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-gamma 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

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Table 1 Clinical characteristics of subjects participating in the association study. Data are mean ± S.D.

<table>
<thead>
<tr>
<th></th>
<th>MSDR</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>55</td>
<td>46</td>
</tr>
<tr>
<td>Sex (F/M)</td>
<td>28/27</td>
<td>21/25</td>
</tr>
<tr>
<td>Age (years)</td>
<td>61 ± 13</td>
<td>64 ± 11</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>31.6 ± 4.6³</td>
<td>23.3 ± 2.7</td>
</tr>
<tr>
<td>WHR; men</td>
<td>0.99 ± 0.06³</td>
<td>0.91 ± 0.05</td>
</tr>
<tr>
<td>WHR; women</td>
<td>0.91 ± 0.08³</td>
<td>0.79 ± 0.06</td>
</tr>
<tr>
<td>Fatmass (%) men</td>
<td>29.6 ± 8.9³</td>
<td>18.9 ± 5.2</td>
</tr>
<tr>
<td>Fatmass (%) women</td>
<td>37.1 ± 5.7³</td>
<td>29.1 ± 4.0</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>2.6 ± 1.8²</td>
<td>1.2 ± 0.5</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>5.9 ± 1.2</td>
<td>5.8 ± 1.0</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l); men</td>
<td>0.89 ± 0.2²</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l); women</td>
<td>1.0 ± 0.3²</td>
<td>1.6 ± 0.5</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>148 ± 20²</td>
<td>137 ± 22</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>82 ± 6.6²</td>
<td>78 ± 9.7</td>
</tr>
<tr>
<td>Fasting blood glucose (mmol/l)</td>
<td>7.5 ± 2.5²</td>
<td>4.9 ± 0.5</td>
</tr>
<tr>
<td>HbAlc (%)</td>
<td>6.6 ± 1.7²</td>
<td>4.9 ± 0.4</td>
</tr>
<tr>
<td>Fasting serum insulin (nmol/l)</td>
<td>13.0 ± 8.1²</td>
<td>8.1 ± 11</td>
</tr>
<tr>
<td>BMR/LBM (kcal/kg LBM)</td>
<td>27.4 ± 3.0 –</td>
<td>–</td>
</tr>
</tbody>
</table>

* P < 0.05, † P < 0.0005, ‡ P < 1 × 10⁻⁵ and § P < 1 × 10⁻¹⁴ vs control subjects.

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.

<table>
<thead>
<tr>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
<th>Intron amplified (product size, bp)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTTGGCCCGCCGCTCGAGCGCG</td>
<td>ACCATGTCAGAATTGTTGCC (1)</td>
<td>1 (357)</td>
<td>64</td>
</tr>
<tr>
<td>TGGTGGCGCCGCTGGGCGGCGG</td>
<td>TCTCTCGGGAATGTCGCTCTG (2)</td>
<td>2 (~1100)</td>
<td>64</td>
</tr>
<tr>
<td>CAGGCAGACTGTAATGCCCAC</td>
<td>TAGGGGGATCTTGGTGGACC (3)</td>
<td>3 (359)</td>
<td>64</td>
</tr>
<tr>
<td>TGGTGGCTGTCATGACCAGTC</td>
<td>TCAATGACCTGTTGACCACAC (5)</td>
<td>4 (~1200)</td>
<td>62</td>
</tr>
<tr>
<td>AGACCGAGATGCGAGCTCAG</td>
<td>TCAGGAAGGACGCTGCTGGG (6)</td>
<td>5 (584)</td>
<td>62</td>
</tr>
</tbody>
</table>
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 NcoI (New England Biolabs, Herts, UK) for 4 h in a total volume of 30 μl using conditions recommended by the manufacturer. NcoI 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 50U 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 1A (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'-TGGGAGTCTTGATGTTGTC-3' as the forward and 5'-AACCTCACAGTTGCGATAGG-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 NH42SO4; 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 (acylamide/bisacylamide 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

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**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.

<table>
<thead>
<tr>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
<th>Exon amplified (product size, bp)</th>
<th>Annealing temperature (°C)</th>
<th>Buffer*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGAGGGACCTGGTTACTATTAGG</td>
<td>AACTTCACAGTGCGATAGG</td>
<td>1A (248)</td>
<td>58</td>
<td>TB</td>
</tr>
<tr>
<td>GTCAAGGCCCCACAGATTGCC</td>
<td>AGGTCACAGGTAAAGCATGTGCC</td>
<td>1B (254)</td>
<td>64</td>
<td>TB</td>
</tr>
<tr>
<td>TACTGCTAAGTTCGGTACAG</td>
<td>TTGGTTGGGCTTGCAAGG</td>
<td>2A (247)</td>
<td>62</td>
<td>NB</td>
</tr>
<tr>
<td>TGTTGGGCTTGGCAATCC</td>
<td>TGGTGTGAACATGGTTCAGG</td>
<td>2B (215)</td>
<td>58</td>
<td>NB</td>
</tr>
<tr>
<td>TCCTGGGGCGATGTCTTG</td>
<td>AGGTGCTTAGAACATGTGGG</td>
<td>2C (256)</td>
<td>60</td>
<td>NB</td>
</tr>
<tr>
<td>AGGTAGAATAAGTAGTGCAAGC</td>
<td>GGGTGAAGACAGAGATAGTCG</td>
<td>3 (308)</td>
<td>58</td>
<td>TB</td>
</tr>
<tr>
<td>CCGTGGGAAGGTGTTGACA</td>
<td>CGGGTCGGCTGGCAAGAG</td>
<td>4 (255)</td>
<td>62</td>
<td>NB</td>
</tr>
<tr>
<td>GAATGATGGTGTAAGATCTTGA</td>
<td>CATGATAGGCGAAGGCGCTG</td>
<td>5 (277)</td>
<td>60</td>
<td>TB</td>
</tr>
<tr>
<td>GGAAAGTTGGAGTGAGGAGTG</td>
<td>TCGAGAAGAGAGGCCTGCGG</td>
<td>6 (165)</td>
<td>64</td>
<td>TB</td>
</tr>
</tbody>
</table>

* TB, tris–HCl buffer for Taq polymerase; NB, ammonium sulphate buffer.
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'-CATCACACCGGGTGACTGGGCG'TTG-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 $\chi^2$ 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.

**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 98 bp upstream of the translation initiation codon, and exon 6 contains a 644 bp non-translated region (5).

**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. Therefore, 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.
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 β3-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
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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