

Functional Variants in the Glutathione Peroxidase-1 (*GPx-1*) Gene Are Associated With Increased Intima-Media Thickness of Carotid Arteries and Risk of Macrovascular Diseases in Japanese Type 2 Diabetic Patients

Tohru Hamanishi,¹ Hiroto Furuta,¹ Hisako Kato,¹ Asako Doi,¹ Masanori Tamai,¹ Hiroko Shimomura,¹ Setsuya Sakagashira,¹ Masahiro Nishi,¹ Hideyuki Sasaki,¹ Tokio Sanke,² and Kishio Nanjo¹

Atherosclerosis in type 2 diabetic patients has been linked to increased oxidative stress. Glutathione peroxidase-1 (*GPx-1*) plays an important role in the antioxidant defense of the vascular wall. To assess the association between variants in the *GPx-1* gene and atherosclerosis, we screened the gene in 184 Japanese type 2 diabetic patients and identified four polymorphisms (-602A/G, +2C/T, Ala⁵/Ala⁶, and Pro198Leu). Among these polymorphisms, -602G, +2T, Ala⁶, and 198Leu were in strong linkage disequilibrium with each other. The patients were divided into two groups on the basis of the codon 198 polymorphism, Pro/Pro ($n = 151$) and Pro/Leu ($n = 33$), to analyze clinical characteristics. The mean intima-media thickness (IMT) of common carotid arteries ($P = 0.0028$) and the prevalence of cardiovascular disease ($P = 0.035$) and peripheral vascular disease ($P = 0.027$) were significantly higher in the Pro/Leu group than in the Pro/Pro group. In vitro functional analyses indicated that the combination of polymorphisms (Ala⁶/198Leu) of the *GPx-1* gene had a 40% decrease in enzyme activity, and the combination of polymorphisms (-602G/+2T) had a 25% decrease in transcriptional activity. These results suggest that functional variants in the *GPx-1* gene are associated with increased IMT of carotid arteries and risk of cardiovascular and peripheral vascular diseases in type 2 diabetic patients. *Diabetes* 53:2455-2460, 2004

Type 2 diabetes is known to be associated with excessively high mortality and morbidity from macrovascular disease (1), and it is important to clarify its pathophysiology. The metabolic abnormalities that characterize diabetes, such as hyperglycemia,

dyslipidemia, and insulin resistance, provoke increased oxidative stress, protein kinase C (PKC) activation, and the activation of the receptor for advanced glycation end products (AGEs). Hyperglycemia directly produces a reactive oxygen species (such as superoxide anion) via increased production from the mitochondrial electron transport chain (2) and glucose autooxidation (3). The reactive oxygen species induce endothelial injury, oxidative modification of LDL, and induction of redox-sensitive genes, including monocyte chemoattractant protein 1 and adhesion molecules such as vascular cell adhesion molecules (4). In addition, oxidative stress activates PKC and increases the production of AGEs (2). The activation of PKC and the activation of the receptor for AGEs also contribute to superoxide generation (5,6). These molecular mechanisms seem to be mainly implicated in the development of atherosclerosis (4).

On the other hand, antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase (GPx), are expressed in the blood vessels (7) and counteract against oxidative stress. One of these enzymes, GPx, which is a soluble selenoprotein, reduces H₂O₂ and organic hydroperoxides to H₂O and the corresponding alcohols using reduced glutathione as an essential co-substrate. To date, five GPx isoenzymes have been identified. GPx-1 (a cytosolic form of GPx) is the most abundant and ubiquitous intracellular isoform (8). GPx-1 mRNA is expressed in vascular endothelial cells where laminar shear stress upregulates the expression and enzymatic activity (9). Recent studies show that the heterozygous deficiency of GPx-1 leads to endothelial dysfunction (10) and to significant structural vascular and cardiac abnormalities (11). These findings suggest that GPx-1 is a key enzyme in protecting vessels against oxidative stress and atherogenesis.

The *GPx-1* gene is located on chromosome 3p21.3 (12) and contains two exons (13). In an attempt to evaluate whether the *GPx-1* gene variants are associated with atherosclerosis in type 2 diabetic patients, we screened genetic variants in all exons, including 5'- and 3'-untranslated regions, intron, and ~1.8 kb of the promoter region of the *GPx-1* gene. We characterized the functional properties and evaluated the association to intima-media thick-

From ¹The First Department of Medicine, Wakayama University of Medical Science, Wakayama, Japan; and the ²Department of Clinical Laboratory Medicine, Wakayama University of Medical Science, Wakayama, Japan.

Address correspondence and reprint requests to Kishio Nanjo, MD, PhD, The First Department of Medicine, Wakayama University of Medical Science, 811-1 Kimiidera, Wakayama 641-8509, Japan. E-mail: k-nanjo@wakayama-med.ac.jp.

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AGE, advanced glycation end product; BAEC, bovine aortic endothelial cell; GPx, glutathione peroxidase; IMT, intima-media thickness; PKC, protein kinase C.

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TABLE 1
List of sequences of primers used

		Product size (bp)	Taq polymerase	Annealing temperature (°C)
Promoter-1 forward	5'-TCAGAAGTCATCTTGCTACCAGTA-3'	866	LA Taq	60
Promoter-1 reverse	5'-ACTGAGTGCCACCCATGAGAACTG-3'			
Promoter-2 forward	5'-AGGAATTGGTTTCACGCTGAGGCA-3'	1177	LA Taq	60
Promoter-2 reverse	5'-CGCGCCGAGAAGGCATACACCGAC-3'			
Promoter-2F-1	5'-ACTTCCTGGCCTAGCTCACCCCTGG-3'			
Promoter-2F-2	5'-GACCAGACATGCCTGCTGCTCCTT-3'			
Exon1-1 forward	5'-CGCTCCGCTGGCTTCTTGACAATTG-3'	384	AmpliTaq Gold	60
Exon1-1 reverse	5'-GACTCTCCGCGCATGGAGCCGACG-3'			
Intorn1-1 forward	5'-GGTACTACTTATCGAGAATGTGGC-3'	510	LA Taq	65
Intorn1-1 reverse	5'-GCACTTCTCGAAGAGCATGAAGTT-3'			
Exon2-1 forward	5'-CTGTTTGTGGTTAGAACGTTTCTC-3'	320	AmpliTaq Gold	60
Exon2-1 reverse	5'-ACCAGGAACCTCTCAAAGTTCCAG-3'			
Exon2-2 forward	5'-CGCCACCGCGCTTATGACCG-3'	187	AmpliTaq Gold	60
Exon2-2 reverse	5'-GCAGCACTGCAACTGCCAAGCAG-3'			
Exon2-3 forward	5'-AAGTTCCTGGTGGGCCCTGACGGT-3'	514	LA Taq	60
Exon2-3 reverse	5'-CAGCTACTCAGGAGGTTGAGGCAG-3'			

Two primers, promoter-2F-1 and -2F-2, were used only for sequencing.

ness (IMT) of the common carotid arteries and macrovascular complications.

RESEARCH DESIGN AND METHODS

A total of 184 unrelated Japanese type 2 diabetic patients were recruited from patients attending the outpatient clinic of the Wakayama University of Medical Science Hospital. In all subjects, the IMT of common carotid arteries was measured with high-resolution B-mode ultrasonography. This group consisted of 102 men and 82 women. The average age was 63.7 ± 9.1 years (range 29–84), BMI was 23.7 ± 3.0 kg/m² (range 15.2–29.7), and age at diagnosis was 44.8 ± 8.5 years (range 25–60). Of the subjects, 24% were treated with diet, 41% with oral agents, and 35% with insulin. Subjects who were GAD antibody positive and/or had started insulin therapy within 3 years of the diagnosis of diabetes were excluded from this study. Diabetes was diagnosed according to the criteria of the World Health Organization. All the participants gave written informed consent before participating in the study. This study was approved by the ethics committee of the Wakayama University of Medical Science.

Screening for polymorphisms in the GPx-1 gene. All exons including 5'- and 3'-untranslated regions, intron, and ~1.8 kb of the promoter region were amplified with a PCR and sequenced in DNA samples from 20 Japanese type 2 diabetic patients. PCR was carried out using AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA) or LATaq polymerase (TaKaRa Biotechnology, Kusatu, Japan). Sequencing was carried out using a Big Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems) on an automated DNA capillary sequencer (model 310; Applied Biosystems). Polymorphisms identified in this screening were designated according to their location from the transcription start site described by Moscow et al. (14). Sequence information of the primers and the conditions for PCR direct sequencing are shown in Table 1.

Genotyping of polymorphisms in the GPx-1 gene. Polymorphisms identified were genotyped in 184 Japanese type 2 diabetic subjects using PCR direct sequencing. Genotyping was performed using primers (promoter-2 forward and promoter-2 reverse) for the A to G polymorphism at -602 and the C to T polymorphism at +2, primers (exon1-1 forward and exon1-1 reverse) for the Ala⁵/Ala⁶ in exon 1, and primers (exon2-2 forward and exon2-2 reverse) for the Pro198Leu missense mutation in exon 2. We estimated the haplotypes using the EH program (<http://linkage.rockefeller.edu>) and calculated linkage disequilibrium coefficients (D' and Δ^2) using the Graphical Overview of Linkage Disequilibrium (GOLD) program (<http://www.sph.umich.edu/csg/abecasis/GOLD>).

Measurement of IMT. The IMT of common carotid arteries defined by Pignoli and Longo (15) was measured with high-resolution B-mode ultrasonography (Power Vision 7000; Toshiba, Tokyo, Japan) using a 7.5-MHz electrical linear transducer. Scanning of the common carotid arteries was performed bilaterally from two different longitudinal projections (i.e., anterior-oblique, lateral) as well as a transverse projection. At each longitudinal projection, the site of the greatest thickness was sought along the arterial walls nearest the skin and farthest from the skin at the common carotid

arteries. Localized thickness of >2.0 mm was excluded from the measurement as plaque lesion. Three determinations of IMT were conducted at the site of the greatest thickness and at two other points: 1 cm upstream and 1 cm downstream from this site. The average of these six determinations (three on each side) was used as the representative value for each individual.

Assessment of complications. Assessment of macrovascular disease was based on each patient's medical records. Cardiovascular disease (myocardial infarction and angina pectoris) and cerebrovascular disease (stroke) were diagnosed according to the criteria used in the Japan Diabetes Complication Study (16). Peripheral vascular disease was diagnosed if there was intermittent claudication with a low ankle-brachial index, i.e., <0.9. Subjects with a systolic blood pressure >140 mmHg and/or with a diastolic blood pressure >90 mmHg or on antihypertensive treatment were defined as hypertensive. Subjects with total cholesterol >5.69 mmol/l (220 mg/dl), HDL cholesterol <1.03 mmol/l (40 mg/dl), and/or triglycerides >1.70 mmol/l (150 mg/dl), or on antihyperlipidemic medication were defined as hyperlipidemic. The existence of nephropathy was determined if a patient had overt proteinuria (two successive urinalyses positive by reagent strip) and/or an elevated serum creatinine level (>1.2 mg/dl). Patients with simple, preproliferative, or proliferative retinopathy were assumed to have retinopathy.

Measurement of GPx enzyme activity in GPx-1-transfected bovine aortic endothelial cells. Two human GPx-1 clones (UI-E-CL1-AEL-E-04-0-UI and UI-CF-EC1-ABK-A-09-0-UI) were obtained from the EST clone collection (Open Biosystems, Huntsville, AL). UI-E-CL1-AEL-E-04-0-UI has a combination of polymorphism and Ala⁵/198Pro, and UI-CF-EC1-ABK-A-09-0-UI has a combination of polymorphism and Ala⁶/198Leu. An 800-bp *Xho*I-*Xho*I fragment, which also contained a selenocysteine insertion sequence in the 3'-untranslated region (17), was subcloned into the plasmid pcDNA3.1 (Invitrogen, Carlsbad, CA), in which expression is driven by a cytomegalovirus promoter.

Bovine aortic endothelial cells (BAECs) were obtained from Dainippon Pharmaceuticals (Osaka, Japan) and maintained in Dulbecco's modified Eagle's medium containing 4,500 mg/l glucose, 10% FBS, and antibiotics (100 units/ml penicillin G sodium and 100 mg/ml streptomycin sulfate). The BAECs (passages 5–10) were seeded into six-well culture plates. Two micrograms each of pcDNA3.1-GPx-1 constructs (pcDNA3.1-GPx-1-Ala⁵/198Pro and pcDNA3.1-GPx-1-Ala⁶/198Leu) or pcDNA3.1 plasmid was transiently transfected to the BAECs using a FuGENE6 transfection reagent (Roche Diagnostics, Mannheim, Germany). After 24 h, the BAECs were washed three times with ice-cold PBS and gently scraped with a lysis buffer (50 mmol/l Tris/HCl: pH 7.5, 5 mmol/l EDTA and 1 mmol/l dithiothreitol). The cell suspension was homogenized with an ultrasonicator (2×5 s) on ice. GPx activity was then determined from the supernatant by coupling the reduction of peroxides (t-butylperoxide) and the oxidation of glutathione using NADPH as a cofactor with a commercially available kit (Cayman, Ann Arbor, MI). The specific activity (per milligram protein) was calculated after protein determination by a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA). In preliminary experiments, a plasmid coding for green fluorescence protein had been transfected under the same condition and indicated that ~20% of cells

demonstrate green fluorescence. The data presented represent the means of five independent transfection experiments per construct. The difference in the averaged activity among three groups was analyzed by one-way ANOVA, and a Fisher's protected least-significant difference test was used for the post hoc test. The difference in the averaged increment of activity between Ala⁵/198Pro and Ala⁶/198Leu-transfected BAECs was analyzed by an unpaired Student's *t* test.

Measurement of promoter activities. DNA fragments corresponding to the basal promoter of GPx-1 (nucleotide -819 to +44) were amplified by PCR using genomic DNA from two patients with different single nucleotide polymorphism combinations (-602A/G and +2C/T, -602A/G and +2C/C) as a template. The PCR products were subcloned into a pGL3-basic firefly luciferase reporter vector (Promega, Madison, WI) in the 5'-3' orientation, and the luciferase reporter plasmids representing three genotypes (-602A/+2C, -602G/+2C, and -602G/+2T) were made.

We transiently transfected 0.25 µg of one of the constructs with 0.01 µg pRL-SV40 vector (renilla luciferase under control of SV40 promoter), as an internal control for transfection efficiency, into the BAECs (passages 5-10) using a FuGENE6 transfection reagent. After 24 h, we collected the cells and measured luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). The luciferase relative activity for each construct was calculated as a fold increase over the activity for the negative control empty vector (pGL3-basic). The data presented represent the means of five independent transfection experiments per construct. The differences in the averaged activity among three groups were analyzed by one-way ANOVA, and a Fisher's protected least-significant difference test was used for the post hoc test.

Statistical analysis. Results are presented as means ± SD or *n* (%). Differences between the type 2 diabetic patient groups divided on the basis of the codon 198 and -602A/G polymorphisms were assessed for statistical significance using an unpaired Student's *t* test, Mann-Whitney's *U* test (when the data were skewed), and a χ^2 test. We used a multivariable regression analysis to estimate the relationship between the GPx-1 genotype (Pro/Pro = 0, Pro/Leu = 1) and the value of IMT and a multivariable logistic regression analysis for comparing the GPx-1 genotype and the prevalence of macrovascular disease with adjustment for common vascular risk factors (age, sex, HbA_{1c}, hyperlipidemia, hypertension, and a smoking habit). All analyses were performed with the StatView program for Windows (version 5.01; SAS Institute, Cary, NC). *P* < 0.05 was considered statistically significant.

RESULTS

Polymorphisms in the GPx-1 gene. All exons, intron, and ~1.8 kb of the promoter region were screened in 20 Japanese type 2 diabetic patients, and four polymorphisms were identified (Fig. 1). These polymorphisms include a G for A substitution at -602 (-602A/G), a T for C substitution at +2 (+2C/T), a six for five polyalanine polymorphism at codon 7-11 (Ala⁵/Ala⁶), and the leucine (CTC) for proline (CCC) amino acid substitution at codon 198 (Pro198Leu) and have been previously reported in the study for patients with lung cancer (18) (-602A/G was described as -592A/G in the article). We found a mistake in the original sequence used for the numbering and changed it to the exact number in this study. The reference single nucleotide polymorphism identification (SNP ID) numbers of -602A/G, +2C/T, and Pro198Leu are rs3811699, rs1800668, and rs1050450, respectively. We further genotyped these polymorphisms in 184 type 2 diabetic patients. Thirty-three patients had -602G, +2T, Ala⁶, and 198Leu

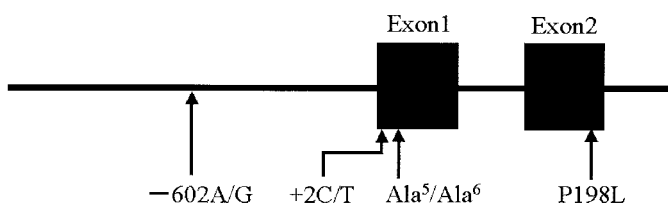


FIG. 1. Schema of genomic structure and polymorphic variants of the GPx-1 gene. Closed boxes indicate exons. Arrows point to the positions of the polymorphic variants identified.

all as the heterozygous state, and four patients had only -602G as the heterozygous state. From these results, we estimated three haplotypes (Table 2). The +2T, Ala⁶, and 198Leu were in complete linkage disequilibrium with each other ($D' = 1.00$, $\Delta^2 = 1.00$). The -602G was also in strong linkage disequilibrium with those polymorphisms ($D' = 1.00$, $\Delta^2 = 0.881$). Genotype distributions did not significantly differ from the Hardy-Weinberg equilibrium expectations. Based on these results, the Pro198Leu was selected as the genetic marker for the detailed clinical analysis.

Patients were divided on the basis of the codon 198 polymorphism into two groups (Pro/Pro and Pro/Leu). Leu/Leu genotype was not identified in this study. These groups were not significantly different with respect to age, maximum BMI, HbA_{1c}, age at diagnosis of diabetes, duration of diabetes, family history of diabetes, mode of treatment, hypertension, hyperlipidemia, and the prevalence of microvascular complications such as nephropathy and retinopathy (Table 3). However, the mean IMT of the common carotid arteries was significantly higher in the Pro/Leu group than in the Pro/Pro group (1.04 ± 0.23 vs. 0.91 ± 0.18 mm, $P = 0.0028$) (Table 4). We also found that the prevalence of cardiovascular disease and peripheral vascular disease was significantly higher in the Pro/Leu group than in the Pro/Pro group (24.2 vs. 10.6%, $P = 0.035$, and 15.2 vs. 4.6%, $P = 0.027$, respectively). The prevalence of cerebrovascular disease was higher in the Pro/Leu group than in the Pro/Pro group but was not statistically significant (15.2 vs. 7.9%, $P = 0.195$) (Table 4). All of the results were virtually unchanged when statistical models were adjusted for common vascular risk factors (age, sex, HbA_{1c}, hyperlipidemia, hypertension, and smoking habit). The regression coefficient using multivariable regression analysis for the comparison between the GPx-1 genotype (Pro/Pro = 0, Pro/Leu = 1) and the value of IMT was 0.127 ($P = 0.0002$). The odds ratios using multivariable logistic regression analysis for the comparison between the GPx-1 genotype (Pro/Pro = 0, Pro/Leu = 1) and the risk of macrovascular complications were as follows: coronary heart disease, 4.07 (95% CI 1.47-11.26, $P = 0.0068$); cerebrovascular disease, 2.59 (95% CI 0.79-8.53, $P = 0.1183$); and peripheral vascular disease, 4.46 (95% CI 1.17-17.05, $P = 0.028$).

When patients were divided into two groups on the basis of the -602A/G polymorphism (A/A and A/G; G/G genotype was not identified), the mean IMT was significantly higher in the A/G group than in the A/A group (1.02 ± 0.22 vs. 0.91 ± 0.18 mm, $P = 0.0052$), but the differences for the prevalence of cardiovascular (21.6 vs. 10.8%, $P = 0.083$), cerebrovascular (13.5 vs. 8.2%, $P = 0.315$), and peripheral vascular (13.5 vs. 4.8%, $P = 0.053$) disease were not statistically significant.

Effect of polymorphisms (Ala⁵/Ala⁶ and Pro198Leu) on the enzyme activity of GPx-1. To determine whether

TABLE 2
Haplotype frequency of GPx-1 gene

	-602	+2	(Ala) repeat	Codon 198	Haplotype frequency
Haplotype A	A	C	(Ala) ⁵	Pro	331 (89.9%)
Haplotype B	G	C	(Ala) ⁵	Pro	4 (1.1%)
Haplotype C	G	T	(Ala) ⁶	Leu	33 (9.0%)

TABLE 3
Clinical characteristics of type 2 diabetic patients divided into groups on the basis of genotype

	Pro/Pro (82.1%)	Pro/Leu (17.9%)	<i>P</i>
<i>n</i> (M/F)	151 (84/67)	33 (18/15)	NS
Age (years)	63.8 ± 9.4	63.4 ± 8.9	NS
Maximum BMI (kg/m ²)	25.9 ± 2.6	26.2 ± 2.3	NS
HbA _{1c} (%)	7.3 ± 1.3	7.5 ± 1.2	NS
Age at diagnosis (years)	44.8 ± 8.6	44.3 ± 7.8	NS
Duration of diabetes (years)	18.7 ± 9.6	19.2 ± 8.1	NS
Family history of diabetes	90 (59.6)	19 (57.6)	NS
Mode of treatment			
Diet or OHA	100 (66.2)	19 (57.6)	NS
Insulin	51 (33.8)	14 (42.4)	NS
Hypertension	62 (41.1)	15 (45.5)	NS
Hyperlipidemia	61 (40.4)	12 (35.3)	NS
Nephropathy	42 (27.8)	11 (33.3)	NS
Retinopathy	65 (43.0)	16 (48.5)	NS

Data are means ± SD or *n* (%). Values compared by an unpaired Student's *t* test, Mann-Whitney *U* test, or χ^2 test. OHA, oral hypoglycemic agent.

the two polymorphisms in the coding region of the *GPx-1* gene (Ala⁵/Ala⁶ and Pro198Leu) would affect its enzyme activity, we constructed two expression vectors, pcDNA3.1-GPx-1 (Ala⁵/198Pro) and pcDNA3.1-GPx-1 (Ala⁶/198Leu), and transiently transfected them into the BAECs. The GPx enzymes express endogenously in the BAECs, and cellular GPx activity in sham-transfected BAECs was 99.6 ± 8.4 units/mg protein. GPx activity significantly increased in both pcDNA3.1-GPx-1 (Ala⁵/198Pro)-transfected (132.5 ± 3.3 units/mg protein, *P* < 0.0001) and pcDNA3.1-GPx-1 (Ala⁶/198Leu)-transfected (119.8 ± 3.7 units/mg protein, *P* = 0.0004) BAECs compared with sham-transfected BAECs (Fig. 2). These levels of overexpression were similar to that observed in a similar experiment described by Weiss et al. (19). When the increment of GPx activity was compared between Ala⁵/198Pro- and Ala⁶/198Leu-transfected BAECs, that of Ala⁶/198Leu was significantly lower than that of Ala⁵/198Pro (20.1 ± 10.6 vs. 32.9 ± 9.5 units/mg protein, *P* = 0.0230).

Effect of polymorphisms (−602A/G and +2C/T) on the transcriptional activity of GPx-1. To investigate whether two polymorphisms, −602A/G and +2C/T, would affect the transcriptional activity of GPx-1, we constructed three kinds of plasmids with an 819-bp basal promoter fragment containing these polymorphisms upstream of a luciferase gene transcriptional unit. In transient transfection into the BAECs, the −602A/+2C construct induced luciferase expression over 1,000-fold relative to a promoter-less pGL3-basic vector (Fig. 3). The relative luciferase activity of the −602G/+2C haplotype decreased significantly compared with that of the −602A/+2C haplotype (986 ± 47 vs. 1,096 ± 76, *P* = 0.0180). The relative luciferase activity of the −602G/+2T haplotype decreased significantly compared with that of the −602A/+2C haplotype (822 ± 76 vs. 1,096 ± 76, *P* < 0.0001) and that of the −602G/+2C haplotype (822 ± 76 vs. 986 ± 47, *P* < 0.0001).

These results indicated that the two polymorphisms, −602A/G and +2C/T, affected transcription of GPx-1.

DISCUSSION

In this study, the mean IMT of common carotid arteries was significantly higher in the Pro/Leu group than in the Pro/Pro group in type 2 diabetic patients. It has been shown that the carotid IMT is positively associated with an increased risk of macrovascular disease (20,21). The prevalences of cardiovascular and peripheral vascular disease in our samples were also significantly higher in the Pro/Leu group than in the Pro/Pro group. We identified four polymorphisms (−602A/G, +2C/T, Ala⁵/Ala⁶, and Pro198Leu) in the human *GPx-1* gene. The 198Leu was in strong linkage disequilibrium with −602G, +2T, and Ala⁶. In vitro functional analyses indicated that the combination of polymorphisms (Ala⁶/198Leu) in the coding region of the *GPx-1* gene had a 40% decrease in enzyme activity, and the combination of polymorphisms (−602G/+2T) had a 25% decrease in transcriptional activity. These results suggest that functional variants in the *GPx-1* gene are associated with increasing risk of atherosclerosis in type 2 diabetic patients.

GPx-1 is a cytosolic isoform of GPx and is expressed in vascular endothelial cells. GPx-1 activity decreased in atherosclerotic plaques excised from patients with carotid artery disease (22). The patients with a selenium deficiency had an increased incidence of coronary artery disease and a decrease in GPx activity, because GPx is a selenocysteine-containing enzyme and a low serum selenium level induces a decrease in GPx enzyme activity (23). In addition, hyperhomocysteinemia is one of the risk factors for atherosclerotic vascular disease. It has been reported that homocysteine inhibited the expression of GPx-1 and lead to an increase in reactive oxygen species that inactivated nitric oxide and promoted endothelial dysfunction (24). The overexpression of GPx-1 rescued this homocysteine-induced endothelial dysfunction (19). Furthermore, heterozygous GPx-1-deficient mice show endothelial dysfunction, such as vasoconstriction to acetylcholine compared with vasodilation in wild-type mice, and an increase in the plasma and aortic levels of the isoprostane iPF2 α -III, a marker of oxidant stress (10). A histological section from the coronary vasculature of these mice shows increased perivascular matrix deposition, an increase in the number of advential fibroblasts, and intimal thickening (11). These findings support the hypothesis that

TABLE 4
IMT and prevalence of macrovascular disease in two groups

	Pro/Pro	Pro/Leu	<i>P</i>
<i>n</i>	151	33	
IMT (mm)	0.91 ± 0.18	1.04 ± 0.23	0.0028
Coronary heart disease	16 (10.6)	8 (24.2)	0.0350
Cerebrovascular disease	12 (7.9)	5 (15.2)	0.1954
Peripheral vascular disease	7 (4.6)	5 (15.2)	0.0267

Data are means ± SD or *n* (%). Values are compared by the χ^2 or Mann-Whitney *U* tests.

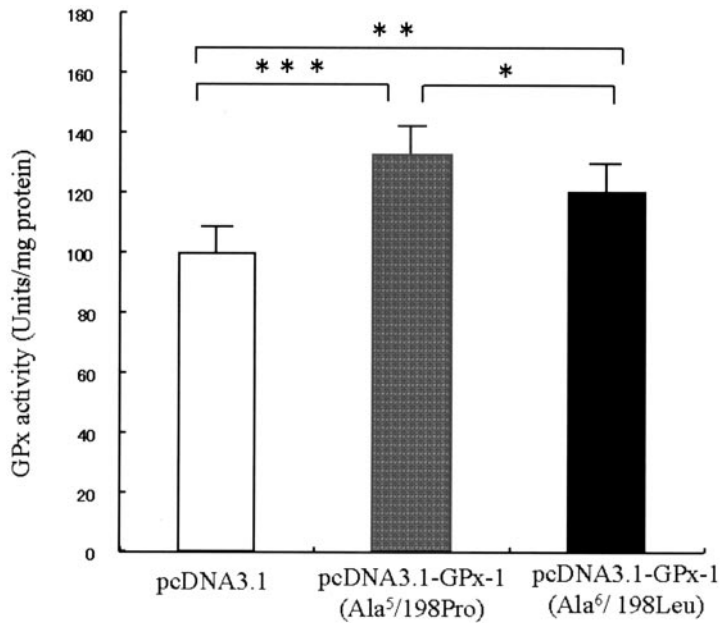


FIG. 2. Effect of polymorphisms (Ala⁵/Ala⁶ and Pro198Leu) on the enzyme activity of GPx-1. Two expression vectors, pcDNA3.1-GPx-1 (Ala⁵/198Pro) and pcDNA3.1-GPx-1 (Ala⁶/198Leu) were constructed and transiently transfected into BAECs. GPx activities were significantly increased in both pcDNA3.1-GPx-1 (Ala⁵/198Pro)-transfected (132.5 ± 3.3 units/mg protein, $***P < 0.0001$) and pcDNA3.1-GPx-1 (Ala⁶/198Leu)-transfected (119.8 ± 3.7 units/mg protein, $**P = 0.0004$) BAECs compared with sham-transfected BAECs. The increment of GPx activity in pcDNA3.1-GPx-1 (Ala⁵/198Pro)-transfected BAECs was significantly higher than that in pcDNA3.1-GPx-1 (Ala⁶/198Leu)-transfected BAECs (20.1 ± 10.6 vs. 32.9 ± 9.5 units/mg protein, $*P = 0.0230$).

a change in the function of Gpx-1 contributes to vascular oxidant stress and the progression of atherosclerosis.

Four polymorphisms identified in this study have been previously reported in a study for patients with lung cancer (18). Linkage disequilibrium among these polymorphisms was also observed in the screening of 17 tumor specimens. The allele frequency of Ala⁶, which was 9.0% in our study, was 27.8% in 42 American cancer patients in the study. The association between the 198Leu variant and erythrocyte GPx activity was examined in 66 subjects from the Finnish/Swedish population (25). The allele frequency of 198Leu was higher (41%) than our result, and no significant association was observed. Because the -602A/G, +2C/T, and Ala⁵/Ala⁶ polymorphisms were not examined in the population, the haplotype configuration was unknown. Although the level of erythrocyte GPx activity is higher in women than in men (26) and smoking reduces the activity (27), the information for sex and smoking was not shown. These factors may affect the result, which is inconsistent with our result. Furthermore, a lack of association between the Leu variant and risk of stroke was reported in a case-controlled study on the Swedish general population from the same group (25). The prevalence of stroke in our study was higher in the Pro/Leu group than in the Pro/Pro group, but it was not statistically significant. On the other hand, the *GPx-1* gene variants were associated with increased IMT. Because it is known that carotid IMT correlates with prevalent and incident stroke (20), the association between the gene variants and risk of stroke requires further clarification in a larger series of individuals. In addition, increased polyol pathway flux in a diabetic condition exacerbates an antioxidant system by GPx. In the pathway, aldose reductase catalyzes glucose to sorbitol, using NADPH as a cofactor. The increased glucose flux in a hyperglycemic environment results in decreased NADPH. Because NADPH is required for regenerating reduced glutathione, which is also a cofactor for GPx, this could enhance the genetic dysfunction of GPx-1 in diabetic patients. It will also be necessary to further evaluate the role of the genetic variants in nondiabetic subjects.

In conclusion, functional variants in the *GPx-1* gene are associated with increased IMT of carotid arteries and risk of cardiovascular disease and peripheral vascular disease in diabetic patients. This information might be helpful in picking up the patients with high genetic risk for macrovascular complications and in preventing them by intensive treatment for other risk factors of atherosclerosis.

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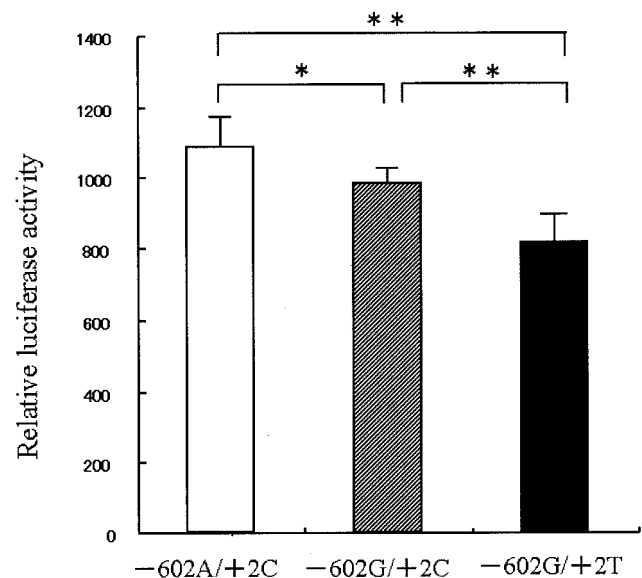


FIG. 3. Effect of polymorphisms (-602A/G and +2C/T) on the transcriptional activity of GPx-1. The relative luciferase activity of the haplotype -602G/+2C was significantly lower than that of the -602A/+2C haplotype ($*P = 0.018$). The relative luciferase activity of the -602G/+2T haplotype was significantly lower than that of the -602A/+2C haplotype ($**P < 0.0001$) and that of the -602G/+2C haplotype ($**P < 0.0001$).

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