13.4 (11.3)	11.9 (10.4)	0.07	13.9 (12.2)	0.74

Values are medians (interquartile range). s, Serum; F, fasting; p, plasma. The *p* value compares subjects heterozygous (AV-55) or homozygous (VV-55) for the codon 55 polymorphism of the UCP2 gene with AA homozygotes

Furthermore, as the present results do not show any relationships between the examined phenotypes and the A/V55 variant, it is unlikely that this polymorphism has a profound influence on the function of the protein.

In the initial mutational screening we studied obese patients with NIDDM. The study population was carefully selected from a NIDDM population of which cDNA from skeletal muscle was available, in order to identify genetic variants implicated in the *common forms* of obesity and NIDDM. Hence, the present investigation indicates that genetic variability of the UCP2 gene is not a common factor contributing to the genetic susceptibility of maturity onset obesity or common NIDDM. However, as we did not have the possibility to perform mutational analysis of the UCP2 gene in the cohort of males with juvenile onset obesity, we cannot from the present study exclude the existence of genetic variants in UCP2 contributing to this specific form of obesity, in which a highly penetrant genotype might be expected. Neither can we exclude the UCP2 gene as implicated in other subtypes of obesity or involved in the multifactorial pathogenesis of NIDDM in subsets of patients, especially patients with severe insulin resistant disorders.

In summary, genetic variability of the human UCP2 gene is not a common factor contributing to NIDDM in obese patients of Danish ancestry and the common A/V55 amino acid polymorphism of the gene is not implicated in the pathogenesis of juvenile or maturity onset obesity or insulin resistance in Caucasians.

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