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*Short communication*

# A polymorphism in the 5' untranslated region and a Met$^{229}$→ Leu variant in exon 5 of the human UCP1 gene are associated with susceptibility to Type II diabetes mellitus

**H. Mori, H. Okazawa, K. Iwamoto, E. Maeda, M. Hashiramoto, M. Kasuga**

[1] Second Department of Internal Medicine, Kobe University School of Medicine, Kobe, Japan

**Abstract**

*Aims/hypothesis.* The cumulative effects of several thrifty factors could contribute to the pathogenesis of Type II (non-insulin-dependent) diabetes mellitus. We screened the human UCP1 gene (*UCP1*) for polymorphisms associated with susceptibility to Type II diabetes.
*Methods.* By using PCR and single-strand conformation polymorphism analysis, *UCP1* were screened for mutations in 25 Type II diabetic subjects and 25 healthy control subjects. The allele frequencies of the detected polymorphisms were determined by PCR and restriction fragment length polymorphism analysis in 320 diabetic subjects and 250 control subjects.
*Results.* An A→C transition in the 5' untranslated region (UTR) of exon 1 (112 bp upstream of the translation initiation codon) and a Met$^{229}$→Leu variant were detected. The allele frequencies for the C variant and for the Leu$^{229}$ variant were higher in the Type II diabetic group than in the control group ($p = 0.017$ and $p = 0.038$, respectively). These polymorphisms were in linkage disequilibrium ($p < 0.00001$). Luciferase assay showed that the former variant in the 5' UTR may affect the promoter activity of *UCP1*.
*Conclusion/interpretation.* Both the A→C polymorphism and the Met$^{229}$→Leu polymorphism of *UCP1* are in linkage disequilibrium and could be one of the diabetes associated single nucleotide polymorphisms (SNPs). [Diabetologia (2001) 44: 373–376]

**Keywords** UCP1, polymorphism, Type II diabetes mellitus, thrifty factor, PCR-SSCP, PCR-RFLP, SNPs, linkage disequilibrium

Genetic factors play an important part in the development of Type II (non-insulin-dependent) diabetes mellitus. Some forms of Type II diabetes are caused by mutations in a single gene but are rare. Relatively common polymorphisms, Trp$^{64}$→Arg of the $\beta_3$-adrenergic receptor and Pro$^{12}$→Ala of PPAR-$\gamma$, have also been associated with Type II diabetes [1, 2]. These single nucleotide polymorphisms (SNPs) could be associated with energy metabolism as "thrifty" factors and the cumulative effects of such factors are possibly responsible for Type II diabetes.

Uncoupling protein 1 (UCP1), abundantly expressed in brown adipose tissue (BAT), regulates energy expenditure in rodents but its importance in adult humans is not known. In humans, BAT is active during the neonatal period but its relative volume decreases with age. As UCP2 is expressed in many tissues and UCP3 is present exclusively in skeletal muscle, major organs of whole-body thermogenesis in adult humans, UCP2 or UCP3 could play a more important part in energy expenditure than UCP1. In-

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*Corresponding author:* Dr. Hiroyuki Mori, Second Department of Internal Medicine, Kobe University School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650–0017, Japan
*Abbreviations:* UCP, Uncoupling protein; *UCP1*, the human UCP1 gene; UTR, untranslated region; SNPs, single nucleotide polymorphisms; PPAR, peroxisome proliferator activated receotor; PCR-SSCP, PCR and single-strand conformation polymorphism analysis; PCR-RFLP, PCR and restriction fragment length polymorphism analysis; BAT, brown adipose tissue; IRS, insulin response sequence.

deed, SNPs in UCP2 and UCP3 were shown to be associated with energy metabolism or obesity in ethnically limited populations [3].

Regulation of the human UCP1 gene (*UCP1*) expression is not clear. It has been shown, however, that UCP1 mRNA is detectable in isolated adult human adipocytes and exposure of cultured preadipocytes to thiazolidinedione resulted in an increase in the amount of UCP1 mRNA, indicating that the regulatory mechanism of the UCP1 expression is maintained and cells of the brown fat lineage are present even in adult humans [4]. Therefore, UCP1 could contribute to energy regulation and adaptive thermogenesis in humans.

Although previous studies detected some SNPs in *UCP1* [5, 6], no association between these polymorphisms and obesity have been reported. We have now screened *UCP1*, as one of the thrifty factors, for mutations associated with the susceptibility to Type II diabetes.

## Subjects and methods

*Subjects.* We first screened 25 (6 men, 19 women) Japanese subjects with Type II diabetes (BMI, 31.6 ± 3.7 kg/m$^2$; age, 58.6 ± 16.7 years) and 25 (3 men, 22 women) normal control subjects (BMI, 21.5 ± 3.9 kg/m$^2$; age 63.5 ± 8.1 years) for mutations in *UCP1* by PCR and single-strand conformation polymorphism analysis (PCR-SSCP). Next, we investigated 320 (180 men, 140 women) Type II diabetic subjects (BMI, 23.1 ± 3.5 kg/m$^2$; age, 62.9 ± 11.8 years) and 250 (145 men, 105 women) healthy control subjects (BMI, 20.9 ± 3.4 kg/m$^2$; age, 76.4 ± 7.9 years) by PCR and restriction fragment length polymorphism analysis (PCR-RFLP) to determine the allele frequencies for the detected SNPs. The diagnosis of diabetes was based on the World Health Organization criteria (1985). Criteria for the selection of normal control subjects included no past history of glucose intolerance, an $HbA_{1c}$ level of less than 5.6%, an age older than 60 years, and no family history of Type II diabetes. All study subjects were unrelated, and they gave their written consent after being informed of the nature of the study. The investigation was conducted in accordance with the guidelines of the Declaration of Helsinki.

*PCR-SSCP.* Specific oligonucleotide primers for amplifying the entire coding regions of *UCP1* including the 5' untranslated region (UTR) of exon1 were synthesized according to the sequence information (GenBank: AC010954 and AC025490, [5, 6]) and labeled with [$\gamma$-$^{32}$P]ATP (ICN, Irvine, Calif., USA). A PCR was done for 30 cycles under suitable conditions with these primers and 50 ng of genomic DNA. The resulting products were subjected to electrophoresis on a 5% polyacrylamide gel, with or without 10% glycerol, and followed by autoradiography. The PCR products that migrated abnormally were subjected to direct sequencing.

*PCR-RFLP.* Allele frequencies for the two SNPs were investigated by PCR-RFLP with PCR primers that amplified the corresponding regions of *UCP1* and contained mismatched bases to create restriction sites (5'-AAGTCCCAGCGGAAGACCGG-3' and 5'-GGCAGCAAACCCGATTCCTG-3' for the A→C polymorphism, and 5'-TATCGCTGGATTTGCGCAAAAGCT-3' and 5'-CTTGAAGAAAGCCGTTGGTCCTTCG-3' for the Met$^{229}$→Leu polymorphism). The resulting PCR products containing these two variants possess restriction sites for *Bst NI* and *Hind* III, respectively. Digested PCR fragments were subjected to electrophoresis on a 10% polyacrylamide gel and visualized by ethidium bromide. The allele frequency for the A→G polymorphism was investigated as reported previously [7].

*Plasmid construction and luciferase assay.* To obtain DNA fragments encompassing the promoter region of *UCP1*, we carried out a PCR with the primers 5'-GACAAGTTCAGAGTGCTCTTG-3' (positions –774 to –754 relative to the translation initiation codon) and 5'-ATCTTCACTCAGAGACTGGAG-3' (positions + 2 to –19) and with genomic DNA of subjects possessing *AA* or *CC* genotypes with the A→C polymorphism as template. The resulting wild-type and mutant DNA fragments were subcloned into the pGL3 luciferase reporter vector (Promega, Madison, Wis., USA), yielding pLUC-WT and pLUC-Mut, respectively. A deletion mutant, pLUC-Del, lacking nucleotides + 2 to –155 was obtained by digestion of pLUC-WT with *Pst* I followed by self-ligation. The COS-7 cells growing in 24-well plates were subjected to transient transfection with 0.5 μg each of luciferase reporter plasmids and a control vector containing the $\beta$-galactosidase by using TransFast Transfection Reagent (Promega). Cells were harvested 48 h after transfection, and luciferase and $\beta$-galactosidase activities were measured.

*Statistical analysis.* Statistical analysis was done with StatView Version 5.0 (SAS Institute, Cary, N. C., USA). All data are presented as means ± SD or proportions. The allele frequencies for SNPs between Type II diabetic and control groups were compared by a standard chi-square test. Other categorical variables were also compared by the chi-square test. To evaluate the statistical significance with multiple testing for three SNPs (Table 1), the $p$ values should be corrected with Bonferroni's correction. As the A→C and the Met$^{229}$→Leu polymorphisms are in strong linkage disequilibrium, they are not regarded as independent tests. Therefore, Bonferroni's correction has to take into account two independent tests (the A→C and the Met$^{229}$→Leu polymorphisms vs the A→G polymorphism). If a $p$ value of less than 0.05 is considered to be nominally significant, this value should be divided by 2 (0.05/2 = 0.025). We therefore considered a $p$ value of less than 0.025 to be truly significant.

## Results

Two SNPs, an A→C nucleotide substitution in exon 1 (+ 112) and an A→T transition in exon 5, were detected by PCR-SSCP. The former is new and the latter results in a Met$^{229}$→Leu substitution which has already been reported [5, 6]. Next, we investigated the genotypes of these SNPs in 320 Type II diabetic subjects and 250 control subjects by PCR-RFLP (Table 1). The genotypes for each of these SNPs were in Hardy-Weinberg equilibrium. The frequency of the *C* allele of the A→C polymorphism was significantly higher in the Type II diabetic group than in the control group ($p$ = 0.017). The frequency of the *Leu* allele of the Met$^{229}$→Leu polymorphism was also higher in the Type II diabetic group than in the control

**Table 1.** Genotypes and allele frequencies for the A→C polymorphism and the $Met^{229}$→Leu polymorphism in *UCP1* for study subjects

| | Type II diabetes ($n$ = 320) | Control subjects ($n$ = 250) |
|---|---|---|
| Genotype | A→C polymorphism | |
| *AA* | 257 | 220 |
| *AC* | 61 | 29 |
| *CC* | 2 | 1 |
| *C* allele frequency (%) | 10.2[a] | 6.2 |
| Genotype | $Met^{229}$→Leu polymorphism | |
| *Met/Met* | 254 | 214 |
| *Met/Leu* | 63 | 36 |
| *Leu/Leu* | 3 | 0 |
| *Leu* allele frequency (%) | 10.8[b] | 7.2 |
| Genotype | A→G polymorphism | |
| *AA* | 83 | 58 |
| *AG* | 156 | 116 |
| *GG* | 81 | 76 |
| *G* allele frequency (%) | 49.7 | 53.6 |

[a] $p = 0.017$, [b] $p = 0.038$ vs control group (chi-square test)

group ($p = 0.038$). These two SNPs were in strong linkage disequilibrium ($p < 0.00001$). We also investigated the allele frequency for the A→G polymorphism in the 5' UTR of *UCP1* that was shown to be related to an increased susceptibility to weight gain over several years [7]. The frequency of the *G* allele did not differ significantly between the Type II diabetic group and the control group (49.7 vs 53.6%, respectively, $p = 0.190$) (Table 1). Among the Type II diabetic subjects, no significant associations of genotype for the three SNPs with clinical characteristics [age at onset of Type II diabetes, body mass index, waist-to-hip ratio, HOMA (homeostasis model assessment), $HbA_{1c}$, total cholesterol and triglyceride] were apparent (data not shown).

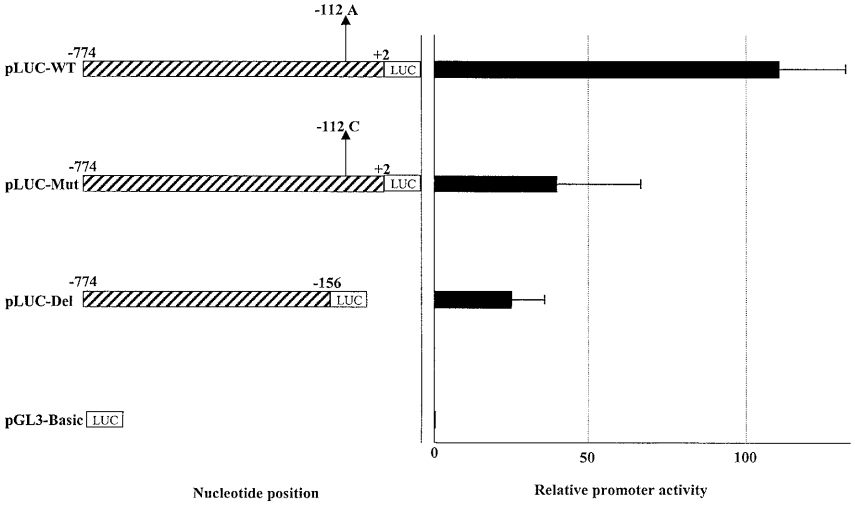
Given that the $Met^{229}$ residue of human UCP1 is replaced by Leu in rodents, the $Met^{229}$→Leu substitution possibly does not affect the function of the protein. We therefore investigated the effect of the A→C polymorphism on the transcriptional activity of the *UCP1* promoter. The A→C polymorphism is localized in the consensus motif of insulin response sequence (IRS: T(G/A)TTT(T/G)(G/T)) [8] and the antisense sequence for the consensus IRS (AAAAA**-**CA) between –116 and –110 bp is altered to AAAA**C**CA by this polymorphism. As there are no available cell lines of BAT, the transcriptional activity was measured using COS cells by transfecting promoter-reporter constructs with or without the A→C polymorphism. The activity of the wild-type (A variant) promoter was about 2.5 and 4 times higher than that of the mutant (C variant) promoter and the deletion construct, respectively (Fig. 1).

## Discussion

In *UCP1* we have identified the A→C polymorphism of 5' UTR and the $Met^{229}$→Leu variant. These two SNPs were in strong linkage disequilibrium and could be associated with susceptibility to Type II diabetes.

The A→C polymorphism in the 5' UTR of *UCP1* alters the consensus motif of IRS [8]. The transcriptional activity of a fragment of the 5' UTR of the mutant *C* allele decreased to approximately 40% compared with that of the corresponding fragment of the wild-type *A* allele, suggesting that this IRS plays an

**Fig. 1.** Effect of the A→C polymorphism at position –112 on the promoter activity of a fragment of the 5' UTR of *UCP1*. Luciferase reporter plasmids containing the indicated fragments of the *UCP1* promoter region were introduced into COS-7 cells together with a plasmid encoding β-galactosidase. The luciferase activity of cell extracts was normalized relative to β-galactosidase activity and then expressed relative to the normalized value for cells transfected with pGL3. Data are means ± SD of values obtained from three independent experiments



important part in the transcription of *UCP1*. It was shown that insulin induced UCP1 gene expression in brown adipocytes at physiological dose [9]. It is therefore possible that this A→C polymorphism impairs the affinity of transcriptional factors for the IRS of *UCP1*. Subsequently, this alternation in the IRS could result in impaired promoter activity, a reduced abundance of UCP1 protein and a consequent tendency towards energy storage. Thus, the A→C polymorphism of *UCP1* might be one of the "thrifty" factors that promote energy storage.

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