Pro198Leu missense polymorphism of the glutathione peroxidase 1 gene might be a common genetic predisposition of distal symmetric polyneuropathy and macrovascular disease in Japanese type 2 diabetic patients

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ABSTRACT

Aims/Introduction: We have previously reported that the Pro198Leu missense polymorphism in the glutathione peroxidase 1 (GPx-1) gene was associated with frequent macrovascular disease (MVD). Our goal was to examine whether the GPx-1 genotype is associated with diabetic neuropathy.

Materials and Methods: We determined the GPx-1 genotype in 173 Japanese type 2 diabetic patients who received medical interviews, physical examinations, nerve conduction studies, quantitative vibratory perception (QVP), head-up tilt and heart rate variability tests by polymerase chain reaction-restriction fragment-length polymorphism. Diabetic sensorimotor distal symmetric polyneuropathy (DSPN) and diabetic autonomic neuropathy (DAN) were evaluated separately. DSPN and DAN were defined by two or more abnormalities of neuropathic leg symptoms, diminished Achilles tendon reflexes or impaired QVP in toes, and two autonomic dysfunctions, respectively. The association of the GPx-1 genotype with DSPN, DAN, MVD and other clinical manifestations was analyzed.

Results: The prevalence of DSPN, impaired QVP and painful leg cramps in patients having a genotype with Pro/Leu at the codon 198 (Pro/Leu type) was significantly higher than those with Pro/Pro type. As a result of multivariate analyses that contained the GPx-1 genotype as an independent variable, the Pro/Leu type was extracted as a significant risk factor of DSPN, QVP impairment and MVD. The statistical significance did not disappear, even after proteinuria, retinopathy and a history of MVD were introduced as independent variables. In contrast, the GPx-1 genotype was not associated with DAN.

Conclusions: The Pro198Leu missense polymorphism of the GPx-1 gene might have a common genetic predisposition to DSPN and MVD. (J Diabetes Invest, doi: 10.1111/j.2040-1124.2011.00127.x, 2011)

KEY WORDS: Glutathione peroxidase 1 gene, Diabetic distal symmetric polyneuropathy, Macrovascular disease

INTRODUCTION

It is well known that macrovascular diseases (MVD), such as myocardial and cerebral infarction, are more common in patients with impaired glucose tolerance[1,2]. Recently, a higher prevalence of polyneuropathy in patients with impaired glucose tolerance, as compared with healthy subjects, has been reported[3]. The similarities in risk factors of diabetic polyneuropathy and MVD have also been reported[4,5]. These findings suggest that there might be a common underlying etiological mechanism in both complications.

One of the most plausible common etiological factors of these complications is the excess of oxidative stress. Elevated reactive oxygen species produced by hyperglycemia induces an oxidation of low-density lipoprotein, and an induction of monocyte chemoattractant protein 1 and adhesion molecules. These mechanisms seem to be mainly implicated in the development of MVD[6]. Excessive oxidative stress is also implicated in the development of diabetic neuropathy[7]. Thus, oxidative stress and the related molecular derangements are widely thought to be a common underlying cause of diabetic complications[8]. We have previously reported that the Pro198Leu missense polymorphism of the glutathione peroxidase 1 (GPx-1) gene is associated with a reduction in transcription and enzyme activity of GPx-1, which is an important anti-oxidative enzyme[9]. Furthermore, we and other investigators have reported significant associations

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Received 13 January 2011; revised 25 February 2011; accepted 29 March 2011

between the polymorphism of the GPx-1 gene and MVD[9,10]. A recent study reported a significant association between a GPx-1 gene variant, which was deferent from our study, and peripheral neuropathy in Caucasian subjects[11]. However, to date, there has been no study to show the association between the GPx-1 gene polymorphism and diabetic neuropathy in Japanese patients. In the present study, we aimed to examine whether Pro198Leu polymorphism in the GPx-1 gene is associated with not only MVD, but also diabetic neuropathy.

Diabetic neuropathy is roughly classified into three types: (i) chronic sensorimotor distal symmetric polyneuropathy (DSPN); (ii) diabetic autonomic neuropathy (DAN); and (iii) focal and multifocal neuropathies, according to a statement by the American Diabetes Association[12]. Although DSPN and DAN are specific complications of diabetes, focal and multifocal neuropathies are not. As we have observed that exacerbating factors of sensory and autonomic functions were different[13], subtypes of diabetic neuropathy, DSPN and DAN were separately evaluated. Additionally, various quantitative neurological functions, such as vibratory perception thresholds, nerve conduction parameters and autonomic functions, were also individually evaluated. Then associations between the GPx-1 gene polymorphism and these subtypes of diabetic neuropathy or quantitative neurological functions were investigated.

MATERIALS AND METHODS

Study Design and Participants

A total of 173 unrelated Japanese type 2 diabetic patients (54 outpatients and 119 hospitalized patients of Wakayama Medical University Hospital) who received serial neurological examinations and agreed to be involved in the genetic study were enrolled after giving written informed consent. The study was approved by the ethics committee of Wakayama Medical University and carried out in accordance with the Helsinki Declaration (revised in 2000). In order to evaluate neurological functions accurately, aged patients (more than 70 years) and patients with severe liver or renal dysfunction, cerebrovascular disease with residual neurological deficits, peripheral arterial disease (second degree of Fontaine classification or more) or other neurological diseases were excluded. Diabetes was diagnosed according to the criteria set by the World Health Organization. Hypertension was defined by a blood pressure > 130/80 mmHg or receiving antihypertensive treatment. Patients with total cholesterol > 5.17 mmol/L (200 mg/dL) and/or triglycerides > 1.70 mmol/L (150 mg/dL) and/or high-density lipoprotein cholesterol < 1.03 mmol/L (40 mg/dL) or those on antihyperlipidemic medication were defined as dyslipidemic. MVD was defined by a previous history of cardiovascular disease and/or stroke without residual neurological deficits. Fair and poor glycemic controls were defined as HbA_{1c} (%) < 8.4% and HbA_{1c} (%) ≥ 8.4%, respectively. The value for HbA_{1c} (%) was estimated as a National Glycohemoglobin Standardization Program (NGSP) equivalent value (%) calculated by the formula HbA_{1c} (%) = HbA_{1c} (Japan Diabetes Society [JDS]) (%) + 0.4%, considering the relational expression of HbA_{1c} (JDS) (%) measured by the previous Japanese standard substance and measurement methods and HbA_{1c} (NGSP)[14].

Genotyping of GPx-1

Genomic DNA was isolated from peripheral blood according to standard procedures. The GPx-1 genotype for the Pro198Leu missense polymorphism in exon 2 was analyzed by polymerase chain reaction-restriction fragment-length polymorphism using *HaeIII* as a restriction enzyme, as previously described[9]. The genotypes with Pro/Leu and Pro/Pro at the codon 198 are described as Pro/Leu type and Pro/Pro type, respectively.

Assessment of Neurological Functions

To evaluate the clinical diabetic neuropathy, the sensorimotor and autonomic symptoms were evaluated. Subjective symptoms were ascertained using five criteria: 'numbness in toe and sole'; 'pain in feet, particularly below the knee: pain in feet'; 'painful leg cramp occurring two or more times in a month: painful leg cramp'; 'dizziness on standing: orthostatic dizziness'; and 'frequent constipation/diarrhea or their alternation: frequent constipation/diarrhea'. In the present study, numbness means an uncomfortable sensation with or without ordinary stimulation and dullness in perception inclusively. Painful muscle cramp was defined as a spasm of the calf muscle with severe pain. Achilles tendon reflex (ATR) in the knee-standing position was also examined bilaterally. Furthermore, four objective and quantitative tests were carried out to assess the sensory, motor and autonomic nerve functions as previously described[15]. All examinations were carried out in a temperature-controlled room at 25°C.

Quantitative Vibratory Perception Threshold

Quantitative vibratory perception threshold (QVP) at 125 Hz was assessed using a vibratory sensation meter (AU-02A; RION Company, Tokyo, Japan), whose output level could be changed from −10 to 40 dB (0 dB ref 0–3 m/s^2)[16]. First, the patient put the plantar aspect of their big toe on a vibrating plate and was shown the vibration output level from minimum to maximum. Then the patient was asked to respond by saying 'buzzing' when they felt vibration during a gradual increase of vibratory stimulation. When the patient responded at the same output level twice or more, we regarded that as the perceptible threshold. Measurements were carried out bilaterally and an average of the two sides was used for analysis.

Autonomic Nerve Function Tests (Head-up Tilt Test and Heart Rate Variability Test)

Sympathetic vasomotor function was evaluated by a head-up tilt test using a tilt table (Sakai, Tokyo, Japan) and an automatic sphygmomanometer (BP-88; Colin Company, Tokyo, Japan). Orthostasis-induced decreases in systolic blood pressure after passive standing for 5 min in a 70° head-up position (ΔBP) were examined.

Parasympathetic cardiovagal function was also evaluated by a heart rate variability test. Coefficients of variation of R-R intervals on electrocardiogram after 15 min resting in the supine position (CVR-R) were determined with an electrocardiograph (Autocardiner FCP-2201; Fukuda Denshi, Tokyo, Japan).

Nerve Conduction Study

Motor nerve conduction velocity (MCV) between the wrist and elbow, compound muscle action potential (CMAP) of the ulnar nerve, sensory nerve velocity (SCV) between the wrist and elbow, and sensory nerve action potential (SNAP) of the median nerve were measured bilaterally using standard methods with an electromyograph (Synax 1200; NEC, Tokyo, Japan). Electric stimuli were produced at supramaximal intensity. The CMAP and SNAP produced by the wrist stimulation were evaluated. Skin temperature was measured at the forearms and was maintained at 32°C.

Decision of Abnormality and Subtypes of Diabetic Neuropathy

QVP, MCV, CAMP, SCV, SNAP and logarithmic CVR-R were distributed normally, values exceeding the range of means ± 2 SD of the age-matched healthy subjects in our institution were judged as impaired. Abnormal ΔBP was defined by the American Autonomic Society criteria[17]. Namely, a fall in systolic blood pressure of more than 20 mmHg and/or a fall in diastolic blood pressure of more than 10 mmHg was judged to be an abnormal value. We then classified various neurological manifestations into two subtypes of diabetic neuropathy, distal symmetric polyneuropathy (DSPN) and diabetic autonomic neuropathy (DAN). DSPN was defined by two or more abnormalities in specific neuropathic leg symptoms (numbness in toes and soles, and/or pain in feet), bilaterally diminished ATR and impaired QVP. Painful leg cramp was not included as a neuropathic symptom. For example, this symptom is recognized as a sign of a circulatory disturbance in the questionnaire of Michigan Neuropathy Screening Instruments (MNSI) announced from the website of the Michigan Diabetes Research and Training Center. Neurological Symptom Score (NSC) in the Mayo Clinic also does not contain painful muscle cramp as a sensory symptom[18]. DAN was diagnosed by the two autonomic dysfunctions, impaired CVR-R and abnormal ΔBP.

Statistical Analysis

All statistical analyses were carried out with the StatView program for Windows (version 5.01; SAS Institute, Cary, NC, USA). Differences of clinical data, neuropathic symptoms, ATR, various nerve function data and subtypes of diabetic neuropathy between the two diabetic groups divided based on the GPx-1 genotype were analyzed by ANOVA and χ^2-test.

Multiple logistic regression analyses were carried out to verify the associations between clinical manifestations of diabetic neuropathy and clinical background factors, including the GPx-1 genotype. DSPN, DAN and painful leg cramps were set as dependent variables for the analyses. Eight clinical background factors (age, sex, duration of diabetes, hypertension, dyslipidemia, glycemic control, body mass index [BMI] and GPx-1 genotype: Pro/Pro = 0, Pro/Leu = 1) were selected as independent variables (model 1). Additional analyses, which added proteinuria and retinopathy as independent variables, were also carried out (model 2). In order to negate the influence of MVD on diabetic neuropathy, another analysis was carried out (model 3) in which the history of MVD was added as an independent variable of model 2. An association between MVD and clinical background factors was also evaluated by the two analyses (model 1 and 2).

Multiple regression analyses were also used to determine independent associations between the GPx-1 genotype and six actual results of nerve function tests using the same three sets of independent variables (model 1, 2, 3). A P-value of <0.05 was considered statistically significant.

RESULTS

GPX-1 Genotype

Genotype frequencies (%) of Pro/Pro type, Pro/Leu type and Leu/Leu type were 86.1, 13.9 and 0 in all diabetic patients, respectively. Genotype distributions did not significantly differ from Hardy–Weinberg equilibrium expectations. The frequency of Pro/Leu type in diabetic patients with DSPN was significantly higher than that in the patients without DSPN (17/79 = 21.5% vs 7/94 = 7.5%, P = 0.0076). The frequencies of Pro/Leu type in diabetic patients with DAN was not significantly different from those in patients without DAN (3/25 = 12.0% vs 21/148 = 14.2%, P = 0.7696).

Relationships Between GPx-1 Genotype and Clinical, Neurological Data

Patients were divided into two groups (Pro/Pro type and Pro/Leu type) based on the codon 198 polymorphism, and clinical and neurological features were then compared. Clinical characteristics of the two diabetic groups, such as age, sex, duration of diabetes, therapy, BMI, hypertension, dyslipidemia, recent HbA_{1c}, proteinuria, retinopathy and history of MVD are shown in Table 1. Though the prevalence of MVD tended to be higher in Pro/Leu type than Pro/Pro type (P = 00510), there was no significant difference in clinical characteristics.

The data of subjective symptoms, ATR, subtypes of diabetic neuropathy and quantitative nerve functions are also shown in Table 1. As a subjective symptom, the prevalence of painful leg cramps in Pro/Leu type was significantly higher than that in Pro/Pro type. Among the two subtypes of diabetic neuropathy, only DSPN showed a significantly higher prevalence in Pro/Leu type compared with Pro/Pro type (70.8 vs 41.6, P = 0.0076). In the quantitative neurological data, statistically significant differences between Pro/Leu type and Pro/Pro type were observed in QVP with a prevalence of impaired QVP. In contrast, there was no significant difference in the autonomic or nerve conduction functions between Pro/Leu and Pro/Pro type.

Table 1 | Comparison of clinical characteristics and neurological functions between two diabetic groups divided based on GPx-1 genotype (n = 173)

	Pro/Leu type	Pro/Pro type	*P*-value
n	24	149	
Clinical characteristics			
Age (year)	55.6 ± 13.0	54.9 ± 10.3	0.8503
Gender (Male/Female)	13/11	85/64	0.7916
Duration of diabetes (years)	12.9 ± 7.8	11.3 ± 7.7	0.3433
Therapy (insulin/OHA/diet and exercise)	1/5/18 (4.2/20.8/75.0)	6/42/101 (4.0/28.2/67.8)	0.7524
BMI (kg/m^2)	23.1 ± 4.2	23.9 ± 3.9	0.3163
Hypertension	10/24 (41.7)	67/149 (45.0)	0.7627
Dyslipidemia	10/24 (41.7)	72/149 (48.3)	0.5445
HbA_{1c} (%)	9.83 ± 2.22	9.11 ± 2.06	0.1134
Proteinuria (no/intermittent/persistent)	16/4/4 (66.6/16.7/16.7)	102/19/28 (68.5/12.7/18.8)	0.8614
Retinopathy (no/simple/pre-, proliferative)	10/4/10 (41.7/16.6/41.7)	78/22/49 (52.4/14.7/32.9)	0.6124
History of macrovascular disease (MVD)	5/24 (20.8)	12/149 (8.1)	0.0510
Subjective symptoms and Achilles tendon reflex (ATR)			
Numbness in toes and soles	9/24 (37.5)	52/149 (34.9)	0.8045
Pain in feet	3/24 (12.5)	16/149 (10.7)	0.7978
Painful leg cramp	14/24 (58.3)	36/149 (24.2)	**0.0006**
Orthostatic dizziness	4/24 (16.7)	27/149 (18.4)	0.8411
Frequent constipation/diarrhea	1/24 (4.2)	13/149 (8.7)	0.4429
Diminished ATRs	19/24 (79.2)	94/149 (64.8)	0.1669
Subtypes of diabetic neuropathy and quantitative nerve functions			
DSPN (Distal symmetric polyneuropathy)	17/24 (70.8)	62/149 (41.6)	**0.0076**
DAN (diabetic autonomic neuropathy)	3/24 (12.5)	22/149 (14.8)	0.7696
QVP (dB)	26.0 ± 7.2	20.4 ± 10.3	**0.0114**
Prevalence of impaired QVP	17/24 (70.8)	56/149 (37.6)	**0.0022**
CVR-R (%)	1.98 ± 0.92	1.96 ± 1.06	0.9591
Prevalence of impaired CVR-R	13/24 (56.5)	68/149 (46.9)	0.3908
ΔBP (mmHg)	7.79 ± 12.49	11.02 ± 14.51	0.3057
Orthostatic hypotension	4/24 (16.7)	35/149 (23.5)	0.4579
MCV (m/s)	50.9 ± 3.9	50.4 ± 6.8	0.7506
Prevalence of impaired MCV	5/24 (20.8)	50/149 (33.6)	0.2141
CMAP (mV)	7.12 ± 3.36	7.10 ± 2.74	0.9734
Prevalence of impaired CMAP	5/24 (20.8)	16/149 (10.7)	0.1599
SCV (m/s)	56.4 ± 5.2	57.3 ± 5.9	0.5187
Prevalence of impaired SCV	10/24 (41.7)	58/149 (38.9)	0.7987
SNAP (μV)	18.4 ± 14.9	21.1 ± 14.3	0.3993
Prevalence of impaired SNAP	8/24 (33.3)	39/149 (26.2)	0.4644

Numbers in parenthesis indicate the percentage. OHA, oral hypoglycemic agents; BMI, body mass index; ATR, Achilles tendon reflex; QVP, quantitative vibratory perception thresholds; CVR-R, correlation coefficient of R-R intervals in electrocardiogram; BP, blood pressure; CMAP, compound muscle action potential; SCV, sensory nerve conduction velocity; SNAP, sensory nerve action potential; Pro/Leu type, genotype with Pro/Leu at the codon 198 of glutathione peroxidase 1 gene; Pro/Pro type, genotype with Pro/Pro at the codon 198 of glutathione peroxidase 1 gene. The value for HbA_{1c} (%) was estimated as an NGSP equivalent value (%) calculated by the formula HbA_{1c} (%) = HbA_{1c} (JDS) (%) + 0.4%. Statistically significant *P*-value was shown by boldfaced type.

Multivariate Analyses

Relationships between the GPx-1 genotype and a subtype of diabetic neuropathy, painful leg cramp and MVD were analyzed by multiple logistic regression analyses and are shown in Table 2. Multiple logistic regression analysis showed that the GPx-1 genotype (Pro/Leu type) was a significant risk factor for DSPN, painful leg cramps and MVD independent from age, sex, duration, hypertension, dyslipidemia, recent glycemic control and BMI (model 1). The GPx-1 genotype (Pro/Leu type) was a significant risk factor for DSPN, painful leg cramps and MVD, even if proteinuria and retinopathy were added as independent variables of the analyses (model 2). In contrast, Pro/Leu type was not identified as a significant risk factor for DAN in the two regression models.

Associations between the GPx-1 genotype and quantitative neurological functions analyzed by multiple regression analyses are shown in Table 3. Though the GPx-1 genotype (Pro/Leu type) was identified as a significant exacerbation factor of QVP independent from age, sex, duration, hypertension, dyslipidemia, recent glycemic control and BMI, no significant relationship between the GPx-1 genotype and other autonomic or nerve

Table 2 | Relationships between the glutathione peroxidase 1 gene polymorphism and subtype of diabetic neuropathy, painful leg cramp, macrovascular disease evaluated by multiple logistic regression analysis

Independent variables	Model 1 dependent variables				Model 2 dependent variables			
	Subtypes of diabetic neuropathy		Painful leg cramp	History of MVD	Subtypes of diabetic neuropathy		Painful leg cramp	History of MVD
	DSPN	DAN			DSPN	DAN		
R^2 (*P*-value)	R^2 = 0.122 (P = 0.0003)	R^2 = 0.137 (P < 0.0001)	R^2 = 0.086 (P = 0.0217)	R^2 = 0.209 (P = 0.0031	R^2 = 0.210 (P < 0.0001)	R^2 = 0.530 (P < 0.0001)	R^2 = 0.102 (P = 0.0202)	R^2 = 0.215 (P = 0.0078)
	Adjusted OR (95% CI) *P*-value	Adjusted OR (95% CI) *P*-value	Adjusted OR (95% CI) *P*-value	Adjusted OR (95% CI) *P*-value	Adjusted OR (95% CI) *P*-value	Adjusted OR (95% CI) *P*-value	Adjusted OR (95% CI) *P*-value	Adjusted OR (95% CI) *P*-value
Age (years)	1.010 (0.976–1.044) 0.5783	**1.039 (1.003–1.076) 0.0345**	0.998 (0.963–1.034) 0.9125	**1.118 (1.022–1.224) 0.0152**	1.010 (0.974–1.047) 0.5809	1.001 (0.934–1.072) 0.9806	0.977 (0.961–1.033) 0.8620	**1.122 (1.023–1.231) 0.0151**
Sex (female: 0, male: 1)	1.054 (0.534–2.077) 0.8800	1.098 (0.552–2.186) 0.7892	0.561 (0.273–1.152) 0.1154	**7.212 (1.609–32.334) 0.0098**	1.099 (0.518–2.329) 0.8059	1.883 (0.458–7.743) 0.3803	0.488 (0.229–1.037) 0.0621	**6.342 (1.375–29.265) 0.0179**
Duration (years) (≥5: 0. 6–15: 1, 16≤: 2)	**1.997 (1.252–3.188) 0.0037**	**2.003 (1.254–3.200) 0.0037**	0.981 (0.598–1.609) 0.9395	1.123 (0.518–2.434) 0.7684	1.343 (0.795–2.270) 0.2706	0.978 (0.409–2.336) 0.9596	1.133 (0.650–1.976) 0.6587	1.031 (0.255–2.335) 0.9413
Hypertension (no: 0, yes: 1)	**2.477 (1.199–5.119) 0.0143**	1.823 (0.881–3.776) 0.1057	0.774 (0.361–1.660) 0.5104	1.590 (0.510–4.961) 0.4244	2.117 (0.958–4.679) 0.0637	2.595 (0.706–9.535) 0.1508	0.753 (0.339–1.674) 0.4864	1.395 (0.429–4.543) 0.5801
Dyslipidemia (no: 0, yes: 1)	**0.412 (0.205–0.830) 0.0130**	**0.463 (0.231–0.931) 0.0308**	0.573 (0.274–1.197) 0.1384	0.801 (0.254–2.524) 0.7050	**0.465 (0.218–0.989) 0.0467**	0.842 (0.212–3.344) 0.8070	0.510 (0.239–1.088) 0.0816	0.741 (0.229–2.393) 0.6160
Glycemic control (∼fair: 0, poor: 1)	1.383 (0.699–2.736) 0.3513	1.664 (0.828–3.344) 0.1526	1.572 (0.756–3.269) 0.2261	0.791 (0.261–3.270) 0.1745	1.728 (0.821–3.638) 0.1496	**9.232 (1.991–43.945) 0.0046**	1.410 (0.669–2.971) 0.3663	0.728 (0.234–2.265) 0.5832
BMI (kg/m^2) (>22: 0. 22–25: 1, 25<: 2)	0.845 (0.552–1.294) 0.4387	**0.572 (0.369–0.885) 0.0122**	0.980 (0.626–1.535) 0.9301	1.624 (0.807–3.270) 0.1745	0.863 (0.547–1.363) 0.5285	**0.158 (0.055–0.456) 0.0006**	0.985 (0.627–1.549) 0.9486	1.606 (0.797–3.236) 0.3330
GPx-1 genotype (Pro/Pro : 0, Pro/Leu :1)	**3.390 (1.252–9.181) 0.0163**	0.891 (0.338–2.351) 0.8157	**4.333 (1.718–10.929) 0.0019**	**3.886 (1.078–14.009) 0.0380**	**3.303 (1.175–9.285) 0.0234**	0.340 (0.045–2.571) 0.2957	**4.653 (1.813–11.943) 0.0014**	**3.782 (1.044–13.703) 0.0428**
Proteinuria (no: 0, intermittent: 1, persistent: 2)					0.694 (0.399–1.205) 0.1941	0.664 (0.302–1.459) 0.3078	1.545 (0.879–2.714) 0.1303	1.350 (0.621–2.936) 0.4494
Retinopathy (no: 0, simple: 1, PPDR∼: 2)					**2.919 (1.764–4.831) <0.0001**	**39.232 (5.792–265.745) 0.0002**	0.659 (0.388–1.119) 0.1228	1.009 (0.491–2.076) 0.9802

BMI, body mass index; CI, confidence interval; DAN, diabetic autonomic neuropathy; DSPN, distal symmetric polyneuropathy; GPx-1, glutathione peroxidase 1 gene; MVD, macrovascular disease, OR, odds ratio; PPDR, preproliferative diabetic retinopathy; R^2, decision coefficient. Statistically significant *P*-value was shown by boldfaced type.

Table 3 | Relationships between Pro198Leu polymorphism of glutathione peroxidase 1 gene and quantitative neurological functions evaluated by multiple regression analysis

Independent variables	Model 1 dependent variables							Model 2 dependent variables						
	Vibration	Autonomic functions		Nerve conduction parameters				Vibration	Autonomic functions		Nerve conduction parameters			
	QVP	CVR–R	ΔBP	MCV	CMAP	SCV	SNAP	QVP	CVR–R	ΔBP	MCV	CMAP	SCV	SNAP
R^2 (*P*-value)	R^2 = 0.182 (*P* < 0.0001)	R^2 = 0.202 (*P* < 0.0001)	R^2 = 0.143 (*P* = 0.0012)	R^2 = 0.101 (*P* = 0.0254)	R^2 = 0.049 (*P* = 0.3987)	R^2 = 0.093 (*P* = 0.0522)	R^2 = 0.286 (*P* < 0.0001)	R^2 = 0.320 (*P* < 0.0001)	R^2 = 0.288 (*P* < 0.0001)	R^2 = 0.266 (*P* < 0.0001)	R^2 = 0.254 (*P* < 0.0001)	R^2 = 0.083 (*P* = 0.1588)	R^2 = 0.182 (*P* = 0.0005)	R^2 = 0.419 (*P* < 0.0001)
	β (*P*-value)	β (*P*-value)	β (*P*-value)	β (*P*-value)	β (*P*-value)	β (*P*-value)	β (*P*-value)	β (*P*-value)	β (*P*-value)	β (*P*-value)	β (*P*-value)	β (*P*-value)	β (*P*-value)	β (*P*-value)
Age (years)	**0.281** (**0.0004**)	**−0.296** (**0.0003**)	0.079 (0.3281)	−0.121 (0.1445)			**−0.326** (**<0.0001**)	**0.288** (**<0.0001**)	**−0.303** (**<0.0001**)	0.088 (0.2425)	−0.133 (0.0823)		−0.080 (0.3241)	**−0.338** (**<0.0001**)
Sex (female: 0, male: 1)	0.062 (0.3950)	0.049 (0.5027)	0.063 (0.4036)	−0.142 (0.0681)			**−0.169** (**0.0157**)	0.072 (0.2984)	0.064 (0.3781)	0.048 (0.5058)	−0.093 (0.2049)		−0.025 (0.7484)	**−0.164** (**0.0122**)
Duration (years) (≧5: 0. 6–15: 1, 16≦: 2)	0.114 (0.1398)	**−0.215** (**0.0015**)	0.178 (0.0247)	−0.061 (0.4510)			**−0.265** (**0.0004**)	−0.062 (0.4192)	−0.118 (0.1406)	0.014 (0.8588)	0.100 (0.2184)		0.084 (0.3350)	−0.089 (0.2228)
Hypertension (no: 0, yes: 1)	0.120 (0.1149)	0.040 (0.5958)	**0.200** (**0.0109**)	−0.064 (0.4235)			−0.069 (0.3355)	0.058 (0.4249)	0.105 (0.1619)	0.122 (0.1052)	0.045 (0.5568)		−0.099 (0.2235)	0.004 (0.9516)
Dyslipidemia (no: 0, yes: 1)	−0.071 (0.3417)	0.037 (0.6153)	−0.129 (0.0899)	0.032 (0.6847)			0.115 (0.1032)	−0.016 (0.8112)	0.006 (0.9343)	−0.090 (0.2120)	0.001 (0.9954)		−0.011 (0.8882)	0.067 (0.3026)
Glycemic control (∼fair: 0, poor: 1)	−0.007 (0.9271)	−0.103 (0.1572)	0.104 (0.1601)	**−0.191** (**0.0135**)			−0.089 (0.1923)	0.025 (0.7038)	−0.117 (0.0938)	0.123 (0.0791)	**−0.196** (**0.0062**)		**−0.246** (**0.0014**)	−0.101 (0.1093)
BMI (kg/m^2) (>22: 0. 22–25: 1, 25<: 2)	−0.072 (0.3443)	0.071 (0.3489)	**−0.205** (**0.0091**)	**0.164** (**0.0429**)			−0.091 (0.2088)	−0.063 (0.3725)	0.062 (0.3891)	**−0.197** (**0.0074**)	**0.163** (0.0286)		0.145 (0.0671)	−0.092 (0.1605)
GPx-1 genotype (Pro/Pro: 0, Pro/Leu: 1)	**0.176** (**0.0145**)	0.036 (0.6111)	−0.111 (0.1292)	0.047 (0.5339)			−0.044 (0.5124)	**0.154** (**0.0196**)	0.050 (0.4617)	−0.129 (0.0596)	0.060 (0.3849)		−0.009 (0.8987)	−0.021 (0.7360)
Retinopathy (no: 0, simple: 1, PPDR∼: 2)								**0.455** (**<0.0001**)	**−0.306** (**0.0006**)	**0.380** (**<0.0001**)	**−0.320** (**0.0003**)		**−0.298** (**0.0017**)	**−0.409** (**<0.0001**)
Proteinuria (no:0, intermittent: 1, persistent: 2)								−0.091 (0.2517)	−0.041 (0.6236)	0.028 (0.7315)	**−0.181** (**0.0322**)		−0.068 (0.4482)	−0.001 (0.9862)

The significant regression formula on compound muscle action potential (CMAP) and sensory nerve velocity (SCV) were not obtained in model 1. The significant regression formula on CMAP was not obtained in model 2. β, Standard regression coefficient; BMI, body mass index; ΔBP, orthostasis-induced decreases in systolic blood pressure at standing; QVP, quantitative vibratory perception thresholds; CVR–R, coefficient of variation of RR intervals on electrocardiogram after 15 min resting; GPx-1, glutathione peroxidase 1 gene; MCV, motor nerve conduction velocity; PPDR, preproliferative diabetic retinopathy; SCV, sensory nerve conduction velocity; SNAP, sensory nerve action potential, R^2, decision coefficient. Statistically significant *P*-value was shown by boldfaced type.

conduction parameters was proven (model 1). Virtually the same result was obtained from the reanalysis to which proteinuria and retinopathy were added as independent variables (model 2).

Table 4 shows the result of the multiple logistic regression and multiple regression analyses that contains a history of MVD as an independent variable (model 3). As in the results of model 1 and 2, significant associations of the GPx-1 genotype with DSPN, painful leg cramp and QVP were also observed in this model.

DISCUSSION

In the present study, we showed the following four major findings. First, the frequency of Pro/Leu type of the GPx-1 genotype in diabetic patients with DSPN was significantly higher than that in the patients without DSPN. Second, the frequencies of painful leg cramp, DSPN and impaired QVP in the patients with Pro/Leu type were significantly higher than those in the patients with Pro/Pro type, respectively. Third, Pro/Leu type was a significant risk factor associated with painful leg cramp, DSPN and history of MVD, but it was not associated with DAN. Fourth, though Pro/Leu type was a significant exacerbation factor of QVP, it had no association with other neurological functions.

Our first finding is that there is a significantly higher frequency of the Pro/Leu type in diabetic patients with DSPN compared with those without DSPN. The genotype frequency (%) of the Pro/Leu type in the Japanese population is quite similar to the present study (15.1 vs 13.9)[19]. So, Pro198Leu polymorphism of the GPx-1 gene seems to be relevant to the development of diabetic complications, but not to the onset of diabetes itself.

Our second finding indicates the possible relationship between the GPx-1 genotype and several manifestations of diabetic neuropathy by univariation analysis. The diabetic patients with Pro/Leu type were susceptible to impaired QVP in the toe, DSPN and painful leg cramps.

Our third and fourth findings confirmed this by multivariate analysis. The statistically significant associations between the GPx-1 genotype and DSPN, painful leg cramp, history of MVD and QVP impairment did not disappear, even if it was adjusted for microangiopathies (proteinuria and retinopathy), which are closely related to diabetic neuropathy[20]. The associations between the GPx-1 genotype and DSPN, painful leg cramp and QVP impairment also kept statistical significance, even after the adjustment for the history of MVD.

On the other hand, we could not observe any significant relationship between the GPx-1 genotype and DAN, autonomic and nerve conduction functions. In general, DSPN and DAN are considered to reflect mainly the large and small diameter nerve fiber dysfunctions, respectively[21]. Thus, the lack of association of the GPx-1 genotype with DAN and autonomic functions might show that the etiological factors of DSPN differ from those of DAN.

In contrast, impairment of nerve conduction is thought to be a reliable marker of DSPN. The amplitude and conduction abnormalities are most prominent in the distal segments of nerves in the legs; the potential for sensory nerve action in the sural nerve is especially sensitive and useful in identifying early abnormalities[22]. As we did not carry out nerve conduction studies in the lower limbs, accurate nerve conduction functions seemed not to be evaluated sufficiently in our study. Associations between the GPx-1 genotype and nerve conduction data in the lower limbs might provide different results. A more plausible explanation of this issue is that the GPx-1 genotype is mainly associated with QVT impairment, which is a part of the manifestation of DSPN. Actually, our data showed a strong association between the GPx-1 genotype and QVP, whereas a significant association with the GPx-1 genotype, diminished ATR and sensory symptoms was not proven. QVP reflects the functions of the peripheral and central nervous system, and it can be impaired by causes other than neuropathy, such as peripheral arterial disease. However, we suppose that the GPx-1 genotype affects the peripheral nerve function of vibratory sensation to some degree, because the patients with clinical peripheral arterial disease were excluded from the present study and the association of the GPx-1 genotype with QVP was independent from the history of MVD. Furthermore, a recent study using DCCT/EDIC participants proved that QVP is a sensitive measure of peripheral neuropathy[23]. Considering all of the aforementioned findings, we might be able to conclude that Pro198Leu polymorphism of the GPx-1 gene might be a candidate for the common genetic predisposition to MVD and DSPN, especially with an impairment of vibratory perception.

Two possible pathophysiological mechanisms of the association between the GPx-1 genotype and QVP impairment can be considered. One possible mechanism is impaired microcirculation in the peripheral nerve caused by vascular endothelial dysfunction elicited through accelerated oxidative stress in patients with a Pro/Leu genotype. We have previously reported that anti-oxidative activity of GPx-1 decreased in the Pro/Leu genotype[9]. Significant relationships between the GPx-1 genotype and painful leg cramps might support this possibility, because painful leg cramps are considered to reflect a circulatory disturbance in the leg and are frequently experienced in cold ischemic conditions. Another possible mechanism is direct nerve damage as a result of elevated oxidative stress. Neurotoxicity of excessive oxidative stress is widely recognized in experimental diabetic neuropathy. At present, the precise mechanism of the harmful effects of Pro/Leu genotype of the GPx-1 gene on QVT impairment is uncertain.

We have also shown a significant relationship between the GPx-1 genotype and the prevalence of painful leg cramps. Because painful leg cramps can be associated with various disorders, such as neurological, muscular, metabolic, endocrine and vascular diseases, a significant association between the GPx-1 genotype and painful leg cramps might not reflect DSPN. Furthermore, the prevalence of painful leg cramps occurring in self-administered questionnaires in 1524 diabetic patients under a primary care physician (25.5%) was not different from that in 501 non-diabetic subjects (29.4%) who underwent a corporate health screening examination (Nakatani M, Sasaki H, Kurisu S, Yamaoka H, Matsuno S, Ogawa K, Yamasaki H, Wakasaki H, Furuta H, Nishi M, Akamizu T, Nanjo K, 2011, unpublished

Table 4 | Relationships between glutathione peroxidase 1 gene polymorphism and subtype of diabetic neuropathy, painful leg cramp and quantitative neurological functions evaluated by multiple logistic regression and multiple regression analysis

Independent variables	Model 3 dependent variables			Model 3 dependent variables						
	Subtypes of diabetic neuropathy		Painful leg cramp	Vibration	Autonomic functions		Nerve conduction parameters			
	DSPN	DAN		QVP	CVR–R	ΔBP	MCV	CMAP	SCV	SNAP
R^2 (*P*-value)	R^2 = 0.210 (*P* < 0.0001)	R^2 = 0.530 (*P* < 0.0001)	R^2 = 0.103 (*P* = 0.0289)	R^2 = 0.320 (*P* < 0.0001)	R^2 = 0.303 (*P* < 0.0001)	R^2 = 0.266 (*P* < 0.0001)	R^2 = 0.254 (*P* < 0.0001)	R^2 = 0.093 (*P* = 0.1411)	R^2 = 0.189 (*P* = 0.0006)	R^2 = 0.419 (*P* < 0.0001)
	Adjusted OR (95% CI) *P*-value	Adjusted OR (95% CI) *P*-value	Adjusted OR (95% CI) *P*-value	β (*P*-value)	β (*P*-value)	β (*P*-value)	β (*P*-value)	β (*P*-value)	β (*P*-value)	β (*P*-value)
Age (years)	1.010 (0.974–1.048) 0.5938	1.002 (0.933–1.076) 0.9619	0.995 (0.959–1.032) 0.7785	**0.292** (**0.0001**)	**−0.278** (**0.0003**)	0.091 (0.2337)	−0.135 (0.0833)	–	−0.096 (0.2446)	**−0.342** (**<0.0001**)
Sex (female: 0, male: 1)	1.095 (0.514–2.335) 0.8136	1.904 (0.455–7.969) 0.3781	0.468 (0.217–1.010) 0.0529	0.076 (0.2664)	0.090 (0.2177)	0.051 (0.4859)	−0.095 (0.2033)	–	−0.039 (0.6258)	**−0.168** (**0.0122**)
Duration (years) (≧5: 0. 6–15: 1, 16≦: 2)	1.343 (0.794–2.269) 0.2713	0.976 (0.408–2.336) 0.9567	1.139 (0.652–1.989) 0.6473	−0.062 (0.4217)	−0.118 (0.1386)	0.014 (0.8577)	0.100 (0.2203)	–	0.083 (0.3434)	−0.089 (0.2237)
Hypertension (no: 0, yes: 1)	2.115 (0.956–4.677) 0.0644	2.598 (0.708–9.531) 0.1499	0.742 (0.333–1.654) 0.4650	0.058 (0.4217)	0.108 (0.1472)	0.123 (0.1046)	0.044 (0.5625)	–	−0.105 (0.1978)	0.003 (0.9595)
Dyslipidemia (no: 0, yes: 1)	**0.466** (**0.218–0.996**) **0.0486**	0.833 (0.206–3.370) 0.7979	0.516 (0.241–1.102) 0.0875	−0.017 (0.8013)	−0.003 (0.9711)	−0.091 (0.2092)	0.001 (0.9866)	–	−0.007 (0.9303)	0.068 (0.2982)
Glycemic control (∼fair: 0, poor: 1)	1.731 (0.821–3.650) 0.1494	**9.470** (**1.968–45.577**) **0.0050**	1.412 (0.672–2.985) 0.3600	0.025 (0.7139)	−0.125 (0.0725)	0.122 (0.0823)	**−0.195** (**0.0066**)	–	**−0.239** (**0.0019**)	−0.100 (0.1148)
BMI (kg/m^2) (>22: 0. 22–25: 1, 25<: 2)	0.862 (0.545–1.364) 0.5263	**0.159** (**0.055–0.457**) **0.0007**	0.978 (0.621–1.540) 0.9235	−0.061 (0.3870)	0.072 (0.3212)	**−0.196** (**0.0082**)	**0.162** (**0.0308**)	–	0.135 (0.0894)	−0.093 (0.1569)
GPx-1 genotype (Pro/Pro: 0, Pro/Leu: 1)	**3.286** (**1.156–9.346**) **0.0257**	0.352 (0.042–2.965) 0.3366	**4.469** (**1.725–11.578**) **0.0021**	**0.157** (**0.0194**)	0.064 (0.3454)	−0.126 (0.0696)	0.058 (0.4070)	–	−0.025 (0.7420)	−0.023 (0.7082)
Proteinuria (no: 0, intermittent: 1, persistent: 2)	0.692 (0.379–1.207) 0.1948	0.670 (0.299–1.503) 0.3314	1.529 (0.869–2.691) 0.1408	−0.090 (0.2624)	−0.030 (0.7158)	0.030 (0.7192)	**−0.182** (**0.0322**)	–	−0.081 (0.3672)	−0.003 (0.9682)
Retinopathy (no: 0, simple: 1, PPDR∼: 2)	**2.921** (**1.764–4.835**) **<0.0001**	**38.537** (**5.553–267.423**) **0.0002**	0.655 (0.384–1.115) 0.1187	**0.455** (**<0.0001**)	**−0.307** (**0.0006**)	**0.380** (**<0.0001**)	**−0.320** (**0.0004**)	–	**−0.293** (**0.0020**)	**−0.409** (**<0.0001**)
History of MVD (no: 0, yes: 1)	1.042 (0.279–3.653) 0.9491	0.890 (0.82–9.632) 0.9235	1.438 (0.421–4.909) 0.5621	−0.018 (0.7950)	−0.128 (0.0736)	−0.018 (0.3834)	0.012 (0.8722)	–	0.086 (0.2765)	0.0181 (0.7868)

The significant regression formula on compound muscle action potential (CMAP) were not obtained in model 3. β, Standard regression coefficient; BMI, body mass index; ΔBP, orthostasis-induced decreases in systolic blood pressure at standing; CI, confidence interval; CVR-R, coefficient of variation of RR intervals on electrocardiogram after 15 min resting; DAN, diabetic autonomic neuropathy; DSPN, distal symmetric polyneuropathy; MCV, motor nerve conduction velocity; MVD, macrovascular disease, OR, odds ratio; PPDR, preproliferative diabetic retinopathy; QVP, quantitative vibratory perception thresholds; R^2, decision coefficient; SCV, sensory nerve conduction velocity; SNAP, sensory nerve action potential. Statistically significant *P*-value was shown by boldfaced type.

data). Therefore, the observed relationship between painful leg cramps and the Pro/Leu genotype might not be exclusively confined to a diabetic population.

As for common risk factors of MVD and diabetic neuropathy, several investigators reported common risk factors, such as obesity, dyslipidemia and hypertension. Most of the reports were epidemiological studies, and diabetic neuropathy was correspondent to the DSPN of the present study, though autonomic or nerve conduction functions were not carefully evaluated[3–5]. It might be speculated that the GPx-1 gene polymorphism could affect the increasing prevalence of DSPN though the deteriorating effect of vibratory perception.

Further studies, such as a prospective observational study, are necessary to confirm the association of the Pro198Leu polymorphism of the GPx-1 gene with diabetic neuropathy.

ACKNOWLEDGEMENT

We thank Ms Keiko Terao and Ms Mayu Miyata for technical support in the neurological examination. We have no conflict of interest in this work.

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