Screening for Variants of the Uncoupling Protein 2 Gene in Japanese Patients With Non–Insulin-Dependent Diabetes Mellitus

Tomoko Shiinoki, Tadashi Suehiro, Yukio Ikeda, Mari Inoue, Toshihiro Nakamura, Yoshitaka Kumon, Yuh Nakauchi, and Kozo Hashimoto

We examined genetic mutations in the coding regions of the uncoupling protein 2 (UCP2) gene in 100 patients with non–insulin-dependent diabetes mellitus (NIDDM). The sequences of each exon-intron boundary were detected by polymerase chain reaction (PCR) using specific primer pairs designed in the cDNA sequence of UCP2 and a cycle-sequence method. Using the specific primer pairs in the intron 5′- or 3′-untranslated region, each exon with its exon-intron boundaries was amplified with the PCR method, and the PCR products were analyzed using a single-strand conformation polymorphism (SSCP) method. One nucleotide substitution in exon 4 was found, which exchanged Ala (gcc) at position 55 of the amino acid sequence for Val (gtc), previously reported in Denmark by Urhammer et al in 1997. The polymorphism was reanalyzed in all patients and 120 normal subjects using a PCR–restriction fragment length polymorphism method. There was no difference in the genotype distribution between patients and normal subjects, and our genotype distribution was similar to the Danish study. Furthermore, there were no clinical differences between genotype groups among the patients. No other mutation including the exon-intron boundary was found in these patients. Genetic mutations of UCP2 may not be commonly associated with obesity or diabetes in Japanese subjects.

UNCOUPLING PROTEIN 1 (UCP1) is located across the inner mitochondrial membrane in brown adipose tissue (BAT)[1] and produces heat by uncoupling-respiration from adenosine triphosphate synthesis.[2] BAT containing UCP1 has an important function in nonshivering thermogenesis in rodents.[3] UCP1 likely does not play a central role in nonshivering thermogenesis in adult humans, because BAT decreases after the newborn stage and is only detected in a limited area of the back.[4] Recently, two novel members of the UCP family have been cloned, UCP2 and UCP3.[5,6] UCP2 is widely expressed in human and rodent tissues including white adipose tissue (WAT), and therefore has been implicated in energy balance in adult humans. On the other hand, UCP3 is highly expressed in skeletal muscle and is a candidate protein for the modulation of respiratory control in skeletal muscle.

Leptin is an adipocyte hormone that regulates body fat and energy metabolism. This hormone induces not only UCP1 mRNA in BAT but also UCP2 mRNA in subcutaneous fat tissue. Leptin may be associated with energy balance through UCP2.[7,8] Thiazolidinediones, antidiabetic agents that enhance the sensitivity of cells to insulin, stimulate UCP2 expression in the pancreatic islets of animals or in cell lines representing WAT and BAT.[9,10] Thiazolidinediones may increase energy expenditure in humans through the expression of UCP2, which is a pharmacological mechanism of these antidiabetic agents. Therefore, the level of UCP2 gene expression is likely involved in the development of diabetes in humans.

These findings suggest that genetic variability of UCP2 is involved in the development of obesity or non–insulin-dependent diabetes mellitus (NIDDM). Urhammer et al[11] reported one polymorphism of the UCP2 gene: Ala at position 55 of the amino acid sequence was substituted with Val (*A/V55*). However, this polymorphism was not implicated in the pathogenesis of juvenile- or maturity-onset obesity or insulin resistance. They identified the mutation in the cDNA of the UCP2 gene from skeletal muscle. Most recently, Otabe et al[12] reported five new UCP2 polymorphisms and a lack of association between these polymorphisms and obesity in French caucasians. One was a common insertion/deletion (Ins/Del) polymorphism in the untranslated region of exon 8.

In this study, we examined genetic mutations in the coding regions of the UCP2 gene including every exon-intron boundary in genomic DNA from patients with NIDDM, and found no common genetic variability of UCP2 involved in the development of NIDDM in Japanese subjects, although polymorphisms of *A/V55* and Ins/Del were detected in the patients and normal subjects.

SUBJECTS AND METHODS

Subjects

The subjects were 100 patients with NIDDM (48 men and 52 women; age, 57 ± 9 years [mean ± SD]; range, 33 to 69) from our outpatient clinic in 1997. NIDDM was diagnosed according to World Health Organization criteria.[13] None of the patients demonstrated ketoacidosis, renal failure (serum creatinine > 2.5 mg/dL), liver disorder, or a recent history of cardiovascular disease. Normal subjects (71 men and 49 women; age, 56 ± 8 years; range, 30 to 68) were recruited from a medical examination center in Kochi.

Methods

Genomic DNA was extracted from whole blood using a commercial kit (SMI test; Sumitomo, Tokyo, Japan). Introns were amplified with the polymerase chain reaction (PCR) method using primer pairs within the sequence of UCP2 cDNA (data for primers not shown), and the sequence of each exon-intron boundary was detected by a cycle-sequence method (DNA Sequencing Kit, BigDye Terminator Cycle Sequencing Ready Reaction; Applied Biosystems, Foster City, CA). We did not examine exons 1 or 2 because they are untranslated exons.[12] Each exon with its exon-intron boundaries was produced by PCR using specific primer pairs (Table 1). Each primer sequence was made in the intron except the sense primer of exon 3 and the antisense primer of

From the Second Department of Internal Medicine, Kochi Medical School, Kochi, Japan.

Submitted June 8, 1998; accepted November 23, 1998.

Address reprint requests to Tomoko Shiinoki, MD, Second Department of Internal Medicine, Kochi Medical School, Kohasu, Okoh-cho, Nankoku, Kochi 783-8505, Japan.

0026-0495/99/4805-0008$10.00/0

Table 1. Sequence of Each Primer, Annealing-Extension Temperature for PCR, and Sequence of Each Exon-Intron Boundary

Primer	Oligo Sequence	Annealing Temperature	Exon-Intron Boundary*
Exon 3			
Sense	5′-CTGGGACGTAGCAGGAAATC-3′	58°C	
Antisense	5′-GAGGGAACAGAGTAGACACC-3′		. . . GGTTACAG · gtgaggggat . . .
Exon 4			
Sense	5′-GTGGGAAGGGCAACATGCTT-3′	64°C	. . . ggccttgcag · ATCCAAGG . . .
Antisense	5′-GCCTACACCCTTGCTCCATA-3′		. . . CTCTGAGC · gtgagtatgg . . .
Exon 5			
Sense	5′-GGTGGTTTTGGTGAGGAGAG-3′	62°C	. . . tcgcccacag · ATGCCAGC . . .
Antisense	5′-TGGGTGAGACCAGAGTATCG-3′		. . . CTGGAAAG · gtgtgtaca . . .
Exon 6			
Sense	5′-TGGTCTCACCCAGGATCTTC-3′	64°C	. . . cctcctacag · GGACCTCT . . .
Antisense	5′-ACCCAGCACCGTCTACCTCA-3′		. . . CATGACAG · gtgagtcatg . . .
Exon 7			
Sense	5′-CCTGCCTTCTGGAATGTTGG-3′	66°C	. . . tccttggcag · ATGACCTC . . .
Antisense	5′-TAGCCAAGAGGCCTGAACTG-3′		. . . TACAAAGG · gtgagcctct . . .
Exon 6			
Sense	5′-TGAATACCAGGCCCAGTGAG-3′	62°C	. . . tctcctctag · GTTCATGC . . .
Antisense	5′-AAGCCAGAGGTGATCAGGTC-3′		
Screening for the *A/V55* amino acid polymorphism			
Sense†	5′-GGGCCAGTGCGACCTACAG-3′	66°C	
Antisense	5′-ATGCGGACAGAGGCAAAGC-3′		

*Capitals show sequence in exon, and lowercase letters show sequence in intron.
†Underscore shows 2-mismatch sequence.

exon 8, which were designed from the cDNA sequence (Genbank Accession No. HSU82819).

PCRs were performed in 50 μL reaction mixture including genomic DNA and 0.5 U Gold Taq (Applied Biosystems). The mixture was subjected to one cycle at 95°C for 9 minutes followed by 40 cycles at 95°C for 45 seconds and each specific annealing-extension temperature for 45 seconds (two-step method).

Genetic mutations of UCP2 in all patients with NIDDM were examined by single-strand conformation polymorphism (SSCP). The PCR product of 8 μL with 12 μL denaturing buffer (96% formamide, 20 mmol/L EDTA · 2Na, 0.1% bromophenol blue, and 0.1% xylene cyanol FF) was heated at 80°C for 5 minutes and loaded onto 6% polyacrylamide gel including 5% glycerol at 4°C, 10°C, and 30°C in Tris-glycine buffer (pH 8.3). After electrophoresis, the gel was stained with ethidium bromide. The sequences of PCR fragments suspected as variants were determined by the cycle-sequence method already described.

The *A/V55* amino acid polymorphism in the UCP2 gene was reanalyzed using a PCR–restriction fragment length polymorphism method among all patients with NIDDM and 120 normal subjects. The PCR fragment was produced using specific primer pairs (Table 1) and 1 μg of the exon 4 product as a template. The sense primer had a two–base pair (bp) mismatched sequence. The PCR product (5 μL) was digested with 2 U *Ecl*HKI (Promega, Madison, WI) and loaded onto a 15% polyacrylamide gel. The fragments were visualized by staining with ethidium bromide.

The Ins/Del polymorphism of exon 8 was determined using primers described by Otabe et al.[12] The PCR was performed as described before, and the deletion (317 bp) and insertion (362 bp) alleles were detected on agarose gel. One sample failed to amplify.

Statistical Analysis

A comparison of variables among the groups was performed using one-way ANOVA. Genotype frequencies were estimated with the chi-square test. *P* values less than .05 were considered significant.

RESULTS

Each sequence of the exon-intron boundary is shown in Table 1.

We detected only one nucleotide substitution in exon 4, which replaced an Ala (gcc) at position 55 of the amino acid sequence with a Val (gtc). This same substitution was described previously.[11] Although we used three different SSCP conditions in 100 patients with NIDDM, other substitutions were not found in any exon.

Figure 1 shows the gel for PCR products digested by *Ecl*HKI for detection of the *A/V55* polymorphism. The distribution of *AA, AV,* and *VV* genotypes did not differ in patients with NIDDM and normal subjects (Table 2). There were no differences in the body mass index (BMI), diabetic control, or plasma lipid concentrations among diabetic groups classified by genotype.

The distribution of the Ins/Del polymorphism in the untranslated region of exon 8 did not differ between the patients and normal subjects (Table 3).

DISCUSSION

Obesity is an important factor for the development of NIDDM. Adipose cells in obesity express tumor necrosis factor

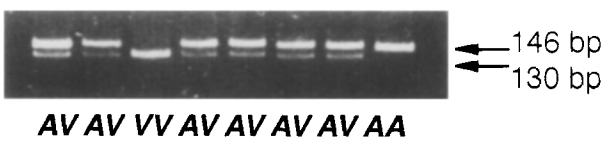

Fig 1. Polyacrylamide gel of PCR products including the area of *A/V55* and digested by *Ecl*HKI.

Table 2. Group Characteristics by Genotype for *A/V55* Polymorphism of the UCP2 Gene in 120 Normal Subjects and 100 Patients With NIDDM

Group	Genotype			P
	AA	*AV*	*VV*	
Normal subjects (n)	28	71	21	NS†
NIDDM patients (n)	30	53	17	
Insulin therapy (n)	7	19	14	NS‡
BMI (kg/m²)*	25.0 ± 6.8	24.1 ± 3.3	24.3 ± 3.5	NS§
HbA_{1c} (%)*	6.6 ± 1.6	7.0 ± 1.8	6.3 ± 0.9	NS§
Total cholesterol (mg/dL)*	197 ± 37	205 ± 45	198 ± 29	NS§
Triglycerides (mg/dL)*	131 ± 62	147 ± 93	128 ± 61	NS§
HDL cholesterol (mg/dL)*	49 ± 12	52 ± 16	48 ± 11	NS§

Abbreviations: HbA_{1c}, hemoglobin A_{1c}; HDL, high-density lipoprotein; NS, not significant.

*Mean ± SD.

†Normals *v* patients, chi-square test.

‡Insulin therapy and non–insulin therapy, chi-square test.

§ANOVA.

Table 3. Group Characteristics by Genotype for Ins/Del Polymorphism in the 3′ Untranslated Region of the UCP2 Gene in 120 Normal Subjects and 99 Patients With NIDDM

Group	Genotype			P
	DD	*DI*	*II*	
Normal subjects (n)	76	38	6	NS†
NIDDM patients (n)	66	30	3	
Insulin therapy (n)	23	6	1	NS‡
BMI (kg/m²)*	24.3 ± 5.2	24.4 ± 3.2	25.2 ± 5.4	NS§
HbA_{1c} (%)*	6.8 ± 1.6	6.9 ± 1.5	5.2 ± 0.4	NS§
Total cholesterol (mg/dL)*	199 ± 35	207 ± 49	188 ± 47	NS§
Triglycerides (mg/dL)*	140 ± 71	144 ± 98	85 ± 59	NS§
HDL cholesterol (mg/dL)*	50 ± 15	52 ± 12	46 ± 19	NS§

NOTE. See Table 2 for abbreviations.

*Mean ± SD.

†Normals *v* patients, chi-square test.

‡Insulin therapy and non–insulin therapy, chi-square test.

§ANOVA.

alpha1, which inhibits the sensitivity of peripheral cells to insulin,[14,15] and free fatty acids released from adipocytes may also be implicated in the insulin resistance of NIDDM patients.[16] Some individuals gain weight and become obese during adolescence or adulthood more easily than others. Although daily meals and exercise influence weight gain, some genetic factors are involved in the development of obesity. UCP2 is abundantly expressed in WAT and is associated with energy balance in humans,[5] and therefore, an abnormality of UCP2 may induce obesity resulting in NIDDM.

Urhammer et al[11] showed a polymorphism (*A/V55*) in the cDNA of UCP2 that may not be involved in obesity or diabetes. Otabe et al[12] reported six polymorphisms including *A/V55* in the genomic DNA of UCP2, three of which were in the 5′ untranslated exons 1 and 2. The Ins/Del in the 3′ untranslated region of exon 8 was a common polymorphism. We detected the sequences of the exon-intron boundary of UCP2 and searched for a genetic mutation of UCP2 in Japanese subjects with NIDDM. Our results show that the same polymorphism of *A/V55* was detected in our Japanese population with a similar distribution as shown by Urhammer et al. The Ins/Del polymorphism was also detected in our subjects, and the frequency of the allele was similar to that in French caucasians. The two common polymorphisms also showed no relationship to the development of NIDDM or clinical conditions of diabetes. In this study, other mutations were not found. These results suggest that genetic factors of UCP2 are not associated with obesity or the development of NIDDM, as Urhammer et al[11] also concluded.

We did not examine the untranslated exons 1 and 2 or the 5′ region upstream from the UCP2 gene. A mutation may exist in the promoter or a regulatory element in the 5′ upstream region. Although we used SSCP with three different conditions of electrophoretic temperature, this method does not always detect a change in the DNA sequence. Thus, some variants may have been missed.

UCP3 may be involved in obesity. Recently, it has been reported that UCP2 and UCP3 genes were located adjacent to one another on mouse chromosome 7,[17] which has been linked to diabetes and obesity in mice.[18] A variant of the UCP3 gene may exist in Japanese subjects, although Urhammer et al[19] showed that variations in the coding region of the human UCP3 gene were not involved in the pathogenesis of juvenile-onset obesity in Danish caucasian subjects.

In conclusion, our findings suggest that UCP2 gene mutations are rare, excluding *A/V55* and Ins/Del polymorphisms, and are not associated with obesity or NIDDM. The role of UCP2 in obesity and diabetes should be examined further, as well as the function of UCP2 in humans.

REFERENCES

1. Kozak LP, Britton JH, Kozak UC, et al: The mitochondrial uncoupling protein gene. Correlation of exon structure to transmembrane domains. J Biol Chem 263:12274-12277, 1988

2. Ricquier D, Casteilla L, Bouillaud F: Molecular studies of the uncoupling protein. FASEB J 5:2237-2242, 1991

3. Himms HJ: Brown adipose tissue thermogenesis: Interdisciplinary studies. FASEB J 4:2890-2898, 1990

4. Garruti G, Ricquier D: Analysis of uncoupling protein and its mRNA in adipose tissue deposits of adult humans. Int J Obes 16:383-390, 1992

5. Fleury C, Neverova M, Collins S, et al: Uncoupling protein-2—A novel gene linked to obesity and hyperinsulinemia. Nat Genet 15:269-272, 1997

6. Boss O, Samec S, Paoloniacobino A, et al: Uncoupling protein-3—A new member of the mitochondrial carrier family with tissue-specific expression. FEBS Lett 408:39-42, 1997

7. Scarpace PJ, Matheny M, Pollock BH, et al: Leptin increases uncoupling protein expression and energy expenditure. Am J Physiol 36:E226-E230, 1997

8. Zhou YT, Shimabukuro M, Koyama K, et al: Induction by leptin of uncoupling protein-2 and enzymes of fatty acid oxidation. Proc Natl Acad Sci USA 94:6386-6390, 1997

9. Shimabukuro M, Zhou YT, Lee Y, et al: Induction of uncoupling protein-2 mRNA by troglitazone in the pancreatic islets of Zucker diabetic fatty rats. Biochem Biophys Res Commun 237:359-361, 1997

10. Camirand A, Marie V, Rabelo R, et al: Thiazolidinediones

stimulate uncoupling protein-2 expression in cell lines representing white and brown adipose tissues and skeletal muscle. Endocrinology 139:428-431, 1998

11. Urhammer SA, Dalgaard LT, Sorensen T, et al: 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. Diabetologia 40:1227-1230, 1997

12. Otabe S, Clement K, Rich N: Mutation screening of the human UCP2 gene in normoglycemic and NIDDM morbidly obese patients. Diabetes 47:840-842, 1998

13. World Health Organization: Diabetes mellitus: Report of a WHO study group. World Health Organ Tech Rep Ser 727: 1-113, 1985

14. Hotamisligil GS, Spiegelman BM: Tumor necrosis factor alpha: A key component of the obesity-diabetes link. Diabetes 43:1271-1278, 1994

15. Hotamisligil GS, Budavari A, Murray D, et al: Reduced tyrosine kinase activity of the insulin receptor in obesity-diabetes. Central role of tumor necrosis factor-alpha. J Clin Invest 94:1543-1549, 1994

16. Marin P, Bjorntorp P: Endocrine-metabolic pattern and adipose tissue distribution. Horm Res 3:81-85, 1993

17. Surwit RS, Wang SY, Petro AE, et al: Diet-induced changes in uncoupling proteins in obesity-prone and obesity-resistant strains of mice. Proc Natl Acad Sci USA 95:4061-4065, 1998

18. Fleury C, Neverova M, Collins S, et al: Uncoupling protein-2: A novel gene linked to obesity and hyperinsulinemia. Nat Genet 15:269-272, 1997

19. Urhammer SA, Dalgaard LT, Sorensen T, et al: Organisation of the coding exons and mutational screening of the uncoupling protein 3 gene in subjects with juvenile-onset obesity. Diabetologia 41:241-244, 1998