

# Association of NAD(P)H Oxidase *p22* *phox* Gene Variation With Advanced Carotid Atherosclerosis in Japanese Type 2 Diabetes

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**OBJECTIVE** — To evaluate the association between the C242T polymorphism of the *p22* *phox* gene, an essential component of NAD(P)H oxidase in the vasculature, with intima-media thickness (IMT) of the carotid artery and risk factors for atherosclerosis in type 2 diabetic subjects.

**RESEARCH DESIGN AND METHODS** — C242T polymorphism of the *p22* *phox* gene was detected by polymerase chain reaction-restriction fragment-length polymorphism in 200 Japanese type 2 diabetic subjects and 215 nondiabetic subjects. We examined the association with this mutation and carotid atherosclerosis as well as the patients' clinical characteristics and the level of 8-hydroxy-2'-deoxyguanosine (8-OHdG) as an index of oxidative DNA damage.

**RESULTS** — The diabetic subjects with the TC+TT genotypes displayed a significantly lower average IMT ( $1.13 \pm 0.31$  vs.  $1.31 \pm 0.34$  mm;  $P = 0.0099$ ) and a not significantly lower serum 8-OHdG level than those with the CC genotype, despite no difference in the risk factors. Stepwise multiple regression analysis showed that the risk factors for increased IMT in the diabetic subjects were systolic blood pressure ( $P = 0.0042$ ) and *p22* *phox* CC genotype ( $P = 0.0151$ ). In nondiabetic subjects, the average IMT of the TC+TT group was not different from that of the CC group ( $0.85 \pm 0.14$  vs.  $0.94 \pm 0.30$  mm,  $P = 0.417$ ). Fasting plasma insulin concentration ( $41.4 \pm 15.6$  vs.  $64.2 \pm 59.4$  pmol/l,  $P = 0.0098$ ) and insulin resistance index of homeostasis model assessment (HOMA-R) ( $1.58 \pm 0.66$  vs.  $2.60 \pm 2.56$ ,  $P = 0.0066$ ) were significantly lower in the TC+TT group than in the CC group.

**CONCLUSIONS** — These results show that the C242T mutation in the *p22* *phox* gene is associated with progression of asymptomatic atherosclerosis in the subjects with type 2 diabetes and is also associated with insulin resistance in nondiabetic subjects.

*Diabetes Care* 26:458–463, 2003

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Received for publication 9 June 2002 and accepted in revised form 30 September 2002.

**Abbreviations:** 8-OHdG, 8-hydroxy-2'-deoxyguanosine; CHD, coronary heart disease; HOMA-R, insulin resistance index of homeostasis model assessment; IMT, intima-media thickness; ROS, reactive oxygen species.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

The production of vascular reactive oxygen species (ROS) is increased in pathophysiological conditions such as atherosclerosis, hypertension, and diabetes (1–3).

NAD(P)H oxidase is a membrane-associated enzyme that catalyzes the 1-electron reduction of oxygen using NAD(P)H as the electron donor. Several studies indicate that NAD(P)H oxidase is the most important source of ROS in intact arteries, rather than arachidonic acid-metabolizing enzymes, xanthine oxidase, or mitochondrial sources (4–8). It has recently been shown that high glucose stimulates ROS production through the protein kinase C-dependent activation of NAD(P)H oxidase in both vascular smooth muscle cells and endothelial cells (9) and in diabetic vessels (10,11).

The *p22* *phox*, cytochrome *b*<sub>558</sub> of vascular NAD(P)H oxidase, plays an important role in the process of O<sub>2</sub><sup>-</sup> production. Expression of *p22* *phox* was markedly increased in the aorta from OLETF rats compared with that of LETO rats (12). Recently, Guzik et al. (11) showed that the *p22* *phox* was significantly increased in human diabetic veins and arteries. This activation of *p22* *phox* may contribute to the acceleration of atherosclerosis in patients with diabetes.

The C242T polymorphism of the *p22* *phox* gene has been demonstrated to be associated with significantly lower basal and NADH-stimulated vascular superoxide production in human blood vessels from patients with atherosclerosis (13). However, controversy still exists regarding the possible anti-atherogenic action of the <sup>242</sup>T allele of the *p22* *phox* gene against coronary heart disease, as shown in several studies (14–21).

We examined the possible effect of this mutation on carotid atherosclerosis in subjects with type 2 diabetes and in healthy nondiabetic subjects, as well as the patients' clinical characteristics and the level of 8-hydroxy-2'-deoxyguanosine

(8-OHdG) as an index of oxidative DNA damage. 8-OHdG is a major product of oxidative DNA damage and is produced by enzymatic cleavage after 8-hydroxylation of the guanine base of DNA (22–26).

## RESEARCH DESIGN AND METHODS

### Subjects

A total of 200 Japanese subjects with type 2 diabetes (127 men and 73 women aged  $58.7 \pm 8.2$  years, duration of diabetes  $12.5 \pm 8.6$  years, mean  $\pm$  SD) diagnosed by the previous World Health Organization criteria in 1985 were recruited from the outpatient diabetes clinic of Osaka University Hospital. Of the diabetic subjects, the disease was controlled in 38 subjects with diet only, in 107 subjects with oral hypoglycemic agents, and in 59 subjects with insulin injections. As a control, we enrolled 215 healthy Japanese nondiabetic volunteers (132 men and 83 women aged  $42.6 \pm 9.6$  years, mean  $\pm$  SD). Subjects with liver disease, renal dysfunction, and/or collagen disease were excluded. The control subjects all met the following criteria: 1) no evidence of liver disease, cardiovascular disease, or impaired renal function; 2) fasting plasma glucose level  $<7$  mmol/l or a HbA<sub>1c</sub> level  $<6.0\%$ . Subjects who had taken lipid-lowering drugs, antihypertensive drugs, or antidiabetic drugs were excluded. Experiments were conducted in accordance with the Osaka University Graduate School of Medicine guidelines. Written consent (informed consent) was obtained from every subject after a full explanation of the study. The Ethics Committee of Osaka University approved this study.

Clinical and laboratory examinations were performed on the subjects. The systolic and diastolic blood pressures used in the analysis were the average of three sitting blood pressure readings. Smoking was defined as current smoking. After at least an 8-h fast, blood samples were collected from the subjects, and serum total and HDL cholesterol, triglyceride, uric acid, creatinine, plasma glucose, and HbA<sub>1c</sub> levels were determined by the Clinical Research Center in Osaka University Hospital following standard laboratory protocols. LDL cholesterol was calculated using the Friedewald formula. For nondiabetic subjects, the insulin resistance index of homeostasis model assessment (HOMA-R) was calculated from

fasting plasma glucose and insulin levels (27).

For diabetic subjects, urinary albumin of a fasting urine specimen and a specimen collected at least 4 weeks later was measured by radioimmunoassay. The two measurements of urinary albumin were averaged. The level was divided by the urinary creatinine concentration and expressed as milligrams per gram of creatinine (28). Diabetic retinopathy was diagnosed by ophthalmologists through funduscopy and/or fluorescein angiography after papillary dilation. Checking for the occurrence of coronary heart disease (CHD: angina pectoris and myocardial infarction) was performed based on the results of the resting 12-lead electrocardiogram and double Master's two-step tests plus either enzyme elevations or the existence of previous symptoms of myocardial infarction in subjects with type 2 diabetes.

### Measurement of 8-OHdG

A competitive enzyme-linked immunosorbent assay was used for the determination of the level of serum 8-OHdG (high sensitive 8-OHdG check; Fukuroi, Shizuoka, Japan) in nonsmoking subjects, as previously described (23–26). To avoid the auto-oxidation of 8-OHdG during the experimental procedure, all solutions were saturated with nitrogen gas, and all tubes were flushed with nitrogen gas just before use. The coefficient of variation for repeated 8-OHdG measurements was 8% over all ranges.

### Measurement of intima-media thickness (IMT)

Ultrasonographic scanning of the carotid artery was performed using an echotomographic system (Toshiba, Tokyo, Japan) with an electrical liner transducer (mid-frequency 8.0 MHz). Scanning of the extracranial common carotid artery, the carotid bulb, and the internal carotid artery was performed bilaterally from three different longitudinal projections (i.e., anterior-oblique, lateral, and posterior-oblique) as well as the transverse projections, as reported in our previous studies (29–32). All of the images were photocopied. The detection limit of this echotomographic system using 8.0 MHz was 0.1 mm. The IMT defined by Pignoli et al. (33,34) was measured as the distance from the leading edge of the first echogenic line to the leading edge of the sec-

ond echogenic line. The first line represented the lumen intimal interface, and the second line was produced by the collagen-containing upper layer of the tunica adventitia. At each longitudinal projection, the site of the greatest thickness including a plaque lesion was sought along the arterial walls nearest the skin and farthest from the skin from the common carotid artery to the internal carotid artery. Three determinations of IMT were conducted at the site of the thickest point and two adjacent points (located 1 cm upstream and 1 cm downstream from this site). These three determinations were averaged. The greatest value among the six averaged IMTs (three from the left and three from the right) was used as the representative value for each individual. All ultrasound scans were performed by an experienced sonographer (K.S.), and an experienced physician (N.K.) performed determination of IMT on the photograph. These two individuals were unaware of the subject's study group and clinical characteristics. The reproducibility of the IMT measurement was examined 1 week later on 25 type 2 diabetic participants by the same sonographer and the same physician. The mean difference in IMT between these two determinations was 0.04 mm (SD 0.06 mm), demonstrating good reproducibility for repeated measurements, as described previously (29–32).

### Genotyping

Genomic DNA was extracted from peripheral blood cells using a DNA extraction kit (QIAamp DNA Blood Kit; Qiagen). The C242T polymorphism at exon 4 of the p22 phox gene was determined by PCR and *RsaI* digestion, as described previously (14). In case of incomplete digestion, a second *RsaI* restriction site was included in the PCR amplification product as an internal control.

The DNA fragment containing the polymorphic site was amplified from genomic DNA by PCR with sense oligonucleotide primer 5'-TGCTTGTGGGTAACCAAGGCCGGTG-3' and antisense oligonucleotide primer 5'-AACACTGAGGTAAGTGGGGGTGGCTCCTGT-3'. The PCR consisted of the initial denaturation step for 5 min at 94°C, 35 cycles of denaturation for 50 s at 94°C, primer annealing for 50 s at 60°C, and primer extension for 30 s at 72°C, followed by a final extension step for 7 min at 72°C in a Gene Amp PCR system 2400

Table 1—Clinical characteristics of the study subjects by p22 phox polymorphism

	Subjects with type 2 diabetes			Nondiabetic subjects		
	CC	TC+TT	P	CC	TC+TT	P
N (men/women)	173 (108/65)	27 (19/8)	NS*	182 (114/68)	33 (18/15)	NS*
Age (years)	58.9 ± 7.8	57.6 ± 10.6	NS†	42.8 ± 9.4	41.2 ± 10.4	NS
Duration of diabetes (years)	12.8 ± 8.6	10.3 ± 8.7	NS	—	—	—
BMI (kg/m <sup>2</sup> )	23.5 ± 4.4	24.5 ± 3.1	NS†	22.7 ± 3.1	22.5 ± 2.9	NS
Systolic blood pressure (mmHg)	134 ± 15	134 ± 15	NS	123 ± 15	121 ± 14	NS
Diastolic blood pressure (mmHg)	77 ± 9	75 ± 10	NS	74 ± 9.8	71 ± 10	NS
Total cholesterol (mmol/l)	5.21 ± 0.91	5.30 ± 0.97	NS	5.21 ± 0.81	5.26 ± 0.83	NS
Triglycerides (mmol/l)	1.46 ± 0.70	1.86 ± 1.11	NS†	1.28 ± 0.74	1.13 ± 0.57	NS
HDL cholesterol (mmol/l)	1.41 ± 0.43	1.44 ± 0.50	NS	1.57 ± 0.36	1.65 ± 0.37	NS
LDL cholesterol (mmol/l)	3.22 ± 0.97	3.06 ± 1.10	NS	2.99 ± 0.69	3.08 ± 0.69	NS
HbA <sub>1c</sub> (%)	7.57 ± 3.03	6.84 ± 1.43	NS†	4.82 ± 0.47	4.66 ± 0.32	NS†
Fasting glucose (mmol/l)	—	—	—	5.33 ± 0.63	5.11 ± 0.39	NS†
Fasting insulin (pmol/ml)	—	—	—	64.2 ± 59.4	41.4 ± 15.6	0.0098†
HOMA-R	—	—	—	2.60 ± 2.56	1.58 ± 0.66	0.0066†
8-OHdG (ng/ml)	1.51 ± 0.75	1.12 ± 0.34	NS	1.29 ± 0.71	1.26 ± 0.27	NS†
Smoking (yes/no)	50/123	7/20	NS*	51/131	10/23	NS*
IMT (mm)	1.31 ± 0.34	1.13 ± 0.31	0.0099	0.94 ± 0.30	0.85 ± 0.14	NS†
CHD (abnormal Q/angina/myocardial infarction)	10/2/7	2/0/1	NS*	—	—	—

Data are means ± SD. \*χ<sup>2</sup> test was performed; †Mann-Whitney U test was performed.

(Perkin-Elmer, Norwalk, CT). After digestion with RsaI (TOYOBO Co.), the samples were separated on 3% agarose gel and visualized with ethidium bromide. Digestion of the PCR products yielded bands of 348 bp in CC homozygotes, 188 and 160 bp in TT homozygotes, and all three bands in heterozygotes. Each genotype was read by two individuals independently; if in conflict, genotyping was repeated.

**Statistical analysis**

Data were expressed as means ± SD. The statistical significance of differences in mean values was analyzed by unpaired Student's t tests, after confirmation of a normal distribution. Mann-Whitney U tests were used when the data were skewed. The significance of differences in frequency was determined by the χ<sup>2</sup> test. The allele frequency of subjects with and without advanced carotid atherosclerosis (IMT ≥ 1.1 mm) was examined by using Fisher's exact test. Forward and backward stepwise multivariate regression analyses were performed using the F value for the inclusion and exclusion of variables of 2.0. The analyses were performed with Stat-View (Abacus Concepts, Berkeley,

CA) and HALBOU (Gendai Sugaku-sha, Kyoto, Japan) software for the Windows operating system. The threshold of statistical significance was defined as P < 0.05.

**RESULTS**

The frequencies of the C242T genotypes for CC, TC, and TT were 86.5, 12.0, and 1.5% in the subjects with type 2 diabetes, compared with 84.7, 13.4, and 1.9% in the control subjects. These distributions were in accordance with Hardy-Weinberg equilibrium. The <sup>242</sup>T allele frequencies in type 2 diabetes and control subjects were 0.08 and 0.09, respectively, and there were no differences between patients and control subjects in allele frequencies. This frequency was approximately one third that of Caucasian populations but did not differ from previous studies of Japanese populations (14,15,21). Therefore, the subjects were divided into two groups (CC and TC+TT) (13–20) for further evaluation.

In diabetic subjects, there was no significant difference between these two groups in sex, age, BMI, smoking habit, duration of diabetes, systolic and diastolic blood pressures, or biochemical examinations such as serum total, HDL, and LDL cholesterol, triglyceride, and HbA<sub>1c</sub> levels

(Table 1). There was no significant difference in the allele frequency according to the diabetic medication in those subjects (data not shown). Despite no difference in the risk factors that can affect atherosclerosis, the CC group displayed significantly higher average IMT values than the TC+TT group (1.31 ± 0.34 vs. 1.13 ± 0.31 mm, P = 0.0099). There was no significant difference in the frequency of diabetic retinopathy, the level of urinary albumin/creatinine ratio (data not shown), or the prevalence of CHD in the genotype groups. In diabetic nonsmoking subjects, 8-OHdG levels of the TC+TT group were not different from those of the CC group (1.12 ± 0.34 vs. 1.51 ± 0.75 ng/ml, P = 0.390).

In nondiabetic subjects, there were no significant differences between the CC group and TC+TT group in sex, age, smoking habit, BMI, blood pressure, or lipid profiles such as total cholesterol, triglyceride, HDL cholesterol, or LDL cholesterol levels (Table 1). Fasting plasma glucose levels of the TC+TT group were not different from those of the CC group (5.11 ± 0.39 vs. 5.33 ± 0.63 mmol/l, P = 0.069). Fasting plasma insulin levels (41.4 ± 15.6 vs. 64.2 ± 59.4 pmol/l, P =

**Table 2—Prevalence of genotype and allele frequencies of p22 phox polymorphism with dichotomizing IMT at 1.1 mm in the subjects**

Genotype	IMT $\geq$ 1.1 mm	IMT <1.1 mm	P
Diabetic subjects			
CC	124 (89.9)	49 (79.0)	0.045
TC+TT	12 + 2 (10.1)	12 + 1 (21.0)	—
T allele frequency	0.058	0.113	—
Nondiabetic subjects			
CC	41 (87.2)	141 (83.9)	0.654
TC+TT	4 + 2 (12.8)	25 + 2 (16.1)	—
T allele frequency	0.085	0.086	—

Data are n (%). Fisher's exact test was performed.

0.0098) and HOMA-R ( $1.58 \pm 0.66$  vs.  $2.60 \pm 2.56$ ,  $P = 0.0066$ ) were significantly lower in the TC+TT group than in the CC group. The average IMT of TC+TT group was not different from that of the CC group ( $0.85 \pm 0.14$  vs.  $0.94 \pm 0.30$  mm,  $P = 0.417$ ). No difference in serum 8-OHdG levels existed between the CC group and the TC+TT group ( $1.29 \pm 0.71$  vs.  $1.26 \pm 0.27$  ng/ml,  $P = 0.725$ ) (Table 1). Between the type 2 diabetic subjects and nondiabetic subjects, serum 8-OHdG levels showed no significant difference ( $1.46 \pm 0.72$  vs.  $1.29 \pm 0.61$  ng/ml,  $P = 0.260$ ).

The 1.1-mm cutoff point was chosen due to the upper limit of IMT in the normal control subjects as previously described (29,30,35) (Table 2). T allele frequencies of C242T polymorphism in the subjects with carotid IMT <1.1 mm were significantly higher than those in subjects with carotid IMT  $\geq$ 1.1 mm (0.113 vs. 0.058). In nondiabetic subjects, on the other hand, T allele frequencies showed no significant difference.

Forward and backward stepwise multivariate regression analyses depicted systolic blood pressure ( $P = 0.0042$ ) and p22 phox CC genotype ( $P = 0.0151$ ) as independent risk factors for an increase in IMT in the subjects with type 2 diabetes within the following variables: sex, age, duration of diabetes, BMI, systolic blood pressure, diastolic blood pressure, smoking habit, HbA<sub>1c</sub>, total cholesterol, triglycerides, HDL cholesterol, creatinine, and p22 phox CC genotype (Table 3).

**CONCLUSIONS**— In the present study, we have shown that the C242T polymorphism of the p22 phox gene is associated with carotid IMT in diabetic patients and is also associated with

plasma insulin and HOMA-R in nondiabetic subjects.

p22 phox is a key component of the cytochrome *b*<sub>558</sub> of the NAD(P)H oxidase, which transfers the electron, and plays an important role in the process of O<sub>2</sub><sup>-</sup> production. The C242T polymorphism of p22 phox gene (amino acid conversion from histidine to tyrosine at a heme-binding site) reduces the activation of NAD(P)H oxidase and is associated with significantly lower basal and NADH-stimulated vascular superoxide production in human blood vessels (13). The diabetic subjects with CC genotype might have more activated NAD(P)H oxidase through the protein kinase C pathway (9–11), although no difference in serum 8-OHdG levels existed between the CC group and the TC+TT group in this study. 8-OHdG is a degradation product of DNA oxidation, and measurement of its serum or urine level provides information on various degrees of oxidative stress in the DNA level (22–24). Therefore, we measured 8-OHdG as one of the clinical markers for vascular oxidative stress (25,26). However, further investigations of clinical markers may assess a possible difference in oxidative stress between the p22 phox C242T genotypes. The carriers

of the CC genotype of this polymorphism manifest a significantly blunted endothelium-dependent dilator response (20). Therefore, the TC+TT diabetic carriers, in whom NAD(P)H oxidase might be reduced even in the diabetic state, might have a reduced ROS production and showed a significantly lower average IMT than the CC diabetic carriers.

In nondiabetic subjects, fasting plasma insulin levels ( $P = 0.0098$ ) and HOMA-R ( $P = 0.0066$ ) were significantly lower in the TC+TT group than in the CC group. The average IMT showed no significant difference between the genotype groups. These results are compatible with the hypothesis that the p22 phox polymorphism affects plasma insulin concentration and HOMA-R in subjects with preserved insulin secretion. There is possible involvement of increased oxidative stress in the pathogenesis and progression of insulin resistance. In an in vitro study, Rudich et al. (36) showed that prolonged oxidative stress impairs insulin-induced Glut4 translocation in 3T3-L1 adipocytes. Also, several clinical trials have reported beneficial metabolic effects of antioxidant supplementation (37–39). However, direct evidence that oxidative stress impairs insulin action in nondiabetic subjects requires further investigation.

The anti-atherogenicity of the <sup>242</sup>T allele of the p22 phox gene, especially the low prevalence of CHD as reported by several studies (13,14,21), is still controversial (15–20). The conflicting results may reflect the ethnic backgrounds of the study populations. In our study, evaluation of CHD was performed based on the results of the resting 12-lead electrocardiogram and double Master's two-step tests plus either enzyme elevations or the existence of previous symptoms of myocardial infarction. To further elucidate the potential association between p22 phox

**Table 3—Risk factors for elevated IMT in the subjects with type 2 diabetes**

Risk factor	$\beta$	F	P
Age (years)	0.0065	3.594	0.0599
BMI (kg/m <sup>2</sup> )	-0.0113	3.470	0.0645
Systolic blood pressure (mmHg)	0.0051	8.471	0.0042
Total cholesterol (mmol/l)	0.0541	3.149	0.0781
p22 phox CC genotype	0.2045	6.053	0.0151

A stepwise multivariate regression analysis was performed.  $R^2 = 0.1502$  for p22 phox polymorphism; CO = 0, TC+TT = 1.

C242T polymorphism and CHD, other studies with coronary angiography would be required.

Our study has shown that the average IMT was significantly lower in the diabetic TC+TT carriers and not significantly lower in the nondiabetic TC+TT carriers. This inconsistency may be related to the fact that the impact of the C242T mutation in the p22 phox gene may be amplified in subjects with certain risk factors such as diabetes. In the present study, a difference regarding age between diabetic subjects ( $58.7 \pm 8.2$  years) and control subjects ( $42.6 \pm 9.6$  years) might exist, although T allele frequency of the p22 phox showed no significance between two groups. Evaluation of carotid artery thickening in populations with various risk factors or Caucasian populations might be helpful in understanding the clinical significance of the p22 phox gene.

**Acknowledgments**— We thank Noriko Fujita for excellent technical assistance. We also thank all the patients who participated in this study and the nursing staff of the Division of Internal Medicine at Osaka University Hospital.

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