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Association of -786T-C mutation of endothelial nitric oxide synthase gene with insulin resistance

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Abstract

Aims/hypothesis. Endothelial derived nitric oxide synthase (*eNOS*) gene polymorphisms affect *eNOS* activity and are associated with abnormal vasomotility and impaired local blood flow. A decrease in local blood flow has been reported to cause insulin resistance. The aim of this study was to examine a possible association of two *eNOS* polymorphisms, Glu298Asp (G894T) in exon 7 and -786T-C mutation with insulin resistance.
Methods. Genotypes of both Glu298Asp and -786T-C mutation were examined by the PCR-RFLP method. Plasma nitrate and nitrite concentrations were also measured.
Results. The allele frequencies of both polymorphisms showed no considerable differences in 233 non-diabetic subjects and 301 patients with Type II (non-insulin-dependent) diabetes mellitus. Non-diabetic subjects with the -786C allele had ($p<0.05$) higher fasting plasma insulin and homeostasis model assessment of insulin resistance than those with the -786T/ -786T genotype. Diabetic subjects with -786C allele showed higher HbA_{1c} than those with the -786T/-786T genotype. A euglycaemic hyperinsulinemic clamp study done on 71 of the 301 patients showed a lower glucose infusion rate in diabetic patients with the -786C allele than those without it. In diabetic patients with the -786C allele, plasma nitrate and nitrite concentrations were lower than in subjects without it ($p=0.026$). No differences were observed between mutant carriers of Glu298Asp and non-carriers among both non-diabetic subjects and Type II diabetic patients.
Conclusions/interpretation. The -786T-C mutation of the *eNOS* gene is associated with insulin resistance in both Japanese non-diabetic subjects and Type II diabetic patients. [Diabetologia (2002) 45:1594–1601]

Keywords Endothelial nitric oxide synthase, polymorphism, glucose infusion rate, homeostasis model assessment of insulin resistance insulin resistance, intima-media thickness.

Endothelial derived nitric oxide (NO), synthesized from L-arginine by the endothelium isoform of NO synthase (*eNOS*), mediates local vasodilation and plays a key role in the regulation of vascular tone. Skeletal muscle glucose uptake is enhanced by insulin-mediated vasodilation and the decrease in local blood flow can result in insulin resistance [1–5]. However, several reports deny a major role of endothelial NO production in determining insulin sensitivity [6, 7].

Recently *eNOS* knockout mice have been reported to be insulin resistant [8]. These mice have also shown a decrease in whole-body and muscle-glucose uptake as well as the simultaneous decrease in the local blood flow [9]. Two major polymorphisms have been found in the human *eNOS* gene: Glu298Asp (G894T) in exon 7 and -786T-C mutation in the 5′-flanking region. Glu298Asp causes a structural change of the *eNOS*

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Abbreviations: NO, Nitric oxide; eNOS, endothelial derived nitric oxide synthase; HOMA IR, homeostasis model assessment of insulin resistance; NOx, nitrate and nitrite; GIR, glucose infusion rate; IMT, intima-media thickness.

protein and reduces *eNOS* activity [10, 11]. In contrast, the -786T-C mutation reduces the promoter activity [12] and thus reduces *eNOS* protein expression and *eNOS* activity [10]. Both Glu298Asp and -786T-C polymorphism have been reported to lead to abnormal vasomotility [13, 14] and to be associated with vasoconstrictive angina [12, 15]. However, the association between both *eNOS* polymorphisms and insulin resistance has not been found.

Subjects and methods

Subjects. We recruited 301 Japanese patients with Type II (non-insulin-dependent) diabetes mellitus aged 30 to 76 years from a group of outpatients at the Osaka University Hospital. The assessment of Type II diabetes was based on World Health Organization (WHO) criteria [16]. Patients were recruited for the study if they met the following inclusion criteria: (i) no episodes of ketoacidosis and absence of ketonuria, (ii) diagnosis of diabetes after 30 years of age, (iii) insulin therapy (if any) started after at least 5 years following diagnosis, (iv) absence of overt diabetic nephropathy or other renal tract disease, and (v) absence of acute stage or signs and syndromes of coronary heart disease, cerebral vascular disease, and peripheral artery disease after careful evaluation of clinical records. The duration of diabetes was 12.0±9.2 years.

We recruited 233 non-diabetic hospital employees as control subjects aged 21 to 66 years without cardiovascular disease, cerebrovascular disease, or peripheral vascular disease. Normal blood glucose concentrations in these subjects were assessed with the 75-g oral glucose tolerance test (OGTT) according to WHO criteria [16]. Written informed consent was obtained from all the subjects enrolled in this study.

At the time of enrolment, the laboratory data, blood pressure measurements, urinary albumin measurements, resting 12-lead ECG and master two-step test results, and intima plus media thickness (IMT) measurements were collected for each patient. Fasting blood was obtained for analysis of serum total cholesterol, HDL cholesterol, serum triglycerides, serum creatinine, blood urea nitrogen, serum uric acid, and HbA_{1c} concentrations by standard laboratory techniques. Urinary albumin of a fasting urine specimen and a specimen collected at least 4 weeks later was measured by radioimmunoassay. The concentration was divided by the urinary creatinine concentration and expressed as milligrams per gram of creatinine [17]. The two measurements of urinary albumin were averaged. Blood pressure was measured with a mercury sphygmomanometer (Model 620; Kenzmedico, Saitama, Japan). After a supine rest of 5 min, three measurements in sitting position were conducted, and the mean value was used. Smoking was estimated as the mean number of cigarettes smoked daily. Checking for the occurrence of angina pectoris and old myocardial infarction (major abnormal Q wave or abnormal QS pattern changes) were based on the results of the resting 12-lead ECG and master two-step tests and the existence of previous symptoms of myocardial infarction. Of the 301 Type II diabetic patients, 73 patients were controlled with diet only, 117 patients with oral agents, and 111 patients with insulin injection.

Assessment of insulin sensitivity. Insulin sensitivity was assessed using fasting insulin and glucose concentrations by homeostasis model assessment of insulin resistance (HOMA IR) for non-diabetic patients [18]. Plasma insulin concentrations were measured by radioimmunoassay (SRL, Tokyo, Japan). Of Type II diabetic patients, 71, from whom agreement was obtained, were subjected to determination of insulin–mediated glucose uptake by the euglycaemic hyperinsulinemic clamp technique using an artificial pancreas (STG22; Nikkiso, Tokyo, Japan) [19, 20]. Briefly, regular insulin (Humalin-R Eli Lilly, Indianapolis, Ind., USA) was infused in a primed continuous manner at a rate of 8.7 $pmol \cdot kg^{-1} \cdot min^{-1}$ for 2 h. Normoglycaemia was maintained by adjusting the rate of a 10% D-glucose infusion based on plasma glucose measurements carried out at 1 min intervals. Glucose infusion rate (GIR) was calculated by averaging the glucose infusion rates achieved over the last 30 min of the clamp as endogenous glucose production is completely suppressed at the increased concentrations achieved. Before the clamp study, the patients were admitted to Osaka University Hospital for at least 2 weeks and were confirmed to be free of glucose toxicity.

Assessment of carotid atherosclerosis. Ultrasonographic scanning of the carotid arteries was done using an echotomographic system (EUB-450; Hitachi Medico, Tokyo Japan) with an electrical linear transducer (midfrequency of 7.5 MHz). Scanning of the extracranial common carotid artery, the carotid bulb, and the internal carotid artery in the neck was carried out bilaterally from three different longitudinal projections (i.e., anterior, lateral, and posterior-oblique) as well as the transverse projection, as reported previously [21–23]. All of the images were photographed. The scanning session lasted an average of 30 min. The detection limit of this echo system using 7.5 MHz was 0.1 mm. The carotid IMT defined by another study [24] was measured as the distance from the leading edge of the first echogenic line to the leading edge of the second echogenic line. The first line represented the lumen-intimal interface and the second line the collagen-containing upper layer of the outer membrane. At each longitudinal projection, the site of the greatest thickness, including plaque, was sought along the arterial walls nearest the skin and farthest from the skin from the common carotid artery to the internal carotid artery. Assessments of IMT were conducted three times at the site of the greatest thickness and at two other points, 1 cm upstream and 1 cm downstream from this site, and these were then averaged. The greatest value among the six averaged IMTs (each three from the left and the right) was used as the representative value for each individual. All scans were conducted by physicians who were unaware of the clinical characteristics of the subjects. Assessment of IMT on the photograph was carried out by a physician who was unaware of the clinical characteristics of the subjects. The reproducibility of the IMT measurements was examined by conducting another scan 1 month later on 20 subjects with Type II diabetes whose IMT values were 0.68 to 1.39 mm. The mean difference in IMT between these two assessments was 0.04 mm, and the standard deviation was 0.09 mm, showing good reproducibility for repeated measurements, as described previously [21–23].

Genomic analyses. Genomic DNA was prepared from blood leukocytes by established methods. The presence of the missense Glu298Asp variant was examined by PCR-RFLP analysis as described by another study [25]. A set of primers was designed to amplify the 248-base pair (bp) fragment encompassing the missense Glu298Asp variant (the sense and antisense primers 5′-AAGGCAGGAGACAGTGGATGGA-3′ and 5′-CCCAGTCAATCCCTTTGGTGCTCA-3′, respectively). The PCR fragments were digested with the restriction enzyme Ban II (Toyobo Tokyo, Japan), separated by electrophoresis using low melting temperature agarose gel (4%, NuSieve GTG AGAROSE, FMC) and visualized by ethidium bromide stain-

Table 1. Genotype distributions and allele frequencies for the Glu298Asp and -786T-C polymorphism of the *eNOS* gene in non-diabetic subjects and Type II diabetic patients

Glu298Asp polymorphism	Genotypes				Allele frequencies	
	N	GG	GT	TT	G	T
Non-diabetic subjects[a]	233	196 (84.1%)	35 (15.0%)	2 (0.9%)	0.916	0.084
Type II diabetic patients	301	256 (85.0%)	42 (14.0%)	3 (1.0%)	0.920	0.080
-786T-C polymorphism	Genotypes				Allele frequencies	
	N	TT	TC	CC	T	C
Non-diabetic subjects[b]	233	194 (83.3%)	35 (15.0%)	4 (1.7%)	0.908	0.092
Type II diabetic patients	301	250 (83.1%)	48 (15.9%)	3 (1.0%)	0.910	0.090

[a] "Non-diabetic subjects vs Type II diabetic patients regarding Glu298Asp mutation of *eNOS* gene": genotypes χ^2=0.14, p=0.93; allele frequencies χ^2=0.11, p=0.74

[b] "Non-diabetic subjects vs type II diabetic patients regarding −786T-C mutation of *eNOS* gene": genotypes χ^2=0.59, p=0.74; allele frequencies χ^2=0.024, p=0.88
χ^2 analysis was carried out

ing. -786T-C mutation was also examined by PCR-RFLP analysis developed specially for this study. A set of primers was designed to amplify the 236 bp fragment encompassing the -786T-C mutation (the sense and antisense primers 5′-ATGCTCCCACCAGGGCATCA-3′ and 5′-GTCCTTGAGTCTGACATTAGGG-3′, respectively). The PCR fragments were digested with the restriction enzyme Msp I (Toyobo Co., Japan), separated by electrophoresis using 20% polyacrylamide gel and visualized by ethidium bromide staining.

NO measurement. We also assessed plasma NO concentrations by measuring the total amount of plasma NO end products (nitrate and nitrite; NOx) as described previously [26, 27]. Specimens of peripheral venous blood from the brachial vein were collected into heparinized tubes after the subjects had been sitting at rest for 15 min in a quiet room maintained at a temperature of 22 °C to 24 °C. The blood was placed immediately in an ice bath and centrifuged within 30 sec for 5 min at 2000 g. The serum fraction was diluted 1:1 with nitrite distilled water and nitrite-free distilled water, and 400 ml of the distilled sample was centrifuged at 2000 g in an ultra-free MC microcentrifuge device to remove substances larger than 10 000 M_r. The filtrate was passed through a copper-plated cadmium column to reduce nitrate and nitrite and then reacted with Griess reagents consisting of 0.1% naphthylethylenediamine dihydrochloride in distilled water and 1% sulphanilamide in 5% H_3PO_4, after which the absorbance was measured at 540 nm to evaluate the total amount of plasma NO end products (nitrate and nitrite). The efficacy of the cadmium column in the conversion of nitrate to nitrite was confirmed to be 100% by measuring both nitrate and nitrite standards before and after sample measurement [26].

Statistical analyses. Data are shown as means ± SD. Deviation from Hardy-Weinberg equilibrium was examined by chi-squared analysis. The clinical characteristics of the subjects were analysed by the genotypes of the Glu298Asp and -786T-C polymorphisms as follows: difference in quantitative variables were examined by one-way ANOVA and differences in frequencies were examined by chi-square analysis. The difference in genotype or allele frequency between non-diabetic subjects and Type II diabetic patients was examined by chi-square analysis. All analyses were conducted using the HALBAU statistical package (Gendai Sugakusha, Kyoto, Japan).

Results

Genotype distributions and allele frequencies in non-diabetic subjects and type II diabetic patients are shown in Table 1. Genotype distributions and allele frequencies of both Glu298Asp and -786T-C polymorphisms in non-diabetic subjects and Type II diabetic patients were in accordance with the Hardy-Weinberg equilibrium. With the Glu298Asp polymorphism, there was no difference of allele frequency between Japanese non-diabetic subjects and Type II diabetic patients. Concerning -786T-C mutation, there is no difference of allele frequency between Japanese non-diabetic subjects and Type II diabetic patients. In each polymorphism, the number of homozygous mutants was so small that we combined the data of homozygous and heterozygous mutants in the following analysis.

The clinical characteristics associated with Glu298Asp polymorphism of the *eNOS* gene in the 233 non-diabetic subjects and 301 Type II diabetic patients are shown in Table 2. In the non-diabetic subjects, no difference was noted between those with and without the mutation with respect to clinical characteristics, such as fasting plasma insulin and HOMA IR. The results of a euglycaemic hyperinsulinemic clamp done on the 71 Type II diabetic patients are shown in Table 3. GIR in those with the mutation showed a tendency of impaired insulin-mediated glucose uptake compared with those without the mutation (4.67±1.04 vs 5.65±1.83 $mg \cdot kg^{-1} \cdot min^{-1}$, p=0.092), although the difference was not statistically significant.

Clinical characteristics associated with -786T-C mutation of the *eNOS* gene in the 233 non-diabetic subjects and 301 Type II diabetic subjects are shown in Table 4. In non-diabetic subjects, those with the mutation showed higher fasting plasma insulin

Table 2. Clinical characteristics according to Glu298Asp (G894T) polymorphism of *eNOS* gene

	Non-diabetic subjects			Type II diabetic patients		
	GG	GT or TT	*p*	GG	GT or TT	*p*
Sex (male/female)	136/60	24/13		183/73	34/11	
Age (years)	43.9±10.5	42.0±11.0	0.315	57.6±10.6	55.8±12.3	0.322
BMI (kg/m^2)	22.5±2.9	22.0±3.0	0.374	23.7±3.9	23.7±4.8	0.980
Systolic BP (mmHg)	122±12	121±13	0.579	133±16	133±18	0.943
Diastolic BP (mmHg)	75±10	74±11	0.386	76±9	75±12	0.277
HbA_{1c} (%)	4.9±0.3	4.9±0.4	0.816	7.7±1.7	8.0±1.8	0.275
Total cholesterol (mmol/l)	5.01±0.76	4.99±0.64	0.894	5.25±0.99	5.20±0.74	0.750
Triglycerides (mmol/l)	1.15±0.51	0.98±0.43	0.070	1.53±0.82	1.39±0.68	0.304
HDL cholesterol (mmol/l)	1.57±0.36	1.55±0.34	0.786	1.33±0.39	1.33±0.35	0.920
Insulin (pmol/l)	43.6±17.0	44.0±17.3	0.901	–	–	
$HOMA_{IR}$	1.66±0.69	1.75±0.74	0.502	–	–	
IMT (mm)	0.96±0.23	0.98±0.24	0.800	1.26±0.44	1.25±0.40	0.888
NOx (μmol/l)	35.3±21.0	34.2±16.3	0.865	25.5±11.3	32.5±19.2	0.132
HT-Risk[a]	32	2	–	60	12	–
HL-Risk[a]	55	6	–	77	12	–
SM-Risk[a]	48	5	–	73	13	–
Nephropathy[a]	–	–		228/28	36/9	–
Retinopathy[a]	–	–		213/43	38/7	–
Treatment of diabetes[a]	–	–		64/101/91	9/16/20	–

[a] χ^2 test
Means ± SD
NOx, plasma nitrate and nitrite levels; HT-Risk, number of subjects with either systolic >160, diastolic pressure >95 mmHg, or taking anti-hypertensive drugs; HL-Risk, number of subjects with either total cholesterol >5.69 mmol/l, triglyceride >1.70 mmol/l, HDL-cholesterol <1.03 mmol/l or taking hypolipidaemic drugs, SM-Risk, number of subjects who smoke more than one pack of cigarettes per day for 20 years; Nephropathy, normoalbuminuria + albuminuria / proteinuria with normal serum creatinine; Retinopathy, no diabetic retinopathy + background diabetic retinopathy/preproliferative diabetic retinopathy + proliferative diabetic retinopathy; Treatment of diabetes, diet therapy / oral agents / insulin injection

Table 3. Results of euglycaemic hyperinsulinaemic clamp among 71 Type II diabetic patients

	Glu298Asp (G894T) polymorphism			-786T-C polymorphism		
	GG	GT or TT	*p*	TT	TC or CC	*p*
Sex (male/female)	48/15	4/4	–	42/15	10/4	–
Age (years)	52±12	49±16	0.494	52±13	49±12	0.562
BMI (kg/m^2)	25.2±5.4	26.0±6.4	0.668	25.8±5.6	25.2±5.4	0.723
HbA_{1c} (%)	6.9±1.6	7.8±0.9	0.221	7.2±1.9	7.8±1.3	0.281
Glucose infusion rate (μmol/kg/min)	31.4±10.2	25.9±5.8	0.092	31.6±10.6	24.3±5.0	0.025

Data are means ± SD

(49.8±16.8 vs 42.4±16.8 pmol/l, p=0.019) and HOMA IR (1.89±0.68 vs 1.63±0.69, p=0.043) than those without the mutation. In Type II diabetic patients, those with the mutation show a higher HbA_{1c} (8.3±2.0 vs 7.6±1.6%, p=0.006) and a lower plasma NOx concentration (20.2±7.5 vs 25.8±8.2 μmol/l, p=0.026) than those without the mutation. The Type II diabetic patients with the -786C mutation showed a lower insulin-mediated glucose uptake than those without the mutation (4.38±0.90 vs 5.68±1.90 $mg \cdot kg^{-1} \cdot min^{-1}$, p=0.025) (Table 3). Those with and without the mutation showed no differences of other clinical characteristics and diabetic microangiopathy and macroangiopathy including the urinary secretion rate of albumin and IMT (1.29±0.37 vs 1.25±0.45 mm, p=0.592) (Table 4).

Discussion

This study showed that -786T-C mutation of the *eNOS* gene is associated with a higher plasma insulin and HOMA IR in non-diabetic subjects. Also, diabetic patients with this mutation possessed a higher HbA_{1c} and a lower plasma NOx concentration. The euglycaemic hyperinsulinemic clamp study showed a lower insulin-mediated glucose uptake in those with -786T-C mutation, although the number of patients

Table 4. Clinical characteristics according to -786T-C polymorphism of *eNOS* gene

	Non-diabetic subjects			Type II diabetic patients		
	TT	TC or CC	*p*	TT	TC or CC	*p*
Sex (male/female)	134/60	26/13		177/69	40/15	0.114
Age (years)	44.1±10.6	41.2±10.6	0.114	57.1±10.9	58.4±10.7	0.434
BMI (kg/m^2)	22.3±2.8	22.8±3.4	0.316	23.7±4.1	23.8±3.9	0.842
Systolic BP (mmHg)	122±13	122±12	0.904	132±17	134±14	0.427
Diastolic BP (mmHg)	75±10	76±11	0.501	76±10	77±9	0.296
HbA_{1c} (%)	4.9±0.3	4.8±0.3	0.419	7.6±1.6	8.3±2.0	0.006
Total cholesterol (mmol/l)	5.03±0.75	4.88±0.66	0.293	5.20±0.95	5.41±0.97	0.136
Triglycerides (mmol/l)	1.11±0.49	1.18±0.54	0.486	1.50±0.80	1.56±0.81	0.623
HDL cholesterol (mmol/l)	1.58±0.36	1.49±0.32	0.129	1.32±0.38	1.39±0.38	0.222
Insulin (pmol/l)	42.4±16.8	49.8±16.8	0.019	–	–	
$HOMA_{IR}$	1.63±0.69	1.89±0.68	0.043	–	–	
IMT (mm)	0.96±0.21	0.96±0.29	0.995	1.25±0.45	1.29±0.37	0.592
NOx (μmol/l)	34.8±20.0	42.0±25.9	0.321	25.8±8.2	20.2±7.5	0.026
HT-Risk[a]	26	8	–	59	13	
HL-Risk[a]	48	13	–	67	22	–
SM-Risk[a]	45	8	–	69	17	–
Nephropathy[a]	–	–		216/30	48/7	–
Retinopathy[a]	–	–		207/39	44/11	–
Treatment of diabetes[a]	–	–		62/94/90	11/23/21	–

[a]χ^2 test

Means ± SD

NOx, plasma nitrate and nitrite levels; HT-Risk, number of subjects with either systolic >160, diastolic pressure >95 mmHg, or taking anti-hypertensive drugs; HL-Risk, number of subjects with either total cholesterol >5.69 mmol/l, triglyceride > 1.70 mmol/l, HDL-cholesterol <1.03 mmol/l or taking hypolipidaemic drugs; SM-Risk, number of subjects who smoke more than one pack of cigarettes per day for 20 years; Nephropathy, normoalbuminuria + albuminuria / proteinuria with normal serum creatinine; Retinopathy, no diabetic retinopathy + background diabetic retinopathy/preproliferative diabetic retinopathy + proliferative diabetic retinopathy; Treatment of diabetes, diet therapy / oral agents / insulin injection

studied was limited due to the difficulty of obtaining informed consent. We did not carry out a euglycaemic hyperinsulinaemic clamp with non-diabetic subjects as this would not have been ethical. Concerning Glu298Asp polymorphism, the non-diabetic subjects showed no difference in clinical characteristics and the Type II diabetic patients showed no difference in the insulin-mediated glucose uptake with the euglycaemic hyperinsulinaemic clamp, although there was a tendency of slightly lower values in the mutant groups.

The mechanisms of these two polymorphisms affecting *eNOS* activity are different. Glu298Asp polymorphism induces the structural change of the *eNOS* protein and reduces *eNOS* activity [10, 11]. In contrast, a different study showed that in -786T-C mutation, replication protein A1 which is known as a DNA binding protein essential for DNA repair and replication, binds only to the mutant allele and reduces the promoter activity of the *eNOS* gene [12, 28]. This could cause -786T-C mutation leading to a profound impairment of vasodilation, which could result in impaired insulin-mediated glucose uptake in the whole body. A recent study indicated that in smokers -786C homozygote shows a decrease of cerebrovascular circulation [29]. Our study showed that the Glu298Asp mutation had a tendency of impaired insulin-mediated glucose uptake (Table 3). A previous report has shown that both -786T-C mutation and Glu298Asp are associated with vasoconstrictive angina [12, 15]. Multiple logistic regression analysis suggests that -786T-C mutation is more closely associated with vasoconstrictive angina than Glu298Asp polymorphism [30]. Our study agreed with this multiple regression analysis. Thus, it is likely that -786-C mutation rather than Glu298Asp polymorphism is associated with development of insulin resistance as well as vasoconstrictive angina, although we cannot exclude the possibility that the Glu298Asp polymorphism could have some less obvious effects on insulin resistance.

In this study, we measured NOx concentration, which is the plasma end products of NO, because the lifetime of NO is quite short and cannot be measured directly as a real time value. The Type II diabetic patients showed a lower plasma NOx concentration than non-diabetic subjects (23.9±8.2 vs 42.3±31.5 μmol/l, p=0.00003). There is increasing evidence that NO synthesis and the vasodilating properties of insulin are impaired in insulin resistant states, such as Type II diabetes and obesity [31, 32]. Thus, our data agree with previous findings [33]. In the 301 Type II diabetic patients, the mutant group of -786T-C polymorphism showed a higher HbA_{1c} than the non-mutant

group. These data agree with the idea that -786T-C polymorphism impairs endothelial NO production, leading to a decrease in insulin-mediated glucose uptake through impaired vasodilatation and poor glycaemic control.

The non-diabetic subjects with the -786T-C mutation showed no difference in NOx concentration. One possibility is that non-diabetic subjects show a large SD of NOx concentration compared to Type II diabetic patients (20.9 vs 8.2 μmol/l, respectively). Especially, non-diabetic females aged 35.4±11.2 years also showed a higher NOx concentration and its wider variation than those of Type II diabetic females aged 58.7±10.5 years (36.7±20.8 vs 17.5±4.1 μmol/l). Oestrogen has been shown to increase eNOS activity [34]. NOx as well as oestrogen could fluctuate during the menstrual cycle in pre-menopausal females. In this study, however, we did not consider the menstrual cycle when we obtained blood samples from female subjects. The other possibility is that non-diabetic females tended to have a higher NOx concentration than non-diabetic males (26.6±14.0 μmol/l). These factors as well as other unknown factors could neglect the effect of eNOS mutation on NOx concentration in non-diabetic subjects.

Regarding Glu298Asp polymorphism of the *eNOS* gene, we found no differences in plasma NOx concentrations, clinical characteristics and insulin-mediated glucose uptake (only in the diabetic patients) between those with and without mutant alleles in both non-diabetic subjects and diabetic patients. Thus, these data agree well with the hypothesis that the Glu298Asp mutation is not strong enough to affect clinical characteristics and insulin-mediated glucose uptake.

We also examined the possible association between two *eNOS* polymorphisms and diabetic nephropathy and retinopathy. There are several reports showing the association of -786T-C polymorphism and diabetic nephropathy [35, 36], and there is a report showing the dissociation of Glu298Asp polymorphism and diabetic nephropathy [37]. In this study, there was no difference in the number of patients taking an ACE inhibitor, which has been reported to reduce urinary protein. We found no association between diabetic microangiopathy and these polymorphisms. We are trying to evaluate the effect of these polymorphisms on the onset of diabetic complications in a larger number of patients.

Another important role of NO in the vasculature is its antiatherogenic effects by scavenging superoxide radicals and suppression of platelet aggregation, leukocyte adhesion and smooth muscle cell proliferation [38–40]. In this study we assessed IMT as an index of early atherosclerosis. There are several reports showing the association of Glu298Asp polymorphism and myocardial infarction [25, 41, 42]. On the other hand, there are inconsistencies according to European reports examining the association of Glu298Asp polymorphism and carotid atherosclerosis [43, 44], and there has been no report about the association of -786T-C polymorphism and carotid atherosclerosis. In this study, we found no relation between these polymorphisms and IMT. These results could be due to genetic differences among ethnic groups and the progression of atherosclerosis might be affected by various factors other than *eNOS* activities such as NADH/NADPH oxidase, inflammatory cytokine, and monocyte chemoattractant protein-1.

Our study shows that the relative allele frequencies of Glu298Asp and -786T-C polymorphism of the *eNOS* gene do not differ between Japanese non-diabetic subjects and Type II diabetic patients. Considering that both insulin resistance and impaired insulin secretion play a major role in the onset of Type II diabetes, one possibility is that -786T-C polymorphism does not affect the susceptibility of Type II diabetes. Another possibility is that non-diabetic subjects were younger than the Type II diabetic patients and thus some subjects with this mutation might have been in a pre-diabetic state.

The frequency of the 894T allele in Japanese non-diabetic subjects (0.084) is similar to that found in Japanese control subjects of other studies [25, 45], but is lower than that found in the control subjects in a European study (0.44) [46]. The frequency of the -786C allele in Type II diabetic patients in this study (0.09) is similar to that found in Japanese control subjects of another study [12, 45], but is lower than that found in American Type I diabetic patients without progressive nephropathy (0.36) [35]. No corresponding data are available for European or American control subjects and Type II diabetic patients such comparative studies are needed.

Those who had both mutant alleles were 4 out of 233 non-diabetic subjects and 3 out of 301 Type II diabetic patients (data not shown) and these frequencies were less than expected (non-diabetic subjects:6.2, Type II diabetic patients:7.6). This might deny the linkage disequilibrium between the Glu298Asp and -786T-C polymorphism of the *eNOS* gene. These findings agree with those of another report [30]. We could not detect any specific clinical characteristics among those with both mutant alleles.

In conclusion, our study shows that -786T-C polymorphism of the *eNOS* gene is associated with insulin resistance both in Japanese non-diabetic subjects and Type II diabetic patients.

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