Molecular Screening of Uncoupling Protein 2 Gene in Patients with Noninsulin-Dependent Diabetes Mellitus or Obesity*

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ABSTRACT

Uncoupling protein 2 (UCP2), a member of the family of mitochondrial carrier proteins, has been implicated in the control of whole-body energy balance. The coding region of the human UCP2 gene has now been shown to comprise six exons, and the sequences of the exon-intron boundaries were determined. With the use of this sequence information, 25 Japanese patients with obesity and noninsulin-dependent diabetes mellitus (NIDDM) and 25 subjects with simple obesity were screened for mutations in the entire coding region of UCP2 by PCR and single-strand conformation polymorphism analysis. Two nucleotide polymorphisms resulting in Ala55→Val and Ala232→Thr substitutions were detected. With the use of PCR and restriction fragment length polymorphism analysis, the allele frequencies for each of these polymorphisms were determined in 210 Japanese patients with NIDDM, 42 obese individuals, and 218 normal control subjects. The frequency of the Val55 allele did not differ significantly among the NIDDM group (46.0%), the obesity group (48.8%), and the normal control group (48.4%). The Thr232 allele was detected in only three subjects, who were heterozygotes and in the NIDDM group (allele frequency, 0.7%). However, expression in yeast of the human wild-type UCP2 protein and UCP2 containing Thr232 revealed no difference in functional activity. These results indicate that the Ala55→Val and Ala232→Thr variants of UCP2 do not play an important role in the pathogenesis of NIDDM or obesity in the Japanese population.

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UCP2 locus on mouse chromosome 7 and human chromosome 11 is linked to obesity and hyperinsulinemia (13). Because mice lacking UCP1 are cold sensitive but not obese, UCP1 likely mediates cold exposure-induced thermogenesis for the purpose of maintaining body temperature (14). In contrast, the wide tissue distribution of UCP2 and the observation that the extent of UCP2 expression in white adipocytes is increased by feeding a high-fat diet (13) indicate that UCP2 may play an important role in determining the basal metabolic rate and, possibly, in resisting the development of obesity. Thus, genetic alterations in the UCP2 gene might contribute to the pathogenesis of obesity and NIDDM.

We have now determined the exon-intron boundaries of human UCP2 and, with the use of PCR and single-strand conformation polymorphism (SSCP) analysis as well as PCR and restriction fragment length polymorphism (RFLP) analysis, screened UCP2 for mutations in subjects with NIDDM or obesity.

Subjects and Methods

Subjects

We first screened 25 (6 male, 19 female) Japanese individuals with obesity and NIDDM [body mass index (BMI), >28.0 kg/m² (mean ± sd, 31.6 ± 3.7 kg/m²); age, 58.6 ± 16.7 yr] and 25 (3 male, 22 female) obese subjects without glucose intolerance [BMI, >30 kg/m² (33.5 ± 3.9 kg/m²); age, 48.2 ± 20.8 yr] for mutations in UCP2 by PCR-SSCP analysis. Next, we investigated 210 (93 male, 117 female) Japanese individuals with NIDDM (BMI, 24.5 ± 4.6 kg/m²; age, 59.8 ± 13.1 yr), 42 (12 male,
Determining the exon-intron boundaries of UCP2

A human full-length UCP2 complementary DNA (cDNA) was obtained by PCR with a human muscle cDNA library as template and two oligonucleotide primers (sense, 5'-CCCTCTATCTGGTCTTTGCTG-3'; antisense, 5'-GGACGGGAAGAGAAGAGAAAG-3') complementary to nucleotide sequences surrounding the start and stop codons of human UCP2 cDNA (13, 15). PCR was performed under standard conditions, and the resulting product of the predicted size was subcloned into the pBluescript II KS(+) (Stratagene, La Jolla, CA) for sequencing.

PCR-SSCP analysis

PCR-SSCP analysis was performed as described previously (16, 17). Briefly, specific oligonucleotide primers for amplifying the coding regions of UCP2, including the exon-intron boundaries, were synthesized and labeled with [γ-32P]ATP (ICN, Irvine, CA) and then used as a probe to screen approximately 106 plaques of a human genomic DNA library in EMBL3 SP6/T7 (Clontech, Palo Alto, CA). Genomic DNA was extracted from each subject as template. The resulting products were subjected to autoradiography.

The sequence of UCP2 open reading frame was shown to consist of six exons, and the framing of UCP2 was confirmed to be identical to the previously described human UCP2 cDNA (13, 15). This PCR product was labeled internally with [α-32P]dCTP triphosphate (ICN, Irvine, CA) and then used as a probe to screen approximately 106 plaques of a human genomic DNA library in EMBL3 SP6/T7 (Clontech, Palo Alto, CA). Genomic DNA was extracted from each subject and digested with restriction endonucleases XhoI and EcoRI. The coding regions of UCP2 were identified by Southern blot analysis with the same probe as that used for screening, and positive fragments were subcloned into pBluescript II KS(+) (Stratagene, La Jolla, CA) for sequencing.

Results

With 32P-labeled human UCP2 cDNA as a probe, we screened approximately 106 clones of a human genomic DNA library and obtained one positive clone that encompassed the entire coding region of UCP2. The open reading frame of UCP2 was shown to consist of six exons, and the sequence of each exon-intron boundary was determined (Table 1). All the splice acceptor and donor sequences conformed to the GT-AG consensus rule for splicing.

The coding sequence of wild-type human UCP2 was isolated from muscle total RNA by RT-PCR and introduced into the vector pYeDP1/8–10, described previously (18). The Ala232 → Thr substitution was introduced into this coding sequence [the codon GCC (Ala) was replaced by ACC (Thr)] by site-directed mutagenesis as described previously (19). The sequence of the UCP2 open reading frame was determined to check for unwanted mutations. The Saccharomyces cerevisiae diploid strain W303 (MATa/a; ade2-10; his3-11,15; leu2-3,112; ura3-1; can1-100; trp1-d1) was transfected with UCP2 expression vectors encoding the wild-type or variant protein or with the empty pYeDP1/8–10 vector (control). The expression of pYeDP-based plasmids in yeast is under the control of the gal-cycl promoter, which is induced by galactose and repressed by glucose (20).

Living transfected yeast cells were labeled with 100 nM DiOC6 (3, 3'-dihexyloxacarbocyanine iodide) (3), a lipophilic cationic cyanine dye that accumulates in mitochondria in a membrane potential-dependent manner (21). Flow cytometric analysis of the labeled cells was performed as described previously (22) with an EPICS ESP instrument (Coulter Electronics), equipped with an argon laser and a standard 76-mm nozzle, in association with a confocal optical system to improve light-scatter resolution.

Construction of expression vectors and flow cytometry

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On the basis of the determined sequences of the exon-intron boundaries and the available sequences of the 5' and 3' untranslated regions of UCP2 cDNA (GenBank accession number, U82819), we designed and synthesized specific oligonucleotide primers for PCR-SSCP analysis (Table 2). The entire protein-coding region, including exon-intron boundaries, was amplified with these primer sets. Each PCR prod...

<table>
<thead>
<tr>
<th>Donor</th>
<th>Acceptor</th>
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<tbody>
<tr>
<td>Exon 1</td>
<td>TTA · CAG gtagggg</td>
</tr>
<tr>
<td>(126 bp)</td>
<td>L</td>
</tr>
<tr>
<td>Exon 2</td>
<td>TCT · GAG · C gtagtag</td>
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<tr>
<td>(211 bp)</td>
<td>S</td>
</tr>
<tr>
<td>Exon 3</td>
<td>TGG · AAA · G gttgtac</td>
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<tr>
<td>(195 bp)</td>
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</tr>
<tr>
<td>Exon 4</td>
<td>ATG · ACA · G gttgatca</td>
</tr>
<tr>
<td>(102 bp)</td>
<td>M</td>
</tr>
<tr>
<td>Exon 5</td>
<td>AAA · GG gttgacct</td>
</tr>
<tr>
<td>(181 bp)</td>
<td>K</td>
</tr>
<tr>
<td>Exon 6</td>
<td>(116 bp)</td>
</tr>
</tbody>
</table>

Sizes of exons 1 and 6 are from transcription start site to last nucleotide of exon 1 and from first nucleotide of exon 6 to stop codon, respectively. Uppercase and lowercase nucleotide sequences indicate exons and introns, respectively. Encoded amino acids are shown in single-letter code below exon sequences.
uct was subjected to dideoxynucleotide sequencing, and its sequence confirmed identical to that of UCP2.

We screened 25 patients with obesity and NIDDM and 25 subjects with simple obesity by PCR-SSCP, and the PCR products that showed abnormal electrophoretic mobility were subjected to direct sequence analysis. We detected two nucleotide substitutions: a C → T transition at nucleotide 164 in exon 2 and a G → A transition at nucleotide 694 in exon 5, which resulted in Ala55 → Val and Ala232 → Thr amino acid substitutions, respectively.

To determine the allele frequencies for each amino acid polymorphism, we investigated 210 patients with NIDDM, 42 obese individuals, and 218 normal control subjects by PCR-RFLP analysis. Specific PCR primers that contained mismatched bases to create restriction endonuclease sites were synthesized. The resulting PCR products corresponding to the Val55 and Thr232 alleles were susceptible to digestion with HincII and Fba I, respectively (Fig. 1).

The frequency of the Val55 variant was 46.0% (60 wild types, 107 heterozygotes, and 43 homozygotes out of 210 subjects) in the NIDDM group, 48.8% (15 wild types, 97 heterozygotes, and 107 homozygotes out of 210 subjects) in the NIDDM group, 48.8% (15 wild types, 97 heterozygotes, and 107 homozygotes out of 210 subjects) in the normal control group, and thus it did not differ significantly among the three groups (P > 0.05, χ-square analysis). Among the NIDDM subjects for whom clinical data were available, no significant associations of genotype with fasting plasma glucose or immunoreactive insulin concentrations, serum concentrations of total cholesterol or triglyceride, BMI, current treatment, or the presence of at least one diabetic complication were apparent (data not shown). Similarly, among the obesity and normal control groups, no significant association of genotype with fasting plasma glucose concentration or BMI was detected (data not shown). Therefore, the Ala55 → Val variant appears to be a common polymorphism and likely does not play an important role in the pathogenesis of NIDDM or obesity.

We detected only three subjects with the Ala232 → Thr variant, all of whom were heterozygotes and in the NIDDM group. These three individuals did not share clinical features associated with a tendency to obesity, hyperinsulinemia, or a positive family history of NIDDM or obesity in common (Table 3). We then compared the activity of the UCP2 protein containing the Ala232 → Thr substitution with that of the wild-type protein with the use of site-directed mutagenesis, expression in yeast cells, and flow cytometry with the membrane potential-sensitive probe DiOC6 (3) (Fig. 2). In these experiments, the two Gaussian curves obtained with the control strain (no expression of foreign protein) incubated in the absence or presence of a chemical uncoupler were used as references. Cells expressing the Ala232 → Thr variant of human UCP2 yielded a broad histogram located between the two reference curves (Fig. 2A), indicating that expression of the human protein reduced the membrane potential of the yeast mitochondria, consistent with its predicted function. The curve obtained with the wild-type protein was virtually identical to that obtained with the Ala232 → Thr variant (Fig. 2B), indicating that there is essentially no difference in the basal uncoupling activities of the two proteins. Therefore,
this polymorphism likely does not influence the function of UCP2 or contribute to the pathogenesis of NIDDM or obesity.

Discussion

With PCR-SSCP analysis, we detected two nucleotide polymorphisms in UCP2 that result in the amino acid substitutions Ala55 → Val and Ala232 → Thr. The Ala55 → Val variant was recently described by Urhammer et al. (23) and shown not to be implicated in the pathogenesis of juvenile or maturity-onset obesity or insulin resistance in a Danish population. Our data also indicate that this polymorphism does not contribute to the pathogenesis of NIDDM or obesity in Japanese. The frequency of the Val55 allele in our Japanese population was similar to that for the Danish population, indicating that the Ala55 → Val substitution represents a common polymorphism in various ethnic groups.

The Ala232 → Thr variant of human UCP2 has not been previously described and was detected only in the NIDDM group. Analysis of the pedigree of one affected proband revealed that this polymorphism does not cosegregate with the onset of NIDDM or obesity (data not shown). Furthermore, the activity in yeast of the human UCP2 protein containing Thr232 was similar to that of the wild-type protein. Although Ala232 of UCP2 is predicted to be located in the fifth transmembrane α helix, near the mitochondrial energy-transfer-protein signature domain (15), this residue is not conserved in human UCP1; the corresponding amino acid in UCP1 is a serine at position 230, based on amino acid sequence alignment of the two proteins (15). Therefore, the Ala232 → Thr polymorphism of UCP2 likely does not affect the function of the protein or play an important role in the pathogenesis of NIDDM or obesity.

Although the two UCP2 variants described in the present study do not appear to represent disease-causing mutations, it is possible that other polymorphisms that affect UCP2 function remain undetected. Because we initially screened only 50 subjects by PCR-SSCP, analysis of larger numbers of individuals is now warranted.

Recently, UCP3 was identified as a candidate mediator of adaptive thermogenesis in humans (15, 24). UCP3 is expressed preferentially and abundantly in human skeletal muscle, an important site of nonshivering thermogenesis in humans. Therefore, UCP3 may be a candidate gene for obesity and NIDDM. Moreover, because genetic alterations in the 5′ flanking regions of the UCP genes may impair gene transcription, the mechanisms of transcriptional regulation of these genes should be further investigated, and important promoter and enhancer sites should be screened for mutations.

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References


