No relationship between identified variants in the uncoupling protein 2 gene and energy expenditure

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Abstract

Objective: The uncoupling protein 2 (UCP2) uncouples respiration from the oxidative phosphorylation in most cell types, predominantly in white fat and skeletal muscle. Since a decreased basal metabolic rate (BMR) would increase the susceptibility to weight gain, genetic alterations in the *UCP2* gene could contribute to the pathogenesis of obesity and the metabolic syndrome (MSDR).

Design and methods: To test this hypothesis, we PCR amplified the introns of the *UCP2* gene and sequenced the exon/intron boundaries. This information was used to construct intronic primers and to screen obese patients with low BMR for mutations in the coding regions of the *UCP2* gene, using the single-strand conformational polymorphism technique. Furthermore, we examined whether there is an association between a biallelic marker in the *UCP2* gene and BMR or MSDR.

Results: The *UCP2* gene is composed of six coding exons, covering 5 kb of chromosome 11q13. One polymorphism, but no mutations, were identified in the coding regions of the *UCP2* gene. There were no significant differences in the allele or genotype frequencies of the Ala55Val polymorphism between 55 patients with MSDR and 46 healthy controls. No association was found between the *UCP2* gene and BMR in patients with MSDR or in healthy controls.

Conclusions: Mutation screening and association studies suggest that mutations in the coding regions of the *UCP2* gene do not affect BMR and do not contribute to increased susceptibility to obesity or MSDR. The results cannot, however, exclude the possibility that variants in regulatory elements of the gene could contribute to the development of obesity or MSDR.

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Introduction

The uncoupling proteins (UCP) represent a family of proteins that uncouple the respiration from the oxidative phosphorylation in the inner mitochondrial membrane (1). For a long time, UCP1 (2, 3) has been a marker for brown fat, a relatively abundant tissue in hibernating animals and newborns but less abundant in adults. The discovery of UCP2 and UCP3 isoforms in white fat and skeletal muscle (4–7) has relaunched the *UCP* genes as candidate genes for obesity, particularly as white fat and skeletal muscle can be expected to contribute more to variations in energy expenditure than the scarce brown fat (8–12).

UCP2 is expressed in most tissues, with the highest mRNA concentrations found in white fat, skeletal muscle and cells of the immune system (4). It is known to be upregulated by leptin (13, 14), high-fat feeding (4) and the peroxisome proliferator-activated receptor- γ agonist troglitazone (15). Recent results suggest that intracellular metabolism of free fatty acids may regulate the expression of UCP2 in adipose tissue (16). The *UCP2* gene is located on chromosome 11q13

(4), a locus that has recently been linked to resting metabolic rate in the Quebec Family Study (17).

Abdominal obesity and dyslipidaemia with high very-low-density lipoprotein-triglyceride and low high-density lipoprotein (HDL)-cholesterol levels are strongly associated with non-insulin dependent diabetes mellitus (NIDDM) (18–25). This particular clinical picture is often referred to as the metabolic syndrome (MSDR; insulin resistance syndrome/syndrome X), which predisposes to the development of atherosclerosis and macrovascular disease (26) and is considered to have a strong genetic background. MSDR clusters in families and represents a genetic adaptation towards an energy-saving phenotype with survival advantage during periods of famine (27). Therefore, genetic alterations in genes influencing energy expenditure seem likely to be involved in the polygenic background of MSDR.

To study whether alterations in the *UCP2* gene contribute to the development of obesity or MSDR, we determined the exon/intron boundary sequences of the *UCP2* gene and screened the coding regions and the exon/intron junctions for mutations in obese subjects with low basal metabolic rate (BMR). Using an amino

Table 1 Clinical characteristics of subjects participating in the association study. Data are means $\pm$ s.d.

	MSDR	Controls
Number	55	46
Sex (F/M)	28/27	21/25
Age (years)	61 $\pm$ 13	64 $\pm$ 11
BMI (kg/m ²)	31.6 $\pm$ 4.6 [‡]	23.3 $\pm$ 2.7
WHR; men	0.99 $\pm$ 0.06 [§]	0.91 $\pm$ 0.05
WHR; women	0.91 $\pm$ 0.08 [†]	0.79 $\pm$ 0.06
Fatmass (%); men	29.6 $\pm$ 8.9 [§]	18.9 $\pm$ 5.2
Fatmass (%); women	37.1 $\pm$ 5.7 [§]	29.1 $\pm$ 4.0
Triglycerides (mmol/l)	2.6 $\pm$ 1.8 [†]	1.2 $\pm$ 0.5
Cholesterol (mmol/l)	5.9 $\pm$ 1.2	5.8 $\pm$ 1.0
HDL cholesterol (mmol/l); men	0.89 $\pm$ 0.2 [*]	1.2 $\pm$ 0.3
HDL cholesterol (mmol/l); women	1.0 $\pm$ 0.3 [§]	1.6 $\pm$ 0.5
Systolic blood pressure (mmHg)	148 $\pm$ 20 [*]	137 $\pm$ 22
Diastolic blood pressure (mmHg)	82 $\pm$ 8.6 [*]	78 $\pm$ 9.7
Fasting blood glucose (mmol/l)	7.5 $\pm$ 2.5 [†]	4.9 $\pm$ 0.5
HbA1c (%)	6.6 $\pm$ 1.7 [†]	4.9 $\pm$ 0.4
Fasting serum insulin (nmol/l)	13.0 $\pm$ 8.1 [†]	8.1 $\pm$ 11
BMR/LBM (kcal/kg LBM)	27.4 $\pm$ 3.0	–

* $P < 0.05$, § $P < 0.0005$, † $P < 1 \times 10^{-5}$ and ‡ $P < 1 \times 10^{-14}$ vs control subjects.

acid polymorphism identified in the second coding exon of the *UCP2* gene, we examined whether this variant is associated with MSDR or decreased BMR.

Materials and methods

Subjects and study design

MSDR was defined in accordance with the recent proposition adopted by the World Health Organization (unpublished, 1997) suggesting that patients fulfilling at least three of the following criteria have MSDR: abdominal obesity, glucose intolerance, elevated blood pressure, elevated plasma triglyceride concentrations, decreased plasma HDL-cholesterol concentrations, microalbuminuria and insulin resistance. In this study, each patient defined as MSDR fulfilled at least three of the following criteria: waist-to-hip ratio (WHR) >0.95 for men and >0.85 for women, systolic/diastolic blood pressure $>160/90$ mmHg, plasma triglycerides >1.7 mmol/l, plasma HDL-cholesterol <1.0 mmol/l for men and <1.1 mmol/l for women, albumin excretion

rate >20 μ g/min and impaired glucose tolerance or NIDDM (28).

Mutation screening Thirty (16 males, 14 females) unrelated Swedish obese patients with low BMR were selected (age 58.2 ± 13 years, body mass index (BMI) 31.1 ± 3.6 kg/m², BMR 25.9 ± 1.8 kcal/kg lean body mass (LBM)). BMR was in the lowest quartile of a control population and BMI was >27 kg/m² for these patients. Twenty-five of them fulfilled the criteria for MSDR as given above.

Association study Fifty-five unrelated patients with MSDR (including the 25 from the mutation screening) and 46 unrelated healthy controls without signs of MSDR were selected from southern Sweden. Clinical characteristics of both study groups are shown in Table 1. Measurements of BMR were available from patients (range 21.1–39.6 kcal/kg LBM) but not from controls.

Assessment of the effect of the Ala55Val genotype on BMR

Fifty-one (26 males, 25 females) unrelated healthy controls with normal glucose tolerance and without known family history of diabetes, were selected from the Botnia region in western Finland. The clinical characteristics of these healthy subjects were: age 45 ± 15 years, BMI 24.0 ± 2.6 kg/m², WHR 0.88 ± 0.06 , fatmass $18.8 \pm 6.5\%$ and BMR 30.7 ± 4.9 kcal/kg LBM (range 24.4–47.5 kcal/kg LBM). Of note, the MSDR patients included in the association study (see above) have significantly lower BMR than these controls (27.4 kcal/kg LBM vs 30.7 kcal/kg LBM, $P < 0.0001$).

Determination of exon/intron boundaries with PCR and direct sequencing

Using information about the exon/intron boundaries of the human *UCP1* gene (2, 3), introns were positioned in the human *UCP2* gene while assuming conserved exon/intron structure between these genes. Exonic primers were constructed on both sides of each intron, and the introns were amplified by PCR (Table 2). The PCR reactions were performed with initial denaturation (96 °C for 1 min) followed by 30 cycles of denaturation (94 °C for 20 s), annealing (62 °C or 64 °C for 30 s; Table

Table 2 Sequences of exonic primers and PCR conditions for amplification of the introns of the *UCP2* gene. All the primer sequences are given in 5' to 3' direction. The number in parenthesis after each primer sequence indicates the exon from which it was derived.

Forward primer sequence	Reverse primer sequence	Intron amplified (product size, bp)	Annealing temperature (°C)
GTTCAGGCCACAGATGTGCC (1)	ACCATGGTCAGAATGGTGCCC (2)	1 (357)	64
TGGTTGCCGGCCTGCAGCG (2)	TCCTCTCGGGCAATGGTCTTG (3)	2 (~1100)	64
CATGCCAGCATTGGGAGCCG (3)	AGGAGGGCATCCTTGATGAGG (4)	3 (359)	64
TGTTGCTCGTAATGCCATTGTC (4)	TCATGTATCTCGTCTTGACCAC (5)	4 (~1200)	62
AGACGAGATACATGAACCTCTGC (5)	TCAGAAGGGAGCCTCTCGGG (6)	5 (584)	62

2) and extension (72 °C for 1 min), followed by final extension (72 °C for 15 min). The reactions were performed in a total volume of 20 µl with Tris buffer for Taq polymerase (10 mmol/l Tris-HCl, pH 8.3; 50 mmol/l KCl; 0.01% w/v gelatin) and 1.5% formamide; 0.13 mmol/l dNTP; 1.5 mmol/l MgCl₂; 0.2 µmol/l of both primers and 0.5 U Taq polymerase (Perkin Elmer, Foster City, CA, USA) using 25 ng genomic DNA as a template. PCR products were sequenced using the ABI PRISM dye terminator cycle sequencing ready reaction kit (Perkin Elmer) and analysed on an automated sequencer (ABI, model 373, Perkin Elmer). Introns 1, 3 and 5 were sequenced bidirectionally in their entirety, whereas only the exon/intron border sequences were sequenced for introns 2 and 4. Exon/intron borders were positioned by applying the GT/AG rule (29).

Determination of the region upstream of the first coding exon by inverse PCR and direct sequencing

Five micrograms genomic DNA isolated from a 50-year-old healthy Caucasian male were cut with 25 U *Nco*I (New England Biolabs, Herts, UK) for 4 h in a total volume of 30 µl using conditions recommended by the manufacturer. *Nco*I was then inactivated at 65 °C for 20 min, and 6 µl of the digest were ligated in a total volume of 230 µl with 50 U T4 DNA ligase (5 U/µl, Appligene, Gaithersburg, MD, USA) at 16 °C for 24 h. In this procedure, the genomic DNA was cut at nucleotide 77 in the second coding exon and at an unknown position upstream of the coding region. Thus, a 2.3 kb long circular fragment containing approximately 2 kb upstream of the first coding exon was generated. Two rounds of PCR were then performed using nested primers; PCR1 (annealing temperature 62 °C, 35 cycles) using the forward primer for fragment 2A (Table 3) and 5'-TGATGAGATCTGCGATGCAGG-3' as the reverse primer and 2.0 µl ligation mixture as a

template; PCR2 (annealing temperature 56 °C, 25 cycles) using 5'-TGGGAGTCTTGATGGTGTG-3' as the forward and 5'-AACTTCACAGTGGCAGTAGG-3' as the reverse primer and 0.5 µl amplification product from PCR1 as a template. PCR reactions were performed as for the exon/intron boundary PCR, but using NH₄⁺ buffer (16 mmol/l (NH₄)₂SO₄; 67 mmol/l Tris, pH 8.8; 0.01% Tween 20) instead of the Tris-HCl and 5% glycerol instead of 1.5% formamide. When sequencing the PCR product from PCR2, the sequence of 295 bp of the region upstream of the first coding exon was acquired.

Single-strand conformational polymorphism (SSCP)

For the SSCP analysis (30), the six coding exons of the UCP2 gene were amplified with intronic primers (Table 3). For exons 1 and 2, overlapping sets of two (exon 1) or three (exon 2) primer pairs were used. A minimum distance of 25 bp between the intronic primer and exon border was used for all fragments. PCR was performed as for the exon/intron boundary PCR, with the following changes: 0.5 µCi [α -³²P]dCTP was added to each reaction; initial denaturation was set at 3 min, cycle denaturation at 30 s, cycle extension at 30 s and final extension at 10 min. See Table 3 for variable annealing temperatures and buffers. The reactions were stopped with 95% formamide buffer (1:1), denatured, cooled and electrophoresed on glycerol-free (35 W for 3.5 h at 4 °C) and 5% glycerol (8 W for 12 h at room temperature), non-denaturing 5% polyacrylamide gels (acrylamide/bisacrylamide 49:1). When differences in band pattern were observed, PCR products were sequenced bidirectionally.

Genotyping the Ala55Val polymorphism in the UCP2 gene

One polymorphism was identified in codon 55 of the

Table 3 Primer sequences and variable conditions for PCR-SSCP analysis of the UCP2 gene. All the primer sequences are given in 5' to 3' direction. The primers for fragments 1A and 1B amplify two overlapping fragments of exon 1. The primers for fragments 2A–2C amplify three overlapping fragments of exon 2. The reverse primer for amplification of exon 6 is located at the end of the coding sequence.

Forward primer sequence	Reverse primer sequence	Exon amplified (product size, bp)	Annealing temperature (°C)	Buffer*
TGAGGGACTTGTTCTATTAGG	AACTTCACAGTGGCAGTAGG	1A (248)	58	TB
GTTCAAGGCCACAGATGTGCC	AGCTACAGGGATAAGCATGTTGC	1B (254)	64	TB
TACTGCTAAAGTCCGGTTACAG	TGGTTGCCGGCCTGCAGCG	2A (247)	62	NB
TGTCTTGGCCTTGACAGATCC	TTGGTGTAGAAGTGTTCAG	2B (215)	58	NB
TCCTCTCGGGCAATGGTCTTG	AGGTGCTTAGGAAGTATGTGG	2C (256)	60	NB
AGGTAGAAAATGAGTGCAAGC	GGGTGAGACCAGAGTATCG	3 (308)	58	TB
CCTCTGGAAGGTGTGTACCA	CGCACCTGCTCCTGGCATG	4 (255)	62	NB
GAATGATGGGTGAAGATCTTGA	CATGCATAGCCAAGAGGCCTG	5 (277)	60	TB
GGAAGTGGGAGGTGGAGGTG	TCAGAAGGGAGCCTCTCGGG	6 (165)	64	TB

* TB, tris-HCl buffer for Taq polymerase; NB, ammonium sulphate buffer.

Figure 1 The exon/intron structure of the coding region of the human *UCP2* gene. Exons are numbered starting from the first coding exon and shown with boxes. Filled boxes represent translated sequences and open boxes untranslated sequences. Introns are illustrated with black lines. Vertical lines indicate the last nucleotide in each exon, with the nucleotide sequence numbered from the first nucleotide in the translation initiation codon. The translation initiation codon is indicated with an arrow. The first coding exon starts 99 bp upstream of the translation initiation codon, and exon 6 contains a 644 bp non-translated region (5).

UCP2 gene changing a GCC (alanine) to a GTC (valine). This polymorphism was genotyped by PCR amplification of the genomic DNA using the forward primer for fragment 2A together with a mismatch primer as the reverse primer (5'-CATCACACCGGTACTGGGCGT^TGTG-3', mismatch underlined) that created a *HincII* site in sequences coding for a valine, followed by digestion with the *HincII* enzyme using conditions recommended by the manufacturer (Appligene), and finally electrophoretic separation on an agarose gel. PCR was performed as for the exon/intron boundary PCR, with the annealing temperature set at 62 °C and the initial denaturation set at 3 min, cycle denaturation at 30 s, cycle extension at 30 s and final extension at 10 min.

Statistical analyses

Differences in allele and genotype frequencies between MSDR patients and control subjects were tested by χ^2 analysis, and differences in clinical characteristics by the Mann-Whitney non-parametric test using the BMDP New System for Windows (Biomedical Data Processing, Los Angeles, CA, USA). A *P* value of <0.05 was considered statistically significant.

GenBank accession numbers

The nucleotide sequences of the *UCP2* gene were submitted to the EMBL Nucleotide Sequence Database with accession numbers AJ223477–AJ223479.

Coding exon no.	5' splice donor	Intron size (bp)	3' splice acceptor	Coding exon no. (exon size/ bp)
			...cttggcttagATTCCGGCAG	1 (225)
1	CGG TTA CAGgtgaggggat.....156.....ggccttgacagATC		CAA GGA	2 (211)
	R L Q ⁴²		I ⁴³ Q G	
2	GGC TCT GAG Cgtgagtatgg....~1000....gccacagAT		GCC AGC ATT	3 (195)
	G S E ¹¹²		H ¹¹³ A S I	
3	CTC TGG AAA Ggtgtgtacca.....80.....cctcctacagGG		ACC TCT CCC	4 (102)
	L W K ¹⁷⁷		G ¹⁷⁸ T S P	
4	CTC ATG ACA Ggtgagtcatg...~1000...tccttggcagAT		GAC CTC CCT	5 (181)
	L M T ²¹¹		D ²¹² D L P	
5	TTC TAC AAA GGgtgagcctct.....369.....tctcctctagG		TTC ATG CCC	6 (759)
	F Y K ²⁷¹		G ²⁷² F M P	

Figure 2 Sequences at the exon/intron junctions of the coding region of the human *UCP2* gene. Exon sequences are given in uppercase letters, with encoded amino acids shown below the nucleotide sequence. Intron sequences are given in lowercase letters. Amino acids that precede, follow or are interrupted by introns are indicated with their codon number. Intron sizes were determined by sequencing (introns 1, 3 and 5) or by size estimation of the PCR product on an agarose gel (introns 2 and 4). When comparing the coding region of the *UCP2* gene with that of the human *UCP1* gene (3), the exon/intron structure was conserved except that exon 2 of the *UCP1* gene is four amino acids shorter and exon 3 is two amino acids longer than corresponding exons of the *UCP2* gene. Thereby, exon 2 of the human *UCP1* gene is interrupted by intron 2 at Thr¹⁰⁹ instead of at His¹¹³. The exon/intron structure of the coding region of the *UCP2* gene is conserved also with the human *UCP3* gene (31), except that exon 3 of the *UCP3* gene contains three additional amino acids and thus is interrupted by intron 3 at Gly¹⁸¹ instead of at Gly¹⁷⁸.

Table 4 Basal metabolic rate (kcal/kg LBM) in MSDR patients and healthy controls according to the Ala55Val genotype of the *UCP2* gene.

Genotype	MSDR (Sweden <i>n</i> = 55)	Controls (Finland <i>n</i> = 51)
Ala/Ala	27.6 ± 3.0	29.0 ± 3.1
Ala/Val	28.2 ± 2.6	30.7 ± 5.2
Val/Val	26.8 ± 2.2	30.6 ± 2.7

Results

The *UCP2* gene was found to be composed of six coding exons covering 5 kb of chromosome 11q13 (Fig. 1). All the exon/intron boundaries and exon sizes (coding exons) were similar to the structures of the human *UCP1* (3) and *UCP3* (31) genes (Fig. 2).

Mutation screening of the *UCP2* gene in 30 obese patients with low BMR revealed one polymorphism (Ala55Val) in codon 55 of the *UCP2* gene, changing an alanine (GCC) to a valine (GTC).

In healthy Finnish controls, the frequency of the alanine allele was 60.8% and that of the valine allele 39.2%. There were no significant differences in BMR between healthy control subjects or MSDR subjects with different Ala55Val genotypes (Table 4). The allele frequencies and genotype distributions of the Ala55Val polymorphism did not differ significantly between 55 subjects with MSDR and 46 healthy controls (Table 5). Genotype frequencies were in Hardy–Weinberg equilibrium in all groups studied.

Discussion

The exon/intron structure of the coding region of the human *UCP2* gene was similar to the structures of the human *UCP1* and *UCP3* genes. Information on the exon/intron boundaries of the six coding exons of the *UCP2* gene allowed us to screen coding exons and

flanking intronic regions for mutations in obese patients with low BMR.

Basal energy expenditure accounts for 60–70% of total energy expenditure (9), the rest being generated during exercise and food-induced thermogenesis. The variability of BMR is largely dependent on processes in non-adipose tissues (60–80%) (9). Nevertheless, white adipose tissue could contribute to the small decrease needed to gain weight over time, and low BMR is a risk factor for weight gain (32).

Mice deficient in brown adipose tissue become obese and hyperinsulinemic (33). Surprisingly, targeted disruption of the *UCP1* gene in mice does not result in obesity, a finding which led to the postulation of other *UCP* genes involved in this mechanism (34). No mutations that increase the susceptibility to obesity have been found in the coding region of the *UCP1* gene in obese humans (35). However, one polymorphism near the *UCP1* gene, at nucleotide position –3826, has been associated with increased weight gain during adult life (36), and the effect of this variant was enhanced when it occurred together with the Trp64Arg mutation in the β 3-adrenergic receptor gene (37).

In the present study, mutation screening of 30 obese patients with low BMR revealed one common variant in exon 2, Ala55Val, but there was no difference in allele frequency between patients with MSDR and healthy controls. This is in accordance with a recent Danish study, in which no association was found between the same polymorphism and juvenile obesity (38). However, this study did not include measures of energy expenditure, a quantitative trait which may be more relevant to the genotype.

In accordance with these findings, highly polymorphic markers flanking the *UCP2* and *UCP3* genes (4, 33) were not linked to BMI or WHR in a study of sibling pairs (39). In contrast to this, the highly polymorphic marker D11S611 was reported to be tightly linked to resting metabolic rate in the Quebec Family Study (17). Consequently, it seems that this chromosomal region links to resting metabolic rate but not to obesity. However, in the present study the Ala55Val polymorphism was associated neither with decreased BMR (in patients with MSDR or in healthy controls) nor with obesity in patients with MSDR. There are several potential explanations for the discrepancy of these findings. First, the Ala55Val variant might not be in linkage disequilibrium with the variants responsible for the linkage found in the Quebec Family Study. Thus, the *UCP2* gene may still be important for the variability of metabolic rate, although in such cases this does not seem to influence susceptibility to obesity. Secondly, regions important for gene regulation were not included in our screening. Thus alleles responsible for the linkage found in the Quebec Family Study could be present in the promoter region or other regulatory parts of the *UCP2* gene or in the very closely located *UCP3* gene. On the other hand, if the linkage in the Quebec Family

Table 5 Allele and genotype frequencies of the Ala55Val polymorphism in Swedish MSDR patients and healthy controls.

Allele or genotype	MSDR % (<i>n</i>)	Controls % (<i>n</i>)
Allele		
Ala	50.0 (55)	53.3 (48)
Val	50.0 (55)	46.7 (42)
Total	100 (110)	100 (90)
Genotype		
Ala/Ala	29.0 (16)	26.1 (12)
Ala/Val	41.8 (23)	52.2 (25)
Val/Val	29.0 (16)	19.6 (9)
Total	100 (55)	100 (46)

Neither allele nor genotype frequencies differ between MSDR patients and controls.

Study was explained by a variant in the promoter of the *UCP2* gene, one would expect to find decreased expression of *UCP2* in obese individuals or in individuals with reduced BMR. However, in a recent study of *UCP2* mRNA levels in adipose tissue and skeletal muscle, no difference was observed between lean and obese subjects and the level of expression did not correlate with BMR (16).

In conclusion, mutation screening and association studies suggest that mutations in the coding regions of the *UCP2* gene do not affect BMR and do not contribute to increased susceptibility to obesity or MSDR. The results cannot, however, exclude the possibility that variants in regulatory elements of the gene could contribute to the development of obesity or MSDR.

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