```
     1                                                          60
SAH  MLRHAKCFQRLAIFGSVRALHKDNRTATPQNF-SNYESMKQDFKLGIPEYFNFAKDVLDQW
KS1        MHWLRKVQGLCTLWGTQMSSRTLYINSRQLVSLQWGHQEVPAKFNFASDVLDHW
KS2  WLRHLVLQALRNSRAFCGSHGKPAPLPVPQKIVATWEAISLGRQL-VPEYFNFAHDVLDVW

     61                                                        120
     TDKEKAGKKPSNPAFWWINRNGEEMRWSFEELGSLSRKFANILSEACSLQRGDRVILILP
     ADMEKAGKRLPSPALWWVNGKGKELMWNFRELSENSQQAANVLSGACGLQRGDRVAVVLP
     SRLEEAGHRPPNPAFWWVNGTGAEIKWSFEELGKQSRKAANVLGGACGLQPGDRMMLVLP

     121                                                       180
     RVPEWWLANVACLRTGTVLIPGTTQLTQKDILYRLQSSKANCIITNDVLAPAVDAVASKC
     RVPEWWLVILGCIRAGLIFMPGTIQMKSTDILYRLQMSKAKAIVAGDEVIQEVDTVASEC
     RLPEWWLVSVACMRTGTVMIPGVTQLTEKDLKYRLQASRAKSIITSDSLAPRVDAISAEC

     181                                          Motif 1      240
     ENLHSKLIVSENSREGWGNLKELMKH--ASDSHTCVKTKHNEIMAIFFT-SGTSGYPKMTAHT
     PSLRIKLLVSEKSCDGWLNFKKLLNQSEASTTHHCVETGSQEASAIYFT-SGTSGLPKMAEHS
     PSLQTKLLVSDSSRPGWLNFRELLRE--ASTEHNCMRTKSRDPLAIYFTKREPPGAPKMVEHS

     241                                                       300
     HSSFGLGLSVNGRFWLDLTPSDVMWNTSDTGWAKSAWSSVFSPWIQGACVFTHHLPRFEP
     YSSLGLKAKMDAG-WTGLQASDIMWTISDTGWILNILCSLMEPWALGACTFVHLLPKFDP
     QSSYGLGFVASGRRWVALTESDIFWNTTDTGWVKAAW-TLFSAWPNGSCIFVHELPRVDA

     301                                                       360
     TSILQTLSKYPITVFCSAPTVYRMLVQNDITSYKFKSLKHCVSAGEPITPDVTEKWRNKT
     LVILKTLSSYPIKSMMGAPIVYRMLLQQDLSSYKFPHLQNCVTVGESLLPETLENWRAQT
     KVILNTLSKFPITTLCCVPTIFRLLVQEDLTRYQFQSLRHCLTGGEALNPDVREKWKHQT

     361                                                       420
     GLDIYEGYGQTETVLICGNFKGMKIKPGSMGKPSPAFDVKIVDVNGNVLPPGQEGDIGIQ
     GLDIRESYGQTETGLTCMVSKTMKIKPGYMGTAASCYDVQIIDDKGNVLPPGTEGDIGIR
     GVELYEGYGQSETVVICANPKGMKIKSGSMGKASPPYDVQIVDDEGNVLPPGEEGNVAVR

     421                                    Motif 2            480
     VLPNRPFGLFTHYVDNPSKTASTLRGNFYITGDRGYMDKDGYFWFVARADDVILSSGYRI
     VKPIRPIGIFSGYVDNPDKTAANIRGDFWLLGDRGIKDEDGYFQFMGRADDIINSSGYRI
     IRPTRPFCFFNCYLDNPEKTAASEQGDFYITGDRARMDKDGYFWFMGRNDDVINSSSYRI

     481                                                       540
     GPFEVENALNEHPSVAESAVVSSPDPIRGEVVKAFVVLNPDYKSHDQEQLIKEIQEHVKK
     GPSKVENALMEHPAVVETAVISSPDPVRGEVVKAFVVLASQFLSHDPEQLTKELQQHVKS
     GPVEVESALAEHPAVLESAVVSSPDPIRGEVVKAFIVLTPAYSSHDPEALTRELQEHVKR

     541
     TTAPYKYPRKVEFIQELPKTISGKTKRN
     VTAPYKYPRKHRLIVIGLLRSRPWGKRLPRKRLIRECF
     VTAPYKYPRKVAFVSELAKDGFWKDPKE
```

Figure 4. Amino acid sequence homology among putative SA gene family. Sequences were obtained from GenBank. *SAH* and *KS1* are derived from NT010441. *KS2* is derived from AK00588. Moreover, 2 other proteins highly homologous to latter half of SA protein have been reported (AC003034). Motif 1 indicates putative AMP-binding domain characteristic of acyl-CoA synthetases; motif 2 indicates putative domain characteristic of acyl-CoA synthetases for medium-chain fatty acids.

allele than in the A allele (Figure 3A). This experiment indicates that this single nucleotide change significantly affected the expression level through affecting splicing of this intron under these experimental conditions. It was recently reported that an intronic sequence variation affects the mRNA expression level in *WNK1*, which causes pseudohypoaldosteronism type II.[17]

The functional significance of I/D polymorphism of *SAH* was assessed by a transient transfection assay with MDCK cells. Two-way ANOVA indicated that the I/D polymorphism, but not the G(119952)A polymorphism, significantly affected promoter activity. The promoter activity of the D allele was about twice that of the I allele in MDCK cells. Although this I/D polymorphism did not affect the mRNA level in peripheral mononuclear cells (see above), this polymorphism may have functional significance in other relevant tissues, such as kidney, liver, and adipose tissues.

Discussion

In the present study, we found that human SA gene product has acyl-CoA synthetase activity for MCFA and that the A/G polymorphism in intron 12 affects BMI, W/H, triglyceride, cholesterol, and blood pressure status. Especially in subjects <60 years old, this genotype strongly affects triglyceride, cholesterol, and blood pressure. The clustering of multiple risk factors, including obesity, hypertension, dyslipidemia, and hyperuricemia, is known as syndrome X or insulin-resistance syndrome.[18,19] These syndromes are likely to be heterogeneous, and SAH, an acyl-CoA synthetase, seems to contribute to a subset of these syndromes.

MCFAs are abundant in milk, coconut oil, and various semisynthetic oils.[20,21] Activation of MCFA takes place mostly in the mitochondrial matrix by acyl-CoA synthetases for MCFA, and most of the MCFA incorporated into hepatocytes is subject to β-oxidation.[20,21] Some of the acetyl-CoA produced during MCFA oxidation is directed toward ketone body production, and the rest is directed to de novo synthesis of long-chain fatty acids (LCFAs), which are then incorporated into triglyceride or other complex lipids.[20,21]

There are ≥5 genes in the SA gene family.[22] Two (*KS1* and *KS2*) are highly homologous to the entire SA protein (Figure 4). *KS1* is localized ≈210 kb upstream of *SAH*. These acyl-CoA synthetases may be functionally linked to specific metabolic pathways, as observed in acyl-CoA synthetases for LCFA in the liver.[23] A higher expression of the SA protein might lead to higher de novo synthesis of LCFA from MCFA, because the G allele was associated with a higher plasma triglyceride level. Preferential deposition of MCFA to triglyceride might lead to a higher accumulation of peripheral triglyceride (visceral obesity) in the long term. The association of the A/G polymorphism with plasma triglyceride was more evident in subjects <60 years old, which may indicate that the primary intermediate is hypertriglyceridemia. Further investigation is needed to clarify the precise mechanisms by which *SAH* influences the phenotypes observed in the present study.

The G allele in intron 12 corresponds predominantly to the D allele in the promoter (Table 4). The estimated haplotype frequencies for IA, IG, DA, and DG are 0.294, <0.01, 0.686, and 0.019. The residuals of plasma triglyceride after adjustment for age and sex (mean±SEM, mmol/L) were −0.047±0.021 (IA/IA), −0.031±0.025 (IA/DA), +0.028±0.022 (DA/DA), and +0.115±0.092 (DG/DA+DG/DG). The profound effects of the intron 12 polymorphism on various phenotypes may be due to additive or synergistic effects of the D allele in the promoter.

The amino acid change (K359N) in exon 8 may not have functional significance, because the other members of this family have Q at this position, which is homologous to N (Table 5). Moreover, this amino acid residue is outside the motifs of this family.[24]

The intron 12 A/G polymorphism affected blood pressure status. Multiple logistic analyses that included BMI and W/H in addition to the genotype, age, and sex as independent variables downplayed the importance of the genotype as a predictor ($P=0.106$ in the total population and $P=0.0436$ in subjects <60 years old). Therefore, the substantial effects of the genotype on blood pressure seem to be conveyed through its effects on BMI and W/H.

In conclusion, human SA, acyl-CoA synthetase for medium-chain fatty acid, contributes to multiple risk factors, including obesity, hypertriglyceridemia, hypercholesterolemia, and hypertension. This intriguing observation opens a new area for future research in multiple-risk-factor syndromes.

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Low Potentiality of Angiotensin-Converting Enzyme Gene Insertion/Deletion Polymorphism as a Useful Predictive Marker for Carotid Atherogenesis in a Large General Population of a Japanese City

The Suita Study

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Background and Purpose—Some previous studies, almost all western, have investigated whether there is a relationship between the insertion/deletion (*I/D*) polymorphism of the angiotensin-converting enzyme (ACE) and carotid atherosclerosis. The results, however, have not been consistently positive. Further, there have been few investigations based on a large, general population. Therefore, the present study aimed to clarify whether ACE gene deletion polymorphism was associated with carotid atherosclerosis in a large Japanese general population with a more homogeneous genetic background than Caucasian populations.

Methods—Subjects aged 30 to 86 years were randomly selected from Suita City, located in Osaka, the second largest urban area of Japan, and included 1894 men and 2137 women. With the aid of high-resolution ultrasonography, carotid atherosclerosis was evaluated using our atherosclerotic indexes of intimal-medial thickness (IMT), plaque number (PN), plaque score (PS), and percentage of stenosis of the carotid artery assessed using high-resolution B-mode ultrasonography. ACE gene *I/D* polymorphism was detected by polymerase chain reaction.

Results—There were no significant differences among the ACE genotypes for age and conventional cardiovascular risk factors, except for systolic blood pressure (SBP) and the percentage of hypertension in men. The values of IMT, PN, and PS as carotid atherosclerotic indexes were not significantly different among genotypes for either sex. After adjusting for age, body mass index, smoking habit, high-density lipoprotein cholesterol, triglycerides, presence of hypertension, presence of diabetes mellitus, and presence of hyperlipidemia, the estimated ORs for the presence of IMT ≥1.10 mm (defined as thickened IMT), according to ACE genotype (*DD* versus *II*, DD+*ID* versus *II*, and *DD* versus *ID*+*II*), for men were 0.80 (95% CI 0.60 to 1.23), 0.89 (0.62 to 1.29), and 0.89 (0.70 to 1.28), respectively. On the other hand, the ORs for women after the same adjustment were 0.92 (95% CI 0.58 to 1.35), 0.93 (0.59 to 1.45), and 0.91 (0.59 to 1.27), respectively.

Conclusions—Our present data suggest that ACE *I/D* polymorphism is not potentially a useful predictive marker for carotid atherogenesis when investigated in a large and homogeneous general Japanese population of 4031 subjects, a finding similar to that in a Caucasian population study, the Perth Carotid Ultrasound Disease Assessment Study, an Australian study based on a general population using 1111 subjects. (***Stroke*. 2001;32:1250-1256.**)

Key Words: angiotensins ■ carotid arteries ■ genetics ■ Japan ■ ultrasonography

Many previous studies have reported that carotid intimal-medial thickness (IMT) correlates with traditional cardiovascular risk factors.[1-11] Also, it has been shown in some population-based[10-15] and intervention[16-18] studies that carotid IMT measured by B-mode ultrasound is a valid and reliable surrogate measure of generalized atherosclerosis, including coronary atherosclerosis. Recently, it has been reported[19] that carotid IMT is a significant predictive value of prevalent and incident stroke as well as coronary heart disease. These findings are increasing the importance of ultrasound studies compared with studies in which the end point is defined only by the presence or absence of clinical disease, ie, stroke or myocardial infarction.

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It is well known that atherosclerosis is a complicated disease influenced by genes and environmental factors, including diet, smoking, and high blood pressure. Certainly, ACE *I/D* polymorphism has been examined as a candidate gene polymorphism for an increased risk of ischemic heart disease[20-26] or other cardiac end points[23,27-29] in previous studies. However, most of those investigations assessed the relationship between ACE genotypes and myocardial infarction risk or increasing risk of hypertension. As a result, few investigations thus far have focused on the relationship between carotid atherosclerosis and gene polymorphism, although several studies[30-35] with relatively small, and often selective, populations have examined the association between ACE *I/D* polymorphism and increased carotid IMT. In particular, no studies based on a large, general population have, to our knowledge, investigated this association, with the exception of one: an Australian study, the Perth Carotid Ultrasound Disease Assessment Study.[36] Further, these results were heterogeneous and were not consistently positive.

The present study aimed to examine the relationship between ACE *I/D* polymorphism and carotid atherosclerosis, including carotid IMT, in a large and relatively genetically homogeneous general Japanese population.

Subjects and Methods

Subject Population

The population for our present study was based on a random sample selected from the residents of Suita, a city located in the second largest urban area in Japan (Osaka). Participants between the ages of 30 and 79 years were arbitrarily selected from the municipality population registry, stratified by sex and age groups of 10 years. The sample consisted of 12 200 men and women, although 3000 men and women were added randomly in the same way in 1996 and 1997. The basic sampling of the population started in 1989 with a cohort study base.

The subjects have visited the National Cardiovascular Center between Tuesday and Thursday every 2 years since then for regular health checkups, and approximately 2500 subjects have been participating in the health checkups every year. In addition to performing a routine blood examination that included total serum cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, glucose levels, blood pressure, and anthropometric measurements, a physician or nurse administered questionnaires covering personal and family history of cardiovascular and other diseases and smoking and drinking habits. In addition, DNA was extracted from an extra 5 mL of blood withdrawn from those who visited the National Cardiovascular Center between May 1996 and February 1998. All subjects were Japanese, and only those who gave informed consent for genetic analysis were enrolled in the present study. The carotid ultrasonic examinations were begun in April 1994, but the examinations were not done on the days when the regular health checkups were performed between Tuesday and Thursday because the examinations were performed by a single physician. As the result, the subjects in the present study included 1894 men and 2137 women, aged 30 to 89 years, who attended regular health checkups and consecutively underwent the ultrasonic examinations and the genetic analysis.

Measurements

The subjects were classified as current smokers if they smoked at least 1 cigarette per day, nonsmokers if they had never smoked, and past smokers if they had stopped smoking for >1 year. Subjects were defined as hypertensive if diastolic blood pressure (DBP) was ≥90 mm Hg and/or systolic blood pressure (SBP) was ≥140 mm Hg or if they were taking antihypertensive medication. Those subjects whose serum total cholesterol levels were ≥5.68 mmol/L (220 mg/dL) or who were taking antihypercholesterolemic medication were defined as having hypercholesterolemia. Those subjects whose fasting blood glucose (FBG) levels were ≥7.00 mmol/L (126 mg/dL) or who were taking antidiabetic medication were defined as diabetic. Subjects who had a history of coronary heart disease or cerebrovascular disease (103 men and 67 women) were excluded from the present analysis. The subjects' blood was sampled after overnight fasting, which resulted in the exclusion of 91 men and 113 women because they did not meet this condition. In total, 194 men and 180 women among the present 4031 subjects were excluded from this analysis. Blood pressure was measured twice in the right arm with a mercury sphygmomanometer, with the subject in a sitting position after taking a short rest. The second measurement was used for the analysis. Body mass index (BMI) was calculated as weight (kilograms) divided by height (meters) squared.

Blood samples drawn from the subjects after a fast of ≥12 hours were collected in EDTA-containing tubes. Total cholesterol and triglycerides levels were assayed enzymatically with a Toshiba TBA-80 mol/L biochemical discrete analyzer. Glucose was assayed enzymatically, and HDL cholesterol was measured after precipitation with heparin and calcium ions with a Toshiba TBA-20R biochemical discrete analyzer. The measurements of total cholesterol, HDL cholesterol, and triglyceride levels were all standardized in accordance with the protocol of the Centers for Disease Control and Prevention.

TABLE 1. Clinical Characteristics of the Present Subjects, by Sex

Variable	Men	Women
No. of subjects	1700	1957
Age, y	60.7±12.1*	58.8±11.6
Body mass index, kg/m²	23.0±2.8*	22.3±3.3
SBP, mm Hg	128.1±19.3*	126.5±20.3
DBP, mm Hg	79.7±10.9*	77.1±10.9
Total serum cholesterol, mmol/L	5.24±0.82*	5.58±0.86
HDL cholesterol, mmol/L	1.41±0.37*	1.68±0.39
Triglycerides, mmol/L	1.53±1.14*	1.17±0.75
FBG, mmol/L	5.66±1.24*	5.29±0.81
Smoking		
Never smoked, %	26.6†	88.9
Past smoker, %	30.0†	2.2
Current smoker, %	43.4†	8.9
Alcohol use		
Never, %	31.5†	72.2
Current, %	68.5†	27.8
Diabetes, %	9.5†	4.2
Hypertension, %	37.4	32.1
Hypercholesterolemia, %	30.3†	48.3

Hypertension indicates SBP ≥140 mm Hg and/or DBP ≥90 mm Hg or antihypertensive medication; diabetes, FBG ≥7.00 mmol/L (126 mg/dL) and/or antidibetic medication; and hypercholesterolemia, serum cholesterol level ≥5.68 mmol/L (220 mg/dL) or antihypercholesterolemic medication. Values are mean±SD.

*$P<0.05$ between men and women by Student t test.

†$P<0.05$ between men and women by χ^2 test.

Carotid Ultrasound

The details of the carotid ultrasonic examination method have been already published.[10] The method used in our present study was the same. We used a high-resolution B-mode ultrasonic machine with 7.5-MHz transducers yielding an axial resolution of 0.2 mm. The

regions between 30 mm proximal from the beginning of the dilation of the bifurcation bulb and 15 mm distal from the flow divider of both common carotid arteries (CCAs) were scanned. All measurements were made at the time of scanning with the instrument's electronic caliper and were recorded as photocopies. The IMT was measured on a longitudinal scan of the CCAs at a point 10 mm proximal from the beginning of the dilation of the bulb. We defined IMT as the mean of the IMTs of the near and far CCA walls at the point of measurement, and defined a mean IMT ≥1.10 mm as thickened IMT. We defined a plaque, a focal IMT thickening, as an area where IMT was ≥1.10 mm and calculated the plaque number (PN) by counting the number of plaques in the bilateral carotid arteries in the scanning area. We also calculated the plaque score (PS) by totaling the maximum thickness of all the plaques in the same area. Finally, we defined stenosis as a condition in which a plaque occupied more than half of the luminal circumference of an artery on a cross-sectional scan, and the degree of stenosis was calculated as a percentage ratio of the area of the plaque to that of the lumen, with the following formula: (Lumen Area−Residual Lumen)/Lumen Area×100. Both areas were measured automatically by the system on a frozen transverse section at the maximal narrowing site. If there was ≥50% stenosis, another skilled ultrasonographer performed color flow Doppler examination to confirm the stenosis.

The intrareader reproducibility of the measurements was assessed for the IMT of the CCA and the PN in 50 subjects just before the start of the ultrasonic examination in the routine health checkups in April 1994, and that matter has been described in the publication.[10] IMT and PN were examined twice at a 1-month interval in a blinded manner for the correlation coefficient between the first and second measurements of IMT ($r=0.87$, $P<0.001$). A paired t test showed no statistically significant difference between the 2 measurements. As for PN, 74% of the first PN coincided with the second ($\kappa=0.68$). Further, the intrareader reproducibility of the measurement for the PS in those same 50 subjects was assessed in the present analysis using the data of PS collected at that time. The first measurement of PS significantly correlated with the second ($r=0.71$, $P<0.01$). A paired t test also showed no statistically significant difference between the 2 measurements.

Determination of Genotype of ACE *I/D* Polymorphism

DNA was extracted from 200 μL of buffy coat separated from fresh blood with the use of a QIAamp Kit (QIAGEN). Template genomic DNA (100 ng) was amplified by polymerase chain reaction with a thermal cycle (Omni Gene; Hybrid). *I/D* polymorphism was determined by agarose gel electrophoresis with ethidium bromide staining, and the *DD* genotype was reconfirmed by insertion allele–specific amplification according to the Lindpainther's protocol[37] with a minor modification. This minor modification does not affect the results and is as follows: The DNA was amplified for 30 cycles with denaturation at 94°C for 1 minute, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute 30 seconds after initial denaturation at 94°C for 5 minutes.

Statistical Analysis

All statistical analysis were performed using the SAS statistical software system and the Statistical Package for the Social Sciences (SPSS Inc). The mean levels of all the numerical values were tested by the Student t test. Those of almost all the categorical values were examined by χ^2 analysis. However, the Fischer exact test was adopted instead of the χ^2 test in case the number of subjects was ≤5. The association between ACE *I/D* polymorphism and clinical variables, carotid atherosclerotic indices (ie, IMT, PN and PS), was tested by 1-way ANOVA. The quantitative effects of covariates for carotid atherosclerotic index, the presence of IMT ≥1.10 mm defined as thickened IMT, were assessed by multiple logistic regression analysis with the aid of SAS. Values of $P<0.05$ were considered significant.

Results

Study Population

Table 1 shows the clinical characteristics of the present subjects by sex. Almost all variables (ie, age, BMI, SBP, DBP, triglycerides, FBG, percentage of current smokers, current alcohol users, diabetes, and hypertension) were significantly higher in men than in women. The levels of total serum cholesterol and HDL cholesterol were significantly higher in women.

TABLE 2. Clinical Characteristics by Sex and ACE Genotype

	Men				Women			
Variable	*DD*	*ID*	*II*	ANOVA *P*	*DD*	*ID*	*II*	ANOVA *P*
No. of subjects	215	791	694		262	849	846	
Age, y	61.8±11.8	60.5±11.9	60.5±12.3	0.32	58.4±11.7	59.0±11.7	58.6±11.5	0.64
Body mass index, kg/m²	23.2±2.9	23.0±2.8	23.0±2.8	0.73	22.7±3.3	22.2±3.4	22.3±3.1	0.07
SBP, mm Hg	131.1±19.9	128.3±18.9	127.0±19.4	0.024	127.0±20.5	126.6±20.0	126.2±20.5	0.84
DBP, mm Hg	80.8±11.3	79.8±10.9	79.3±10.8	0.18	77.8±11.5	77.2±11.2	76.8±10.5	0.42
Total serum cholesterol, mmol/L	5.19±0.86	5.25±0.84	5.24±0.80	0.61	5.57±0.92	5.57±0.83	5.59±0.86	0.89
HDL cholesterol, mmol/L	1.38±0.38	1.42±0.38	1.42±0.36	0.43	1.65±0.37	1.65±0.39	1.67±0.39	0.44
Triglycerides, mmol/L	1.50±0.99	1.60±1.30	1.45±0.96	0.027	1.16±0.62	1.20±0.85	1.16±0.67	0.52
FBG, mg/dl	5.56±1.09	5.66±1.17	5.71±1.36	0.34	5.36±0.94	5.30±0.79	5.27±0.77	0.25
Smoking, %	39.5	45.0	42.8	0.69	9.0	8.7	9.3	0.95
Alcohol use, %	66.0	70.8	66.6	0.18	33.2	27.8	26.1	0.09
Diabetes, %	8.8	9.0	10.4	0.59	6.1	4.5	3.3	0.74
Hypertension, %	47.9	37.8	33.7	0.017	34.7	31.3	32.0	0.74
Hypercholesterolemia, %	31.2	31.6	28.7	0.45	50.0	47.9	48.1	0.84
Percentage of IMT ≥1.10 mm defined as thickened IMT, %	7.4	9.5	9.5	0.62	3.8	5.4	4.6	0.52

Hypertension indicates SBP ≥140 mm Hg and/or DBP ≥90 mm Hg or antihypertensive medication; diabetes, FBG ≥7.00 mmol/L (126 mg/dL) and/or antidiabetic medication; and hypercholesterolemia, serum cholesterol level ≥5.68 mmol/L (220 mg/dL) or antihypercholesterolemic medication. Values are mean±SD.

TABLE 3. Genotype Frequency of ACE *I/D* Polymorphism by Sex and Age Group

	Men				Women			
Age Group, y	n	*DD*, %	*ID*, %	*II*, %	n	*DD*, %	*ID*, %	*II*, %
30–44	204	9.8	47.1	43.1	254	14.2	42.1	43.7
45–54	313	14.1	44.4	41.5	444	13.7	42.1	44.1
55–64	476	10.5	48.9	40.5	594	13.3	42.8	43.9
65–74	472	13.6	48.7	37.7	483	12.2	45.1	42.7
75–89	235	15.7	39.6	44.7	182	14.8	45.6	39.6
Total	1700	12.6	46.5	40.8	1957	13.4	43.4	43.2

Clinical Characteristics by Sex and ACE *I/D* Polymorphism

For men, there were significant differences ($P<0.05$) in SBP, triglycerides, and the percentage of hypertension among the 3 genotypes. Also, SBP and the percentage of hypertension were highest in the *DD* genotype (Table 2). On the other hand, for women, no significant differences were found among the 3 genotypes with respect to age, BMI, SBP, DBP, total serum cholesterol, HDL cholesterol, triglycerides, FBG, the percentage of smoking, alcohol use, diabetes, and hypertension (Table 2).

Genotype Distribution of ACE *I/D* Polymorphism

The frequencies of the *D* and I allele were 35.9% and 64.1% for men and 35.1% and 64.9% for women. There were no significant differences in genotype frequencies of ACE *I/D* polymorphism among age groups for either sex (Table 3). According to Hardy-Weinberg's expectation, there was no significant deviation in ACE genotype distribution for men ($\chi^2=0.12$, $P=0.94$) for women ($\chi^2=2.23$, $P=0.33$), or for total subjects ($\chi^2=0.71$, $P=0.70$). In the present subjects, there was no significant difference in ACE genotype distribution ($\chi^2=3.64$, $P=0.16$) between men and women.

Carotid Atherosclerotic Indexes by Sex and ACE *I/D* Polymorphism

Figure 1 shows the mean IMT values of both sexes, by ACE *I/D* polymorphism, adjusted for age, pack-years of smoking, alcohol consumption, SBP, serum total cholesterol level, HDL cholesterol, triglycerides, and FBG. There were no significant differences among the 3 genotypes by 1-way ANOVA for either sex. Figures 2 and 3 show the mean values for PN and PS, adjusted for age, pack-years of smoking, alcohol consumption, SBP, serum total cholesterol level, HDL cholesterol, triglycerides, and fasting blood glucose. The results were roughly similar to those shown in Figure 1, and no significant differences were found among the 3 genotypes by 1-way ANOVA. Table 4 shows the distribution and percentage of 2 grades of stenosis (stenosis of 25% to <50% and stenosis ≥50%) by sex and ACE *I/D* polymorphism. There was no significant difference among the 3 genotypes with respect to the percentage of these 2 grades of stenosis for men. On the other hand, for women, the percentage of these 2 grades of stenosis of *DD* was lower, though not significantly, than that of *ID* and *II* by Fischers exact test.

Distribution of the Mean IMT Divided Into 6 Classes by Sex

The prevalence of IMT ≥1.10 mm, defined as thickened IMT, was significantly ($P<0.05$) higher in men than in women (9.5% for men and 4.9% for women; Figure 4).

Association Between Presence of IMT ≥1.10 mm and ACE *I/D* Polymorphism

After being adjusted for age, BMI, smoking habit, drinking habit, HDL-C, TG, presence of hypertension, presence of diabetes mellitus, and presence of hypercholesterolemia, the estimated odds ratios for the presence of IMT ≥1.10 mm (defined as thickened IMT), according to ACE genotype (*DD*

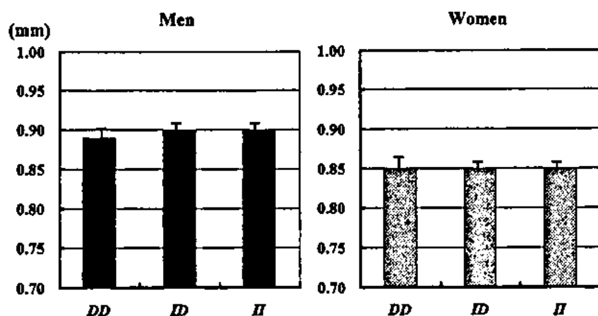

Figure 1. IMT values (±SE), by ACE polymorphism and sex, adjusted for age, pack-years of smoking, alcohol consumption, SBP, serum total cholesterol level, HDL cholesterol, triglycerides, and FBG. The differences of IMT values among genotypes were analyzed by 1-way ANOVA and Scheffé test. There were no significant differences among genotypes for either sex.

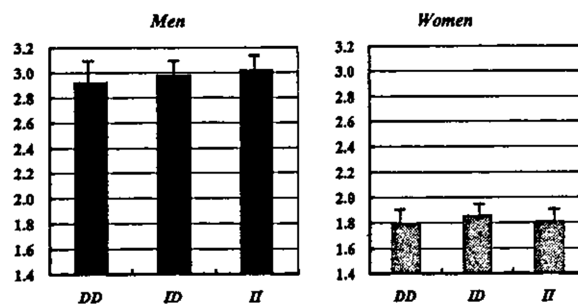

Figure 2. PN values (±SE), by ACE polymorphism and sex, adjusted for age, pack-years of smoking, alcohol consumption, SBP, serum total cholesterol level, HDL cholesterol, triglycerides, and FBG. Differences of PN values among genotypes were analyzed by 1-way ANOVA and Scheffé test. There were no significant differences among genotypes for either sex.

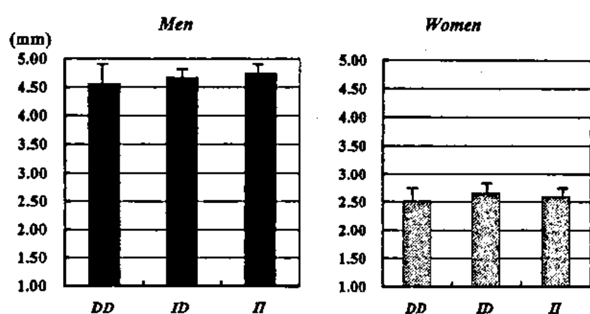

Figure 3. PS values (±SE), by ACE polymorphism and sex, adjusted for age, pack-years of smoking, alcohol consumption, SBP, serum total cholesterol level, HDL cholesterol, triglycerides, and FBG. Differences in IMT values among genotypes were analyzed by 1-way ANOVA and Scheffé test. There were no significant differences among genotypes for either sex.

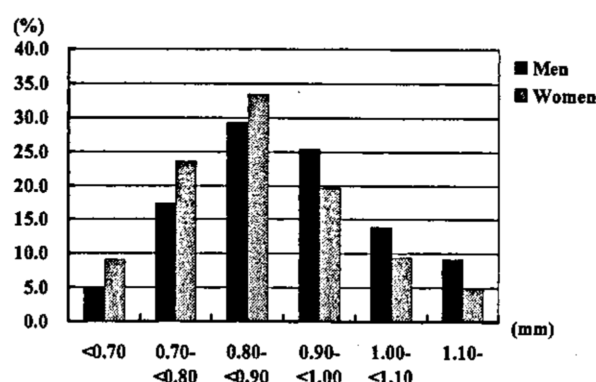

Figure 4. Distribution of the mean IMT divided into 6 classes by sex. The prevalence of IMT ≥1.10 mm (thickened IMT) was significantly ($P<0.05$) higher in men than in women.

versus *II*, *DD*+*ID* versus *II*, and *DD* versus *ID*+*II*), for men were 0.80 (95% CI 0.60 to 1.23), 0.89 (0.62 to 1.29), and 0.89 (0.70 to 1.28), respectively. These odds ratios for women after the same adjustment were 0.92 (95% CI 0.58 to 1.35), 0.93 (0.59 to 1.45), and 0.91 (0.59 to 1.27), respectively (Figure 5). In other words, there was no association between the presence of IMT ≥1.10 mm (thickened IMT) and ACE *I/D* polymorphism in either sex.

Discussion

Investigation of the effect of the *D* allele of ACE has thus far mainly focused on IHD.[20-26] Further, a recent meta-analysis[38] of 15 studies showed that there was a positive, but weak, relationship between the *D* allele and increased risk of myocardial infarction, although it is necessary to bear in mind that most of these results were heterogeneous and drawn from smaller, positive case-control studies. However, a large case-referent study[39] using the Copenhagen City Heart Study of 10150 subjects has recently reported that there was no significant association in the development of myocardial infarction or any other manifestations of IHD. Furthermore, according to a recent review[40] of meta-analyses examining the cause and effect relation between ACE *I/D* polymorphism and cardiovascular-renal risk among 49 959 subjects, there was no significant association of ACE *I/D* polymorphism with hypertension, but there was potentiality of the ACE *I/D* polymorphism as a useful marker of atherosclerotic cardiovascular complications and diabetic nephropathy.

However, there have been few studies[30-36] that focused on the relationship between ACE *I/D* polymorphism and carotid IMT, early atherosclerotic changes, or the nearly established surrogate end point of generalized atherosclerosis. In particular, there have been few studies based on a large and homogeneous randomly sampled population, except for one, the Perth Carotid Ultrasound Disease Assessment Study.[36] Even in this large study, which comprised 1111 subjects, the *D* allele was not found to be associated with either thickened IMT or carotid plaque formation, although some studies[30, 33-35] with small sample size or selection bias showed positive association. Also, there have been no studies showing a relationship between the *D* allele and carotid plaque or stenosis.

The present study, to our knowledge, is the first report to show the lack of a relationship between ACE *I/D* polymorphism and carotid IMT based on a large, homogeneous, randomly selected general population. Also, our present data showed that the *D* allele was not associated with the presence of IMT ≥1.10 mm (thickened IMT), irrespective of whether it was considered a dominant, codominant, or recessive gene polymorphism in either sex. Some studies[41-45] found an association between asymptomatic extracranial carotid lesions and asymptomatic brain infarction, which is thought to be a risk factor for symptomatic brain infarction. From this evidence, there is little possibility that ACE *I/D* polymorphism is a risk factor for ischemic stroke in Japan, although a previous Japanese study[46] with relatively small sample size (228 hypertensive and 104 normotensive individuals) showed that there was a close relationship between the ACE *D* allele and ischemic stroke in Japanese hypertensives and that the *D* allele may be an independent risk factor for the development of cerebrovascular disease in hypertensive patients.

It is also well known that the frequency of gene polymorphism is different among races. With regard to ACE *I/D*

TABLE 4. Distribution and Percentage of Subjects for Three Grades of Stenosis, by Sex and ACE *I/D* Polymorphism

	Men			Women		
Stenosis	*DD*, n (%)	*ID*, n (%)	*II*, n (%)	*DD*, n (%)	*ID*, n (%)	*II*, n (%)
<25%	186 (86.5)	667 (84.3)	581 (83.7)	254 (96.9)	792 (93.3)	792 (93.6)
25% to 50%	18 (8.4)	73 (9.2)	60 (8.6)	5 (1.9)	47 (5.5)	36 (4.3)
≥50%	11 (5.1)	51 (6.4)	53 (7.6)	3 (1.1)	10 (1.2)	18 (2.1)

% indicates percentage in each genotype.

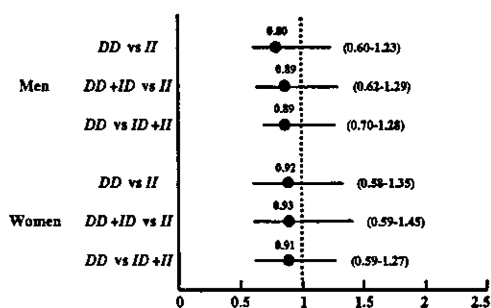

Figure 5. Multiple adjusted odds ratios for the presence of IMT ≥1.10 mm (thickened IMT) according to ACE genotype, codominant (*DD* versus *II*), dominant (*DD/ID* versus *II*), and recessive (*DD* versus *ID/II*). There were no significant differences among all the classified groups of genotypes in for either sex. Values in parentheses indicate confidence intervals.

polymorphism, the *D* allele frequency among the present Japanese subjects was significantly lower than that in the Caucasian individuals of the Copenhagen City Heart Study[39] or the Perth Carotid Ultrasound Disease Assessment Study.[36] The advantages of the present study are that the participants were randomly selected from urban Japanese residents, which resulted in an all-Japanese, homogeneous population. Our previous studies[10,11] (the Suita Study) have already shown that there was strong relationship between carotid IMT and various cardiovascular risk factors such as hypertension, smoking, and hypercholesterolemia. Thus, our previous and present data strongly suggest that the impact of exposure from traditional cardiovascular risk factors (such as age, hypertension, smoking, and hyperlipidemia) on carotid atherogenesis may be so much greater than that of gene polymorphisms, especially the ACE *I/D* polymorphism. However, the combination of some gene polymorphisms may be a risk factor for carotid atherogenesis, although this possibility should be further investigated.

In conclusion, our present data, based on a large, homogeneous general population of 4031 subjects, showed that there was little genetic influence of ACE *I/D* polymorphism on carotid atherogenesis and suggested that ACE *I/D* polymorphism might not be a potentially useful predictive marker for increased risk of carotid atherosclerosis of the Japanese. These results are similar to those of a Caucasian population study, the Perth Carotid Ultrasound Disease Assessment Study,[36] an Australian study based on a large general population of 1111 subjects.

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Original Article

Lack of Association between Genetic Polymorphism of *CYP11B2* and Hypertension in Japanese: The Suita Study

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Aldosterone synthase (*CYP11B2*) is a key enzyme in the biosynthesis of aldosterone. Recently, a polymorphism in the 5′-flanking region of the *CYP11B2* gene [T(−344)C] has been reported to be associated with blood pressure and plasma aldosterone levels. We investigated the association between this polymorphism and hypertension in a large population-based sample of 4,000 Japanese. The genotype distribution in hypertensive subjects (*n*=1,535) was compared to that in normotensive subjects (*n*=2,514). In subjects not receiving antihypertensive medication, the influence of the genotype on blood pressure values adjusted for clinical covariates was analyzed. All analyses were performed separately for men and women. The genotype distribution did not differ between hypertensive and normotensive subjects in either men (frequency of C allele: 30.3% *vs.* 31.4%, *p*=0.48) or women (31.5% *vs.* 31.7%, *p*=0.93). There were no significant differences in systolic blood pressure, diastolic blood pressure, or pulse pressure among the three genotypes in either men or women who had not received hypertensive medication. Our data suggest that the T(−344)C polymorphism of *CYP11B2* is unlikely to influence blood pressure status in the Japanese population. (*Hypertens Res* 2001; 24: 105-109)

***Key Words*: aldosterone, blood pressure, cytochrome P-450 *CYP11B2*, genetics, hypertension**

Introduction

Aldosterone synthase (*CYP11B2*), a cytochrome P450 enzyme mainly located in the zona glomerulosa of the adrenal cortex, is a key enzyme in aldosterone synthesis (*1, 2*). Aldosterone controls the sodium balance and thus influences blood pressure regulation. Rare mutations of this gene are associated with either markedly elevated aldosterone levels and arterial hypertension or insufficient aldosterone synthesis and sodium wasting (*3-6*). Therefore, genetic variants of the *CYP11B2* gene that affect its expression may contribute to the development of salt-sensitive hypertension.

Several common polymorphisms have recently been described in the *CYP11B2* gene (*7, 8*). The Lys173Arg polymorphism has been suggested to be linked to low-renin hypertension in Chilean hypertensives (*8*). The T(−344)C polymorphism, which is at a putative binding site for the steroidogenic transcription factor (SF-1), has recently been reported to be associated with left ventricular mass in young Finnish adults without clinical heart diseases (*9*).

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We previously reported that the 173Arg allele completely corresponded to the (−344)C allele, and that the (−344)C allele might be a genetic marker for low-renin hypertension in Japanese (*10*). The (−344)C allele was reported to be associated with higher systolic blood pressure in Finns (*11*). The (−344)C allele was reported to be associated with elevated levels of plasma aldosterone, but not in French (*12*).

On the other hand, the (−344)T allele has also been reported to be associated with hypertension in French (*13*) and Scottish (*14*), low-renin hypertension in Japanese (*15*), higher aldosterone level in French (*16*), higher excretion rate of tetrahydroaldosterone in Scottish (*14*), and higher urinary aldosterone excretion and higher 11-deoxycortisol responses to adrenocorticotropin stimulation in Finns (*11*).

Some groups have reported negative results: the T (−344)C polymorphism was not associated with blood pressure in Japanese (*17*), with blood pressure, serum aldosterone level, or cardiac size or function in Germans (*18*), or with salt sensitivity or increased activity of the renin-angiotensin system in young normotensive German men (*19*).

To settle these inconsistent results, we investigated the association between the T(−344)C polymorphism of the *CYP11B2* gene and hypertension in a large sample of the general population in Japan.

Methods

Subjects

The selection criteria and study design of the Suita Study have been described previously (*20, 21*). The sample consisted of 14,200 men and women aged 30 to 79 years stratified by gender and 10-year age groups who were selected randomly from the municipal population registry. The basic sampling of the population started in 1989 with a cohort study base, and 51.7% (n=7,347) of the subjects had paid their initial visit to the National Cardiovascular Center by February 1997. The subjects have visited the National Cardiovascular Center every 2 years for regular health checkups. DNA from leukocytes was collected from subjects who visited the National Cardiovascular Center between May 1996 and February 1998. All of the subjects were Japanese, and only those who gave their written informed consent for genetic analysis of the aldosterone synthase gene were enrolled in the present study (n=4,049). The committee on genetic diagnosis and genetic therapy of the National Cardiovascular Center approved the present study.

Measurements

Systolic blood pressure and diastolic blood pressure values were the means of two physician-obtained measurements (recorded 3 min apart) determined by the first and fifth Korotkoff phases, respectively, in the right arm of the seated subject with a mercury column sphygmomanometer. The pulse pressure was the difference between the systolic and diastolic blood pressure. Hypertension was defined as a systolic blood pressure of ≥140 mmHg, a diastolic blood pressure of ≥90 mmHg, or current use of antihypertensive drugs. Body height and weight were used to calculate body mass index (BMI) (weight (kg)/height^2 (m^2)). Data were collected on clinical variables including age, alcohol consumption, smoking habit, presence of cerebrovascular accident, presence of ischemic heart disease, diabetes mellitus, proteinuria, and use of antihypertensive drugs.

Genotype Determination

DNA was isolated from peripheral leukocytes according to standard procedures (*22*). The T(−344)C polymorphism of the *CYP11B2* gene was genotyped using the polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analysis method described previously (*10*). The designed primers were 5′-CAGGAG GGATGAGCAGGCAGAGCACAG-3′ (sense, from −677 to −651), and 5′-CTCACCCAGGAACCTGCTCT GGAAACATA-3′ (antisense, from −71 to −42). The PCR profile included 34 cycles of denaturing at 94°C for 45 s, annealing at 58°C for 45 s, and polymerization at 72°C for 45 s. The amplified fragments were digested with Hae III restriction enzyme (Takara Shuzou, Otsu, Japan) and subjected to electrophoresis on 2.0% agarose gels. Fragments of 404 bp (T allele) and of 333 bp and 71 bp (C allele) were detected.

Statistical Analysis

All analyses were performed separately for men and women. The difference in genotype or allele distribution between hypertensive and normotensive subjects was examined by a χ^2 analysis. The association between the *CYP11B2* T(−344)C polymorphism and clinical variables was examined by one-way ANOVA. In subjects not receiving antihypertensive medication, multiple linear regression analyses were performed to predict the blood pressure values with other covariates (age, BMI, alcohol consumption, and waist/hip (W/H) ratio). All statistical analyses were performed using the JMP statistical software package (SAS Ins. Inc., Cary, USA).

Table 1. Characteristics of Men and Women Subjects by *CYP11B2* Genotype

Characteristic	*CYP11B2* genotype		
	TT	*TC*	*CC*
Men (n=1,915)	(n=916)	(n=813)	(n=186)
Age (years)	60.8±12.1	61.1±12.2	61.6±12.3
Body mass index (kg/m^2)	22.9±2.7	23.1±2.9	23.2±3.1
Waist-to-hip circumference ratio	0.90±0.05	0.91±0.06	0.90±0.06
Alcohol consumption (ml/d)	26.3±27.2	24.3±26.4	25.2±24.3
Smoking habit (%)	40.7	38.1	40.3
Ischemic heart disease (%)	4.9	4.1	3.8
Diabetes mellitus (%)	20.3	23.3	23.1
Proteinuria (%)	6.6	7.3	9.2
Use of antihypertensive drugs (%)	18.7	17.7	18.3
Women (n=2,134)	(n=1,017)	(n=885)	(n=232)
Age (years)	58.5±11.8	59.1±11.7	59.5±12.0
Body mass index (kg/m^2)	22.4±3.1	22.2±3.3	22.3±2.9
Waist-to-hip circumference ratio	0.88±0.08	0.88±0.07	0.89±0.08
Alcohol consumption (ml/d)	5.0±11.8	5.2±10.6	4.2±8.7
Smoking habit (%)	9.1	7.7	8.2
Ischemic heart disease (%)	3.3	2.5	2.6
Diabetes mellitus (%)	12.2	14.9	16.8
Proteinuria (%)	3.9	3.7	6.5
Use of antihypertensive drugs (%)	16.6	15.7	14.7

Values are mean±SD. *TT* indicates homozygotes for T allele; *TC*, heterozygotes; *CC*, homozygotes for C allele.

Results

Clinical Characteristics and *CYP11B2* Genotype and Allele Frequencies

The overall frequencies of the genotypes *TT*, *TC*, and *CC* were as follows: in men, 47.8%, 42.5%, and 9.7%, respectively; in women, 47.6%, 41.5%, and 10.9%; and overall, 47.7%, 42.0%, and 10.3%. The allele frequencies for T and C were as follows: in men, 69.1% and 30.9%, respectively; in women, 68.4% and 31.6%; and overall, 68.7% and 31.3%. The observed genotype frequencies were in agreement with those predicted by Hardy-Weinberg equilibrium.

Table 1 summarizes the clinical characteristics of men (n=1,915) and women (n=2,134) at the index examination according to the *CYP11B2* genotype. There were no significant differences among the three genotypes with regard to any characteristics in either men or women.

Relationship between the *CYP11B2* Genotype and Blood Pressure

The genotype distribution and the allele distribution of the *CYP11B2* T(−344)C polymorphism in hypertensive subjects were compared to those in normotensive subjects for both men and women (Table 2). No significant differences in the genotype distribution or the allele distribution were observed between hypertensive subjects and normotensive subjects in either men or women.

The influence of the *CYP11B2* genotype on residuals of blood pressure values after adjusting for age, BMI, W/H, and alcohol consumption is shown in Table 3. No significant influence of the genotype on blood pressure was observed.

Discussion

In the present study, we investigated the association between the T(−344)C polymorphism in the promoter region of the human *CYP11B2* gene and blood pressure in a population-based sample (the Suita Study) consisting of 4,049 subjects. Our results indicate that the genotype distribution did not differ between hypertensive and normotensive subjects, and that the genotype had no significant effects on blood pressure values in either men or women.

The association between the T(−344)C polymorphism of the *CYP11B2* gene and hypertension has been investigated by several researchers with conflicting results (*10–19*). These discrepancies may be due to the low statistical power of relatively small-scale association studies. We have also reported an association between the C (−344) allele and low-renin hypertension (*10*). However, we did not find such an association between this polymor-

Table 2. Genotype and Allele Distribution of *CYP11B2* T(−344)C Polymorphism in Hypertensive Subjects and Normotensive Subjects

	Men				Women			
	Hypertensive subjects		Normotensive subjects		Hypertensive subjects		Normotensive subjects	
	n	%	*n*	%	*n*	%	*n*	%
Genotype								
TT	377	49.0	539	47.0	363	47.3	654	47.8
TC	318	41.4	495	43.2	323	42.2	562	41.1
CC	74	9.6	112	9.8	80	10.5	152	11.1
		χ^2=0.76, p=0.68				χ^2=0.36, p=0.84		
Allele								
T	1,072	69.7	1,573	68.6	1,049	68.5	1,870	68.3
C	466	30.3	719	31.4	483	31.5	866	31.7
		χ^2=0.49, p=0.48				χ^2=0.0071, p=0.93		

TT indicates homozygotes for T allele; *TC*, heterozygotes; *CC*, homozygotes for C allele.

Table 3. Blood Pressure of Subjects Not Receiving Antihypertensive Medication by *CYP11B2* Genotype

	CYP11B2 genotype			ANOVA
	TT	*TC*	*CC*	*p*
Men (n=1,556)	(n=745)	(n=669)	(n=152)	
Systolic blood pressure (mmHg)				
Unadjusted	125.9±18.5	126.1±18.0	125.2±18.3	0.87
Age-adjusted	126.1±17.1	125.9±16.5	125.1±17.5	0.82
Fully adjusted*	126.1±16.6	126.0±16.0	125.0±17.3	0.76
Diastolic blood pressure (mmHg)				
Unadjusted	80.1±10.7	80.2±10.3	80.3±11.0	0.98
Age-adjusted	80.1±10.6	80.2±10.2	80.3±11.1	0.99
Fully adjusted*	80.1±10.1	80.2±9.8	80.2±10.8	0.99
Pulse pressure (mmHg)				
Unadjusted	45.8±12.9	45.9±12.9	44.9±12.9	0.71
Age-adjusted	45.9±11.4	45.8±11.4	44.8±11.2	0.56
Fully adjusted*	45.9±11.3	45.8±11.3	44.8±11.3	0.55
Women (n=1,792)	(n=848)	(n=746)	(n=198)	
Systolic blood pressure (mmHg)				
Unadjusted	124.6±18.9	125.3±19.7	125.8±19.8	0.65
Age-adjusted	125.0±17.1	125.1±18.1	125.0±18.0	0.99
Fully adjusted*	124.9±16.7	125.2±17.9	125.0±17.8	0.96
Diastolic blood pressure (mmHg)				
Unadjusted	77.8±10.0	77.7±10.8	77.2±11.7	0.76
Age-adjusted	77.9±9.8	77.6±10.6	77.0±11.6	0.56
Fully adjusted*	77.8±9.5	77.7±10.2	77.0±11.3	0.63
Pulse pressure (mmHg)				
Unadjusted	46.9±13.2	47.6±13.4	48.6±14.9	0.21
Age-adjusted	47.2±11.7	47.4±12.0	48.0±13.3	0.66
Fully adjusted*	47.2±11.6	47.4±12.0	48.0±13.3	0.66

Values are the mean±SD. *TT* indicates homozygotes for T allele; *TC*, heterozygotes; *CC*, homozygotes for C allele. *Multiple linear regression, adjusted for age, body mass index, waist-to-hip circumference ratio, and alcohol consumption.

phism and hypertension in the present study. These different results may be due to the different sampling methods: hospital-based sampling *vs.* population-based sampling. A population of only several hundred subjects may be too small to draw statistically certain conclusions. However, since the present study population was derived from an epidemiological cohort study, certain laboratory tests, such as for plasma renin and aldosterone levels, had not been performed. This is a drawback of the present study population. It is still possible that the *CYP11B2* T(−344)C polymorphism affects aldosterone levels, but not blood pressure levels at large.

Considering the sample size, the *CYP11B2* T(−344)C polymorphism is unlikely to play an important role in the pathogenesis of hypertension in Japanese. However, the present observations do not necessarily exclude the possibility that *CYP11B2* is involved in hypertension. It is possible that some other polymorphism(s) in *CYP11B2* may influence blood pressure. For this reason, it will be necessary to identify a large number of polymorphisms throughout *CYP11B2* and to perform association studies between these polymorphisms and blood pressure in future studies.

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Diabetologia

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·kg^{-1}·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/m²)	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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