Uncoupling Protein 2 Promoter Polymorphism −866G/A Affects Its Expression in β-Cells and Modulates Clinical Profiles of Japanese Type 2 Diabetic Patients

Miyoshi Sasahara, Masahiro Nishi, Hiromichi Kawashima, Kazuya Ueda, Setsuya Sakagashira, Hiroto Furuta, Eisaku Matsumoto, Tadashi Hanabusa, Hideyuki Sasaki, and Kishio Nanjo

Common uncoupling protein 2 (UCP2) promoter polymorphism −866G/A is reported to be associated with its expression in adipose tissue and the risk of obesity in Caucasians. On the other hand, several studies suggested that UCP2 expression in β-cells is an important determinant of insulin secretion. In the Japanese population, morbid obesity is very rare, and insulin secretion capacity is relatively low as compared with Caucasians. Because UCP2 would link to insulin secretion and obesity, it might explain this ethnic difference. Here, we report that the UCP2 promoter with the A allele showed higher promoter activity in the INS-1 β-cell line. The frequency of the A allele is higher in our Japanese study than that in Caucasians. Type 2 diabetic patients with the A allele need insulin therapy earlier and showed higher frequency of insulin treatment. Moreover glucose-induced early insulin secretion is significantly lower in patients with the A allele. However, there was no difference in allele frequency between obese and lean type 2 diabetic patients. In conclusion, UCP2 promoter polymorphism −866G/A does not affect obesity in Japanese type 2 diabetic patients but affects its transcription in β-cells and modulates glucose-induced insulin secretion and eventually insulin requirement in Japanese type 2 diabetic patients. Higher A allele frequency in the Japanese population might partly explain the ethnic difference of insulin secretion capacity. *Diabetes* 53:482–485, 2004

The major defects in type 2 diabetes are those in insulin secretion and insulin sensitivity (1). Obesity is obviously an important factor for insulin sensitivity or resistance. Morbidly obese subjects are very rare in the Japanese population, and mean BMI in Japanese type 2 diabetic patients is also smaller than that in Caucasians (2). On the other hand, insulin secretion capacity is reported to be lower in Japanese (3,4). These ethnic differences may be explained by differences in genetic background associated with both obesity and insulin secretion. Uncoupling protein 2 (UCP2) is a subtype of the UCP family that mediates mitochondrial proton leak (5). Thus, higher expression of UCP2 might lead to the consumption of excess energy and a lessening of obesity. However, higher expression of UCP2 in β-cells would lead to a decrease in ATP production and a defect in insulin secretion. Several lines of evidence, including knockout mice, have also indicated an important role of UCP2 in insulin secretion (6–9). Because UCP2 is related to both obesity and insulin secretion, it will be a strong candidate gene for type 2 diabetes. UCP2 promoter polymorphism −866G/A has been reported to associate with its expression in adipose tissue and obesity in Caucasians (10). Therefore, we have investigated the promoter activity of each allele of UCP2 in an INS-1 β-cell line and the relationship between this polymorphism and clinical profiles of Japanese type 2 diabetic patients.

RESEARCH DESIGN AND METHODS

A total of 413 type 2 diabetic patients and 172 nondiabetic control subjects were recruited for the study after giving their written informed consent (Table 1). This study was approved by the ethics committee of Wakayama Medical University. To eliminate type 1 diabetes or mature-onset diabetes of the young, the following patients were excluded: patients diagnosed before 25 years of age or patients receiving insulin therapy within 3 years from the onset of diabetes. Patients with severe liver or renal dysfunction were also excluded. Nondiabetic subjects were defined as >60 years of age, HbA_{1c} <5.6%, and no positive family history of diabetes. Diagnosis of diabetes was made according to the criteria of World Health Organization (11).

Clinical assessment of diabetic patients. Diabetic retinopathy was diagnosed by the ophthalmologist's ocular fundi examination. Patients with simple preproliferative or proliferative retinopathy were assumed to have retinopathy. With regard to diabetic nephropathy, patients with persistent proteinuria were defined as having nephropathy. Patients with a history of nondiabetic renal diseases were excluded. Patients with clinical symptoms or diminished Achilles' tendon reflex were diagnosed as having neuropathy. Hypertension was diagnosed according to the following criteria; systolic blood pressure >140 mmHg and/or diastolic blood pressure >90 mmHg, or use of antihypertensive drugs. Hyperlipidemia was diagnosed according to the following criteria: use of antihyperlipidemic drugs, serum total cholesterol >220 mg/dl, serum triglyceride >150 mg/dl, or HDL <40 mg/dl. Estimation of the intimal-medial thickness (IMT) of the carotid artery was carried out using ultrasonography as previously described (12). These clinical characteristics of diabetic patients are shown in Table 2.

Frequently sampled intravenous glucose tolerance test. After an overnight fast, blood samples were collected at −10, −1, 0, 4, 6, 8, 10, 15, 19, 22, 24, 30, 40, 70, 90, and 180 min from an antecubital vein, and plasma glucose and immunoreactive insulin (IRI) were measured. At time 0 min, 300 mg/kg body wt of glucose was injected into the contralateral vein. At time 20 min, 0.05 units/kg body wt of insulin was injected. Insulin sensitivity (S_i) and glucose effectiveness at basal insulin (S_g) were calculated by minimal model method (13). Glucose-induced total insulin secretion was evaluated as the integral area of IRI above the basal level during the first 19 min (ΣIRI19). Glucose-induced early insulin secretion was evaluated as the increment of IRI

From the First Department of Medicine, Wakayama Medical University, Wakayama, Japan.

Address correspondence and reprint requests to Masahiro Nishi, MD, PhD, the First Department of Medicine, Wakayama Medical University, 811-1 Kimiidera, Wakayama 641-8509, Japan. E-mail: mnishi@wakayama-med.ac.jp.

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IMT, intimal-medial thickness; IRI, immunoreactive insulin; RLU, relative luciferase unit; UCP2, uncoupling protein 2.

TABLE 1
Characteristics of subjects

	Type 2 diabetes	Nondiabetic
n	413	172
Age (year)	65.51 ± 10.12	72.49 ± 7.70
Sex (M/F)	248/165	56/116
Maximum BMI (kg/m^2)	27.39 ± 4.4	24.72 ± 3.12
HbA_{1c} (%)	7.42 ± 1.48	4.99 ± 0.39
Age of onset diabetes (years)	47.42 ± 9.94	—
Duration of diabetes (years)	18.21 ± 9.74	—
UCP2 genotype (−866G/A)		
G/G (%)	28.1	29.1
G/A (%)	49.6	52.3
A/A (%)	22.3	18.6

at 4 min from basal level (ΔIRI4). Subjects with poor glycemic control (HbA_{1c} >8.0%) and obese subjects (BMI >30 kg/m^2) were excluded from this analysis to avoid the effects of glucose toxicity or obesity on insulin sensitivity and secretion.

Typing of UCP2 genotype. Genomic DNA was isolated from peripheral blood according to standard procedures. The UCP2 genotype was analyzed by PCR–restriction fragment–length polymorphism method using *Mlu*I as previously described (10). Primers used were 5′-AGGCGTCAGGAGATGGACCG-3′ as forward primer and 5′-CACGCTTCTGCCAGGAC-3′ as reverse primer.

Cell culture and luciferase assay. INS-1 cells (14) were cultured in RPMI-1640 supplemented with 10% FCS, 50 μmol/l 2-mercaptoethanol, 1 mmol/l pyruvic acid, 25 mmol/l HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin. The DNA fragment of the UCP-2 promoter region with −866G or A (from −1,318 to +125) was amplified by PCR. pGL3-G (−866G) or pGL3-A (−866A) were constructed by inserting each fragment into *Sac*I and *Xho*I site of the pGL3-Basic vector (Promega, Madison, WI). Both strands were sequenced, and no PCR errors were confirmed. pGL3-Basic, pGL3-G, or pGL3-A (500 ng/well each) together with pRLSV40 (10 ng/well; Promega) were transfected into INS-1 cells in 12-well plates using FuGENE-6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN). At 24 h after transfection, media were changed to those with a glucose concentration of 5.6, 11.1, or 22.2 mmol/l, respectively. At 48 h after transfection, cells were lysed, and the activity of firefly and *Renilla* luciferase were measured by a Lumat LB9507 luminometer (EG & G, Berthold, Germany). Relative luciferase units (RLUs) were calculated as the ratio of pGL3-Basic to luciferase activity.

Statistical analysis. Categorical variables (sex, insulin therapy, retinopathy, nephropathy, neuropathy, hypertension, and hyperlipidemia) were compared by χ^2 analysis. Differences between continuous variables (age, max BMI, HbA_{1c}, duration of diabetes, age at onset of diabetes, and average IMT) were evaluated by Mann-Whitney's *U* test. Logistic regression analysis was used to determine the independent association between genotype status (G/G versus G/A + A/A) in UCP2 gene and clinical characteristics (sex, family history of diabetes, max BMI, age at onset of diabetes, duration of diabetes, and HbA_{1c}). Cumulative survival ratios free from insulin therapy were obtained by Kaplan-Meier analysis. *P* values <0.05 were considered to be statistically significant. All analyses were performed using the StatView software program version 5.0 for Windows.

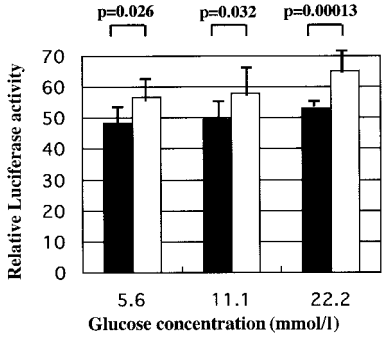


FIG. 1. Comparison of promoter activity according to UCP-2 genotype. Promoter activities of pGL3-G with the UCP-2 −866G allele (■) and pGL3-A with the UCP-2 −866 A allele (□) were compared. Relative luciferase activities were calculated as the ratio of pGL3-Basic (empty vector) to luciferase activity. Data are as means ± SD (*n* = 8). The results are representative of three independent experiments.

RESULTS

Luciferase assay. The promoter activity of pGL3-A was significantly higher than that of pGL3-G (57.8 ± 7.3 vs. 49.4 ± 6.8 RLU, $P = 0.032$) in a glucose concentration of 11.1 mmol/l. Similar results were obtained under glucose concentrations of 5.6 or 22.2 mmol/l, respectively (Fig. 1).

Allele frequency of UCP2 promoter polymorphism. The allele frequency of UCP2 promoter polymorphism −866G/A in nondiabetic subjects and type 2 diabetic patients are shown in Table 1. There are no significant differences of allele frequency between type 2 diabetic patients and nondiabetic subjects.

UCP2 genotype and clinical manifestation. Between type 2 diabetic patients with the A allele and those without the A allele, there are no significant differences in terms of age; sex; max BMI; duration of diabetes; HbA_{1c} level; incidences of diabetic microangiopathy, hypertension, and hyperlipidemia; and average IMT (Table 2). However, the patients with the A allele showed significantly earlier onset of the disease (46.79 ± 10.15 vs. 49.02 ± 9.21 years of age, $P = 0.042$) and higher frequency of insulin therapy (48.1 vs. 31.9%, $P = 0.0027$) (Table 2).

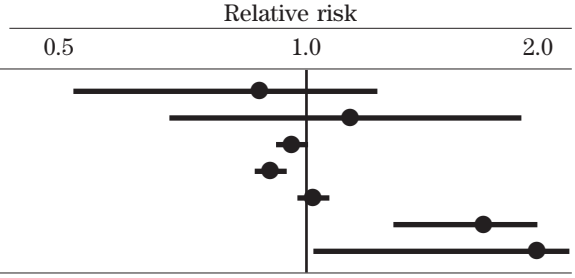
Logistic regression analysis and Kaplan-Meier analysis. Logistic regression analysis for insulin treatment

TABLE 2
Clinical characteristics of diabetic subjects

	G/G	G/A + A/A	*P* value
n	116	297	
Age (year)	66.20 ± 9.44	65.25 ± 10.37	NS
Sex (M/F)	70/46	178/119	NS
Maximum BMI (kg/m^2)	27.61 ± 4.50	27.31 ± 4.37	NS
HbA_{1c} (%)	7.46 ± 1.43	7.40 ± 1.50	NS
Duration of diabetes (year)	17.69 ± 9.65	18.41 ± 9.78	NS
Onset of diabetes (year)	49.02 ± 9.21	46.79 ± 10.15	0.042
Insulin therapy (%)	31.9	48.1	0.0027
Retinopathy (%)	49.4	44.2	NS
Nephropathy (%)	15.3	15.2	NS
Neuropathy (%)	38.5	36.4	NS
Average IMT (mm)	0.95 ± 0.17	0.96 ± 0.19	NS
Hyperlipidemia (%)	47.4	55.4	NS
Hypertension (%)	54.6	53.1	NS

TABLE 3
Logistic regression analysis

Modulator	*P* value
Sex (men: 1; women: 0)	0.6454
Family history (positive: 1; negative: 0)	0.5149
Maximum BMI (kg/m^2)	0.4689
Onset age (years)	<0.0001
Duration (years)	0.0017
HbA_{1c} (%)	<0.0001
Genotype (G/G: 0; G/A + A/A: 1)	0.0126



Dependent factors were: insulin therapy (insulin: 1, diet/OHA: 0). Modulators were: sex, family history of diabetes, maximum BMI, onset age of diabetes duration of diabetes, HbA_{1c}, and UCP-2 genotype. Subjects were Japanese type 2 diabetic patients (n = 413).

revealed that, like onset age, duration of the disease, and HbA_{1c}, the UCP2 genotype affected insulin treatment independently (P = 0.0126) (Table 3). Kaplan-Meier analysis also revealed that patients with the A allele needed insulin therapy significantly earlier after onset of diabetes than those without the A allele (P = 0.0161) (Fig. 2).

Frequently sampled intravenous glucose tolerance test. Both glucose-stimulated early insulin secretion estimated by ΔIRI4 and glucose-stimulated total insulin secretion estimated by ΣIRI19 were significantly lower in patients with A alleles than in patients with the G/G genotype (P = 0.009 and P = 0.046, respectively) (Table 4). After adjustment of BMI, HbA_{1c}, sex, age, and duration and family history of diabetes, there was still a significant difference in ΔIRI4 (P = 0.016). However, in ΣIRI19, the difference was not significant (P = 0.124). There were no significant differences in S_i or S_g.

DISCUSSION

UCP2 is expressed in many tissues, including adipose tissue and pancreatic β-cells. Underexpression of UCP2 in adipose tissue would lead to obesity. Esterbauer et al. (10) reported that the G allele of UCP2 promoter polymorphism −866G/A, which associates with lower expression in adipocytes, was a risk factor for obesity in middle-aged Caucasians. However, UCP2-null mice did not show the obese phenotype. In this study, we also could not find a positive correlation between UCP2 promoter polymorphism −866G/A and the maximum BMI in Japanese type 2 diabetic patients. This could be attributable to ethnic differences. In the Japanese population, genetic factors other than UCP2 could be important for obesity, although further study will be necessary. UCP2 exists in pancreatic β-cells and might regulate ATP levels in β-cells. Higher expression of UCP2 in β-cells will cause defects in insulin secretion via a lowering of ATP. If the promoter polymorphism (−866G/A) of UCP2 affects expression in β-cells as in adipocytes, it might correlate with insulin secretion. To answer this question, first we have analyzed the promoter activity of each allele in INS-1 cells. As expected from the report in adipocytes (10), the A allele showed significantly higher promoter activity than G alleles. Krempler et al. (15) also reported on the promoter activity of this promoter polymorphism using INS1-E cells. In their experiment, there was no difference under the basal state. However, after transfection of either PAX6A or PAX6B, promoter activity of the A allele was significantly higher than that of G allele. The difference between their results and ours may be explained by the expression level of PAX6 in the cell lines used. Because the INS1-E cell line was clonally selected from the parental INS-1 cell line based on its improved insulin secretion (16), INS1-E cells may lose some PAX6 expression during the selection process. Therefore, INS-1 cells may express more PAX6 than INS1-E cells and show higher promoter activity in the A allele without transfection of PAX6. Further study will be needed to clarify this difference. However, both their data and ours clearly showed higher promoter activity in the A allele than in the G allele, although PAX6 was transfected in their experiment and not transfected in ours. If it is true in human β-cells, people with the A allele will have higher expression of UCP2 in their β-cells. There are several reports showing the close relationship between UCP2 expression in β-cells and its insulin secretion. UCP2 knockout mice were reported to have higher ATP content in their islets and showed higher insulin secretion (6,7). Overexpression of UCP2 in β-cells by adenovirus caused impaired insulin secretion (8). Chronic exposure of β-cells to fatty acids induced UCP2 expression, and this induction may be the cause of impaired glucose-induced insulin secretion (9). Thus, it is highly probable that higher expression of UCP2 in pancreatic β-cells leads to a lower amount of ATP and eventually to lower insulin secretion. To support this hypothesis, in our study, glucose-induced early insulin secretion assessed by ΔIRI4 and ΣIRI19 is

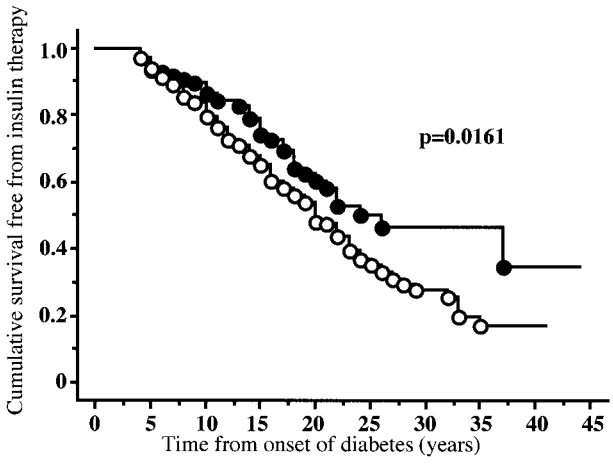


FIG. 2. Kaplan-Meier analysis of cumulative survival ratios free from insulin therapy. Patients were grouped according to UCP2 genotype (●, G/G; ○, G/A + A/A). Patients receiving insulin therapy within 3 years from their onset were excluded.

TABLE 4
Frequently sampled intravenous glucose tolerance test

	G/G	G/A + A/A	*P* value
n	28	109	
Sex (M/F)	18/10	61/48	NS
Age (years)	56.7 ± 17.2	58.7 ± 13.7	NS
BMI (kg/m^2)	23.08 ± 2.82	23.9 ± 3.11	NS
HbA_{1c} (%)	6.59 ± 0.69	6.68 ± 0.75	NS
Δ IRI4 (μU/ml)	19.9 ± 32.8	8.2 ± 16.5	0.009 (0.016)*
ΣIRI19 (min · μU/ml)	269.2 ± 445.4	136.2 ± 268.1	0.046 (0.124)*
S_I (10^{-4}/min · μU/ml)	2.30 ± 1.49	2.25 ± 1.77	NS
S_G (10^{-2}/min)	2.02 ± 0.64	1.89 ± 0.65	NS

*P value after adjustment by BMI, HbA_{1c}, sex, age, duration, and family history of diabetes.

significantly lower in patients with the A allele than in those without the A allele. Therefore, earlier onset of the diabetes and higher incidence of insulin therapy in patients with the A allele is thought to be caused by their lower insulin secretion capacity. Logistic regression analysis revealed that the UCP2 genotype, as well as HbA_{1c}, duration and onset age, was independently associated with insulin requirement in Japanese type 2 diabetic patients. Kaplan-Meier analysis for the accumulation of non-insulin treatment also clearly revealed the earlier requirement of insulin therapy in Japanese type 2 diabetic patients with the A allele. Recently, Sesti et al. (17) also reported impaired β-cell function in subjects with the A/A genotype.

The frequency of the UCP2 genotype was not significantly different between diabetic patients and nondiabetic control subjects in our study. Krempler et al. (15) reported higher A allele frequencies in diabetic patients than in obese control subjects (41.3 vs. 31.2%) . However, our control subjects were relatively thin (mean maximum BMI 24.72 kg/m^2), and the A allele frequency in lean Caucasian control subjects was reported to be much higher (38.2%) than in those who were obese (10). Therefore, interpretation of this difference leads to at least two possibilities: the first is a difference of BMI, and the second is difference of ethnicity. The frequency of the A allele was relatively higher in our study compared with previous Caucasian studies (47.1 vs. 41.3% in diabetic patients and 44.8 vs. 31.2–38.2% in control subjects) (10,15). This might be attributable to ethnic difference. In the Japanese population, insulin secretion capacity is reported to be lower (3,4). This Japanese ethnic characteristic is at least partly explained by the higher frequency of the A allele in the UCP2 genotype. However, further study is necessary to fully clarify this ethnic difference.

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