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Association of myeloperoxidase G-463A gene polymorphism with diabetic nephropathy in Japanese type 2 diabetic subjects

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Abstract. It is possible that myeloperoxidase (MPO) contributes to the pathogenesis of diabetic nephropathy through the production of reactive oxygen species and HOCl/OCl⁻. In this study, we examined the relationship between renal damage and *MPO* G-463A gene polymorphism that is associated with its transcription activity in diabetic patients. We evaluated the association between *MPO* G-463A polymorphism and the prevalence of proteinuria and estimated GFR (eGFR) in 1448 Japanese type 2 diabetic subjects. The prevalence of macroalbuminuria was higher as the number of G alleles increased (GG (7.6%), GA (3.8%), AA (0.0%), *p* for trend=0.0269). The number of G alleles was significantly associated with macroalbuminuria (odds ratio 2.12, 95%CI 1.06-4.24, *p*=0.0344) even after adjustment for conventional risk factors. Inversely, eGFR was lower as the number of G alleles increased (GG (76.7±20.7 mL/min/1.73m²), GA (81.0±22.8 mL/min/1.73m²), AA (92.0±23.1 mL/min/1.73m²), *p* for trend=0.0025) and the number of G allele was an independent risk factor for a low eGFR (β =-0.072, *p*=0.003). We also examined the association between MPO expression and several stages of renal damage in a high-fat diet-induced diabetic mouse model. The proteinuria-induced increase in MPO expression was markedly enhanced in diabetic mice, and MPO expression was significantly correlated with the severity of kidney damage. In conclusion, it is likely that the G allele of the *MPO* G-476T polymorphism is a susceptibility allele for renal injury in type 2 diabetic patients.

Keywords: Diabetes, Myeloperoxidase, Polymorphism, Single nucleotide polymorphisms (SNP), Nephropathy

MYELOPEROXIDASE (MPO), a member of the mammalian heme peroxidase superfamily, is stored within the azurophilic granules of circulating neutrophils, monocytes, and some tissue macrophage populations [1, 2]. The catalytic activity of MPO results in the generation of various reactive oxidants and diffusible radical species [1]. Although MPO-derived reactive oxygen species (ROS) play an important role in killing

invading parasites and pathogens, they could also promote host tissue injury through oxidative modification of proteins, lipids, and nucleic acids [3-6], leading to a wide range of chronic inflammatory diseases including kidney disease. In addition, MPO generates hypochlorous acid/hypochlorite (HOCl/OCl⁻) from hydrogen peroxide in the presence of chloride ions, which may cause dysfunction of cells in different compartments of the kidney through the generation of a variety of chlorinated protein and lipid adducts.

Indeed, previous studies implied that MPO plays roles in the pathogenesis of several renal diseases such as acute immune complex crescentic glomerulonephritis [7], membranous glomerulonephritis [8], and ischemia/reperfusion injury [9]. It is possible that MPO also contributes to the pathogenesis of diabetic nephropathy through the production of ROS and HOCl/OCl⁻, since increased production of ROS plays an important role

Submitted Sep. 11, 2012; Accepted Nov. 21, 2012 as EJ12-0345
Released online in J-STAGE as advance publication Dec. 24, 2012
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Abbreviations: Cr, creatinine; FFA, free fatty acid; HFD, high-fat diet; MPO, Myeloperoxidase; ND, normal diet; ROS, reactive oxygen species

in the pathogenesis of renal injury in diabetes mellitus [10-13]. However, so far, very little information has been obtained about the association between MPO and renal dysfunction in diabetic patients.

The G-463A gene polymorphism (rs2333227), a G/A substitution at SP1 transcription factor binding site in *MPO* gene, is associated with its transcription activity and the G allele and GG genotype have been reported to be associated with increased MPO expression [14, 15]. However, there have been very few studies on the association of this polymorphism and the risk of diabetic nephropathy.

In this study, we examined the relationship between this polymorphism and kidney damage in a cohort of Japanese type 2 diabetic patients. In addition, we investigated whether MPO expression might be related to kidney damage in type 2 diabetes, using a high-fat diet (HFD)-induced diabetic mouse model.

Materials and Methods

Subjects

All the Japanese type 2 diabetic subjects who periodically attended the outpatient clinics of diabetes in 7 participating hospitals (Osaka University Medical Hospital, Ehime Prefectural Central Hospital, Ehime Prefectural Imabari Hospital, Imamura Clinic, Ishibashi Clinic, Naka Memorial Clinic, Kawai Clinic) during 1 year (January-December period in 2005) in Japan were asked to participate in this study. We considered subjects eligible when they had type 2 diabetes diagnosed by diabetologists based on Japan Diabetes Society's criteria. After all, a total of 1448 subjects (males, 61.7%; age, 60.6±9.7 years (mean±SD); HbA1c 7.3±1.3%) were enrolled in the present study. The patients' characteristics and their medication are listed in Table 1. The study protocol was approved by the committees on the ethics of human research of Osaka University Graduate School of Medicine. A written informed consent was obtained from all the participants after a full explanation of the study.

Clinical and biochemical analysis

Fasting blood samples were collected and HbA1c, serum total and HDL cholesterol, and serum triglyceride levels were measured using standard laboratory protocols. HbA1c was measured by high-performance liquid chromatography using a set of calibrators assigned by the Japan Diabetes Society (normal range

4.3-5.8%). According to correlation analysis, HbA1c values in Japan were estimated to be 0.4% lower than those measured by the National Glycohemoglobin Standardization Program (NGSP) [16]. To standardize HbA1c values to NGSP units, 0.4% was added to the measured values and expressed as HbA1c-equivalent NGSP units in the current study. Serum MPO levels were measured in randomly selected 174 subjects using enzyme-linked immunosorbent assay kits (R&D Systems, Inc, Minneapolis, USA). The inter-assay and intra-assay variability of the MPO test was 8.3% and 7.7%, respectively. The determination of hypertension (defined as systolic blood pressure ≥ 130 mmHg or diastolic blood pressure ≥ 80 mmHg or having been treated for hypertension) and dyslipidemia (defined as serum LDL cholesterol ≥ 120 mg/dL or serum triglyceride (TG) ≥ 150 mg/dL or HDL-cholesterol < 40 mg/dL or having been treated for dyslipidemia) was based on the Japan Diabetes Society's criteria. Smoking status was evaluated as follows; a value of 0 and 1 were assigned to subjects when Brinkman's Index (the number of cigarettes per day x smoking years) was less than 200 and greater than 200, respectively. Urinary albumin and creatinine (Cr) concentrations were determined using early morning spot urine. Nephropathy was graded as follows: normoalbuminuria, urinary albumin excretion less than 30mg/g Cr; microalbuminuria, 30 to 300mg/g Cr; or macroalbuminuria, more than 300mg/g Cr. GFR was estimated using the new equation proposed by the Japanese Society of Nephrology as follows: $eGFR (mL/min/1.73 m^2) = 194 \times Cr^{-1.094} \times Age^{-0.287} \times 0.739$ (if female) [17].

Genetic analysis

Venous blood was collected from each subject and genomic DNA was isolated with a DNA isolation kit (Qiagen). The genotypes of the *MPO* gene G-463A polymorphism were determined with a fluorescence- or colorimetry-based allele-specific DNA-primer probe assay system as previously described [18]. The average genotyping success rate was 99.7% and the genotyping accuracy was 100%, which was determined from the genotype concordance between 500 duplicate samples.

Animal protocol

All procedures were performed in accordance with the guidelines of the Research Center for Animal Life Science of Shiga University of Medical Science. Six-week-old male C57BL/6 mice were purchased from

Table 1 Clinical characteristics of the subjects in two genotype groups

<i>G-463A</i> polymorphism of the <i>myeloperoxidase (MPO)</i> gene	Total (n = 1448)	G/G (n = 1204)	G/A or A/A (n = 244)	<i>p</i> value
Gender (male / female)	892 / 556	755 / 449	137 / 107	NS
Age (years)	60.6 ± 9.7	60.8 ± 9.6	59.7 ± 9.7	NS
Smoking status (B.I.<200 / ≥200)	746 / 702	607 / 597	139 / 105	NS
Presence of obesity (%)	562 (38.8%)	463 (38.5%)	99 (40.6%)	NS
Body mass index (kg/m ²)	24.4 ± 3.7	24.4 ± 3.7	24.5 ± 3.5	NS
HbA _{1C} (%)	7.7 ± 1.3	7.7 ± 1.3	7.7 ± 1.3	NS
Duration of diabetes (years)	11.1 ± 8.0	11.2 ± 8.0	11.0 ± 8.3	NS
Presence of hypertension (%)	1053 (72.7%)	877 (72.8%)	176 (72.1%)	NS
Systolic blood pressure (mmHg)	131 ± 14	131 ± 14	130 ± 13	NS
Diastolic blood pressure (mmHg)	75 ± 10	74 ± 9	75 ± 9	NS
Presence of dyslipidemia (%)	1064 (73.5%)	883 (73.3%)	181 (74.2%)	NS
Total-cholesterol (mg/dL)	201 ± 35	200 ± 35	207 ± 38	0.004
HDL-cholesterol (mg/dL)	56 ± 17	56 ± 17	56 ± 17	NS
Triglyceride (mg/dL)	107 (26 - 2233)	107 (26-1662)	105.5 (29-2233)	NS
Ln-triglyceride (mg/dL)	4.71 ± 0.58	4.71 ± 0.57	4.72 ± 0.63	NS
Cr (mg/dL)	0.86 ± 2.28	0.89 ± 2.49	0.73 ± 0.27	NS
eGFR (mL/min/1.73m ²)	77.5 ± 21.1	76.7 ± 20.7	81.4 ± 22.9	0.0016
eGFR <60 mL/min/1.73m ² (%)	270 (18.6%)	236 (19.6%)	34 (13.9%)	0.038
Urinary albumin excretion index (mg/gCr)	16.3 (0-7605)	16.1 (0-7605)	16.9 (0.5-3573)	NS
Ln-UAE (mg/gCr)	3.10 ± 1.46	3.11 ± 1.47	3.04 ± 1.38	NS
Proteinuria (normo/micro/macro)	951 / 397 / 100	791 / 322 / 91	160 / 75 / 9	-
Albuminuria >30mg/gCr (%)	497 (34.3%)	413 (34.3%)	84 (34.4%)	NS
Albuminuria >300mg/gCr (%)	100 (6.9%)	91 (7.6%)	9 (3.7%)	0.030
Antidiabetic drugs (yes / no)	1232 (85.1%)	1024 (85.0%)	208 (85.2%)	NS
Antihypertensive drugs (yes / no)	558 (38.5%)	467 (38.8%)	91 (37.3%)	NS
Antihyperlipidemic drugs (yes / no)	347 (24.0%)	296 (24.6%)	51 (20.9%)	NS
Serum MPO (ng/mL)	287 (12-3371) (n=174)	320 (12-3371) (n=151)	212 (46-743) (n=23)	0.049
Ln-serum MPO (ng/mL)	5.74 ± 0.97	5.79 ± 0.99	5.35 ± 0.78	0.039

Data are shown as numbers, means ± SD, or median (range). All the subjects were divided into two groups based on the major allele's recessive genetic models (*GG* genotype vs. *GA* + *AA* genotype) and the parameters were compared among the groups. Quantitative data between the groups were compared by the 2-tailed unpaired *t* test or Mann-Whitney test, depending on the distribution pattern of the data. Categorical data were analyzed with the χ^2 test or Fisher's exact test. Nephropathy was graded as follows: normoalbuminuria, urinary albumin excretion less than 30mg/g Cr; microalbuminuria, 30 to 300mg/g Cr; or macroalbuminuria, more than 300mg/g Cr. *p*-values over 0.05 were displayed as NS (not significant).

B. I., Brinkman's Index; Ln-, log-transformed; UAE, Urinary albumin excretion index; MPO, myeloperoxidase

CLEA Japan (Tokyo, Japan) and housed in cages under a 12-h light/12-h dark cycle. The normal diet (ND; 10% of total calories from fat) and high-fat diet (HFD; 60% of total calories from fat) were purchased from Research Diets (New Brunswick, NJ). In order to establish a mouse model of obese type 2 diabetes and microalbuminuria, we used the following protocol. After 2 weeks of acclimatization, the mice were fed with an ND or HFD for an additional 12 weeks, and then they were placed in metabolic balance cages to measure 24-h urinary albumin excretion [19]. At the 12-week point, the mice were sacrificed and urinary

albumin concentrations and serum cystatin C levels were measured with a commercially available ELISA kit Albuwell (Exocell, Philadelphia, PA) and Cystatin C (mouse) ELISA Kit (ALEXIS Biochemicals, CA). The urinary albumin was expressed as the total amount excreted during the 24-h urine collection [19].

To determine the effects of HFD-induced diabetes on proteinuria-induced renal injury, after 2 weeks of acclimatization, a second cohort of mice were maintained on an ND or HFD for 6 weeks, and then intraperitoneally injected with PBS (control) or free fatty acid (FFA)-non-depleted bovine serum albumin (protein-

overload) for 11 days, as previously described [19]. For both of the animal experiments undertaken, each group contained eight mice. At the end of the experimental period, each of the animals' body weights, blood glucose and glycated hemoglobin levels were measured, as previously described [19]. Urinary protein concentrations were measured using the Bradford method. Kidney samples were also removed and collected as previously reported [19].

RNA extraction and quantitative real-time PCR

Total RNA was isolated from the kidney of each group of mice to synthesize cDNA. The iQSYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) was used for real-time PCR (ABI Prism TM 7500 Sequence Detection System; Perkin-Elmer Applied Biosystems, Foster City, CA). The mRNA expression levels were quantified using the standard curve method and adjusted for the mRNA expression of β -actin as an internal control [19]. The following primer sequences were used (forward and reverse): β -actin, CGTGCGTGACATCAAAGAGAA and TGGATGCACAGGATTCCAT; neutrophil gelatinase-associated lipocalin (Ngal), TACCCTGTATGGAAGAA CCAAGGA and CGGTGGGGACAGAGAAGATG; F4/80, CTTTGGCTATGGGCTTCCAGTC and GCAAGGAGACAGAGTTTATCGTG; myeloperoxidase (MPO), GTGTCAAGTGGCTGTGCCTATC and AGTGGGGCTTCGTCTGTTGT.

Cell culture study

SV40-transformed murine mesangial, immortalized murine podocyte and murine proximal tubular cell lines were cultured as described previously [19]. After a 12-hour starvation period, cells were stimulated with FFA-non-depleted albumin (30 mg/mL) for 9 hours. Total RNA was isolated and cDNA was synthesized by reverse transcriptional PCR. cDNA was used for standard PCR to check the expression of MPO and β -actin.

Statistical analysis

All the subjects were divided into two groups based on the major allele's recessive genetic models (*GG* genotype *vs.* *GA + AA* genotype) and the parameters were compared between the groups. Quantitative data between the groups were compared by the 2-tailed unpaired *t* test or Mann-Whitney test, depending on the distribution pattern of the data. Categorical data were analyzed with the χ^2 test or Fisher's exact test. In the

major allele's additive genetic model (*GG* genotype < *GA* genotype < *AA* genotype or *GG* genotype > *GA* genotype > *AA* genotype), the associations between the polymorphism and variables were evaluated with the Pearson's univariate test or the Cochran-Armitage test.

Multiple logistic regression analyses were performed to evaluate the relationship between the presence of macroalbuminuria and the number of G alleles or the *GG* genotype of *MPO* G-463A polymorphism. Following variables were included in the model: gender, age, duration of diabetes, HbA1c, body mass index (BMI), presence of hypertension, and presence of dyslipidemia. Forward and backward stepwise multivariate regression analyses were also performed to evaluate the relationship between eGFR and the following variables: gender (female=0, male=1), age, smoking status, duration of diabetes, HbA1c, BMI, presence of hypertension, presence of dyslipidemia, and the number of G alleles of *MPO* G-463A polymorphism.

Results

Association of *MPO* G-463A polymorphism and macroalbuminuria in T2DM subjects

The prevalence of the G-463A genotypes was as follows: *GG*, 83.1%; *GA*, 16.3%; *AA*, 0.6%; and the genotype distribution was in Hardy-Weinberg equilibrium. Since the number of subjects with *GA* and *AA* genotypes was low, we combined the subjects with *GA* and *AA* genotypes, and performed a statistical analysis between two groups: the subjects with *GG* and those with *GA* or *AA* genotypes. There were no associations between this polymorphism and clinical characteristics including gender, age, smoking status, BMI, HbA1c, duration of diabetes, presence of hypertension, presence of dyslipidemia, and administration of drugs (Table 1). However, serum MPO levels were significantly higher in subjects with *GG* genotype (*n*=151) as compared to those with *GA* or *AA* genotype (*n*=23) (median (range) 320 (12-3371) and 212 (46-743) ng/mL, respectively, *p* = 0.049).

Among a total of 1448 subjects, 397 subjects were defined as having microalbuminuria and 100 subjects were defined as having macroalbuminuria. There were no significant associations between this polymorphism and the prevalence of patients with albuminuria >30mg/gCr. However, the prevalence of macroalbuminuria (>300mg/gCr) was higher as the number of G alleles increased (*GG* (7.6%), *GA* (3.8%), *AA* (0.0%),

p value for trend = 0.0269) and was significantly higher in the subjects with GG genotype as compared to those with GA or AA genotype (7.6% vs. 3.7%, $p=0.0297$) (Table 1).

When subjects were divided into the subjects with the macroalbuminuria (+) group ($n=100$) and the macroalbuminuria (-) group ($n=1348$), the frequency of G allele (95.5% vs. 91.0%, $p=0.0289$), BMI (25.3 ± 4.9 vs. 24.3 ± 3.6 kg/m², $p=0.0134$), duration of diabetes (13.4 ± 7.8 vs. 11.0 ± 8.0 years, $p=0.0036$), and presence of hypertension (86.0% vs. 71.7%, $p=0.0020$) were significantly higher in the former as compared to the latter (Table 2). Next, a multiple logistic regression analysis was performed to evaluate the relationship between the presence of macroalbuminuria and

the following variables: the GG genotype of *MPO* G-463A polymorphism, gender, age, duration of diabetes, HbA_{1c}, BMI, presence of hypertension, and presence of dyslipidemia. This analysis revealed that the subjects with GG genotype had a significantly higher risk of macroalbuminuria as compared to the subjects with AA or GA genotype (OR 2.11 with 95%CI 1.04-4.29, $p=0.038$) even after adjustment for conventional risk factors (Table 3). In addition, another multiple logistic regression model showed that the number of G alleles of *MPO* G-463A polymorphism was significantly associated with macroalbuminuria even after adjustment for conventional risk factors (Odds ratio (OR) for 1-point increase in the number G allele = 2.12 with 95%CI 1.06-4.24, $p=0.034$) (data not shown).

Table 2 Clinical characteristics of the study subjects with and without proteinuria

	proteinuria (-) (n = 1348)	proteinuria (+) (n = 100)	<i>p</i>
Gender (male / female)	824 / 524	68 / 32	NS ^a
Age (yr)	60.5 ± 9.7	62.2 ± 9.4	NS
Smoking (B.I. < 200 / ≥200)	698 / 650	48 / 52	NS ^a
Body mass index (kg/m ²)	24.3 ± 3.6	25.3 ± 4.9	0.0134
HbA _{1c} (%)	7.7 ± 1.3	8.0 ± 1.5	NS
Duration of diabetes (yr)	11.0 ± 8.0	13.4 ± 7.8	0.0036
Presence of hypertension (%)	967 (71.7%)	86 (86.0%)	0.0020 ^a
Systolic blood pressure (mmHg)	130 ± 14	137 ± 16	<0.001
Diastolic blood pressure (mmHg)	75 ± 10	77 ± 11	NS
Presence of dyslipidemia (%)	988 (73.3%)	76 (76.0%)	NS
Presence of obesity (%)	517 (38.4%)	45 (45.0%)	NS
Total-cholesterol (mg/dL)	201 ± 35	206 ± 39	NS
HDL-cholesterol (mg/dL)	56 ± 17	53 ± 15	NS
Triglyceride (mg/dL)	106 (26 - 2233)	127.5 (38 - 516)	NS
Ln-triglyceride (mg/dL)	4.70 ± 0.58	4.81 ± 0.54	NS
eGFR (ml/min/1.73m ²)	78.2 ± 20.2	68.7 ± 30.0	<0.001
Administration of			
Antidiabetic drugs (%)	976 (72.4%)	76 (76.0%)	NS ^a
Antihypertensive drugs (%)	493 (36.4%)	65 (65.0%)	<0.001 ^a
Anti-hyperlipidemic drugs (%)	319 (23.7%)	28 (28.0%)	NS ^a
Allele frequency (%)			
G	91.0	95.5	0.029 ^a
A	9.0	4.5	0.029 ^a
Genotype frequency (%)			NS (MDM)
GG, GA, AA	82.6 / 16.8 / 0.6	91.0 / 9.0 / 0.0	0.030 (MRM)
			0.027 (MAM)
Serum MPO (ng/mL)	193 (12-3371) (n=158)	301 (54-1991) (n=16)	NS

Data are shown as numbers (%) or means ± SD. In the major allele's dominant and the recessive genetic models, the associations between the polymorphism and variables were evaluated with the χ^2 test^(a). In the major allele's additive genetic model, the associations between the polymorphism and variables were evaluated with the Cochran-Armitage test. p values over 0.05 were displayed as NS (not significant).

B. I., Brinkman's Index; MDM, major allele's dominant model; MRM, major allele's recessive model; MAM, major allele's additive model; Ln-, log-transformed; MPO, myeloperoxidase.

Table 3 Multivariate logistic regression analysis to identify independent determinants for proteinuria

Parameters	Model 1		Model 2	
	Odds ratio (95%CI)	<i>p</i> value	Odds ratio (95%CI)	<i>p</i> value
Gender (male)	1.36 (0.87 – 2.13)	NS	1.36 (0.87 – 2.13)	NS
Age (years)	1.02 (0.99 – 1.04)	NS	1.02 (0.99 – 1.04)	NS
Duration of diabetes (years)	1.03 (1.00 – 1.06)	0.024	1.03 (1.00 – 1.06)	0.024
HbA1c (%)	1.17 (1.01 – 1.36)	0.043	1.17 (1.01 – 1.36)	0.043
Body mass index (kg/m ²)	1.08 (1.02 – 1.14)	0.008	1.08 (1.02 – 1.14)	0.007
Presence of hypertension	2.15 (1.19 – 3.86)	0.011	2.15 (1.19 – 3.86)	0.011
Presence of dyslipidemia	1.02 (0.63 – 1.67)	NS	1.03 (0.63 – 1.67)	NS
-463G allele	2.12 (1.06 – 4.24)	0.034	—	—
-436GG genotype	—	—	2.11 (1.04 – 4.29)	0.038

Multivariate logistic regression analysis was done for 1448 type 2 diabetic patients to select variables significantly associated with an increase in the risk of proteinuria. The threshold of statistical significance was defined as $p < 0.05$. Model 1; gender, age, duration of diabetes, HbA1c, body mass index, presence of hypertension, presence of dyslipidemia, and the number of -436G allele were included as independent variables.

Model 2; gender, age, duration of diabetes, HbA1c, body mass index, presence of hypertension, presence of dyslipidemia, and the number of -436GG genotype were included as independent variables.

NS, not significant

Inversely, eGFR was lower as the number of G alleles increased (GG (76.7 ± 20.7 mL/min/1.73m²), GA (81.0 ± 22.8 mL/min/1.73m²), AA (92.0 ± 23.1 mL/min/1.73m²), p value for trend = 0.0025) and was significantly lower in subjects with GG genotype compared to those with GA or AA genotype (76.7 ± 20.7 vs. 81.4 ± 22.9 mL/min/1.73m², $p=0.0016$) (Supplementary Table). In univariate correlation analysis, eGFR was significantly correlated with the number of G alleles ($r=-0.088$, $p=0.0008$), age ($r=-0.364$, $p<0.0001$), duration of diabetes ($r=-0.197$, $p<0.0001$), HbA1c ($r=0.174$, $p<0.0001$), BMI ($r=0.053$, $p=0.0441$) and presence of hypertension ($r=-0.145$, $p<0.0001$) (Table 4). To examine whether the number of G allele is a determinant of eGFR independent of conventional risk factors, we performed a stepwise multivariate regression analysis with gender, age, smoking status, duration of diabetes, HbA1c, BMI, presence of hypertension, presence of dyslipidemia, and the number of G alleles of *MPO* G-463A polymorphism as independent variables and eGFR as objective variables. This analysis showed that the number of G allele was an independent risk factor for a low eGFR ($\beta=-0.072$, $p=0.003$) (Table 4). We also performed a dichotomous trait analysis for CKD stage 3 (eGFR < 60 mL/min/1.73m² vs. eGFR ≥ 60 mL/min/1.73m²). This analysis revealed that the frequency of G allele (93.7% vs. 90.7%, $p=0.028$) and GG genotype (87.4% vs. 82.2%, $p=0.038$) were significantly higher in the subjects with low eGFR (<60) as compared to those with preserved eGFR (≥ 60) (Table

5). However, a multiple logistic regression analyses showed that neither the number of G allele nor GG genotype was independently associated with low eGFR after adjustment for conventional risk factors (OR 1.45; 95%CI 0.98-2.15, $p=0.062$ and OR 1.42; 95%CI 0.95-2.12, $p=0.090$, respectively) (Table 6).

Furthermore, we also performed a series of analyses in which the patients with shorter duration of diabetes (less than 10 years) were excluded, since the control (nephropathy free) groups must include patients who will develop overt nephropathy later on. The results of these analyses were similar to those of the original analyses. The prevalence of macroalbuminuria (>300mg/gCr) was significantly higher in the subjects with GG genotype as compared to the subjects with GA or AA genotype (10.4% vs. 4.3%, $p=0.037$) (Supplementary Table 1 and 2). In addition, multiple logistic regression analyses showed that the number of G allele (OR 2.63; 95%CI 1.63-6.70, $p=0.043$) as well as GG genotype (OR 2.64; 95%CI 1.03-6.76, $p=0.044$) were independently associated with macroalbuminuria even after adjustment for conventional risk factors (Supplementary Table 3). Inversely, eGFR was significantly lower in subjects with GG genotype compared to those with GA or AA genotype (72.3 ± 20.5 vs. 80.2 ± 25.1 mL/min/1.73m², $p<0.001$) (Supplementary Table 1). The frequency of G allele (94.5% vs. 90.8%, $p=0.029$) and that of GG genotype (89.0% vs. 81.9%, $p=0.028$) were significantly higher in the subjects with low eGFR as compared to those with preserved eGFR

Table 4 Correlations between eGFR and variables

	Univariate*		Multivariate	
	<i>r</i>	<i>p</i>	β	<i>p</i>
Gender (male / female)	-0.074	0.0046	-0.079	0.001
Age (yrs)	-0.364	<0.001	-0.304	<0.001
Smoking (yes / no)	-0.025	NS	NI	—
Duration of DM (yrs)	-0.197	<0.001	-0.088	0.001
HbA1c (%)	0.174	<0.001	0.122	<0.001
BMI (kg/m ²)	0.053	0.0441	NI	—
Presence of hypertension	-0.145	<0.001	-0.101	<0.001
Presence of dyslipidemia	-0.028	NS	NI	—
MPO -463G	-0.088	0.0008	-0.072	0.003
<i>R</i> ²	—	—	0.176	—

*Pearson's univariate correlation coefficients. A stepwise multivariate regression analysis was performed. Gender: male = 1, female = 0; Smoking: yes = 1, no = 0. Presence of hypertension: yes = 1, no = 0; Presence of dyslipidemia: yes = 1, no = 0; β : partial regression coefficient. NS, not significant; NI, not included; BMI, body mass index.

Table 5 Clinical characteristics of the study subjects with and without low eGFR (< 60mL/min/1.73m²)

	preserved eGFR ($\geq 60\text{mL/min/1.73m}^2$) (n = 1178)	low eGFR (< 60mL/min/1.73m ²) (n = 270)	<i>p</i>
Gender (male / female)	716 / 462	176 / 94	NS ^a
Age (yr)	59.7 $\pm$ 9.6	64.6 $\pm$ 8.6	<0.001
Smoking (B.I. < 200 / ≥ 200)	606 / 564	140 / 126	NS ^a
Body mass index (kg/m ²)	24.4 $\pm$ 3.7	24.4 $\pm$ 3.4	0.0134
HbA _{1C} (%)	7.8 $\pm$ 1.3	7.4 $\pm$ 1.1	<0.001
Duration of diabetes (yr)	10.6 $\pm$ 7.8	13.5 $\pm$ 8.6	<0.001
Presence of hypertension (%)	819 (69.5%)	234 (86.7%)	<0.001 ^a
Systolic blood pressure (mmHg)	130 $\pm$ 14	135 $\pm$ 16	<0.001
Diastolic blood pressure (mmHg)	75 $\pm$ 9	77 $\pm$ 11	0.001
Presence of dyslipidemia (%)	858 (72.8%)	206 (76.3%)	NS
Presence of obesity (%)	460 (39.0%)	102 (37.8%)	NS
Total-cholesterol (mg/dL)	202 $\pm$ 35	199 $\pm$ 36	NS
HDL-cholesterol (mg/dL)	56 $\pm$ 17	53 $\pm$ 15	0.006
Triglyceride (mg/dL)	105 (26 - 2233)	115.5 (35 - 798)	NS
Ln-triglyceride (mg/dL)	4.70 $\pm$ 0.58	4.76 $\pm$ 0.55	NS
Administration of			
Antidiabetic drugs (%)	866 (73.5%)	186 (68.9%)	NS ^a
Antihypertensive drugs (%)	419 (35.6%)	139 (51.5%)	<0.001 ^a
Anti-hyperlipidemic drugs (%)	271 (23.0%)	76 (28.1%)	NS ^a
Allele frequency (%)			
G	90.7	93.7	0.028 ^a
A	9.3	6.3	0.028 ^a
Genotype frequency (%)			
GG, GA, AA	82.2 / 17.1 / 0.7	87.4 / 12.6 / 0.0	0.038 (MRM)

Data are shown as numbers (%) or means $\pm$ SD. In the major allele's dominant and the recessive genetic models, the associations between the polymorphism and variables were evaluated with the χ^2 test (^a). In the major allele's additive genetic model, the associations between the polymorphism and variables were evaluated with the Cochran-Armitage test.

p-values over 0.05 were displayed as NS (not significant).

B. I., Brinkman's Index; MRM, major allele's recessive model Ln-, log-transformed.

Table 6 Multivariate logistic regression analysis to identify independent determinants for low eGFR ($< 60 \text{ mL/min/1.73 m}^2$)

Parameters	Model 1		Model 2	
	Odds ratio (95%CI)	<i>p</i> value	Odds ratio (95%CI)	<i>p</i> value
Gender (male)	1.22 (0.91 – 1.63)	NS	1.22 (0.91 – 1.64)	NS
Age (years)	1.05 (1.03 – 1.07)	<0.001	1.05 (1.03 – 1.07)	<0.001
Duration of diabetes (years)	1.03 (1.01 – 1.05)	0.002	1.03 (1.01 – 1.05)	0.002
HbA1c (%)	0.80 (0.70 – 0.91)	0.001	0.80 (0.70 – 0.91)	0.001
Body mass index (kg/m^2)	1.02 (0.98 – 1.07)	NS	1.02 (0.98 – 1.07)	NS
Presence of hypertension	2.48 (1.68 – 3.65)	<0.001	2.48 (1.68 – 3.65)	<0.001
Presence of dyslipidemia	1.23 (0.89 – 1.72)	NS	1.23 (0.89 – 1.72)	NS
-463G allele	1.45 (0.98 – 2.15)	0.062	—	—
-436GG genotype	—	—	1.42 (0.95 – 2.12)	0.090

Multivariate logistic regression analysis was done for 1448 type 2 diabetic patients to select variables significantly associated with an increase in the risk of low eGFR ($< 60 \text{ mL/min/1.73 m}^2$). The threshold of statistical significance was defined as $p < 0.05$.

Model 1; gender, age, duration of diabetes, HbA1c, body mass index, presence of hypertension, presence of dyslipidemia, and the number of -463G allele were included as independent variables.

Model 2; gender, age, duration of diabetes, HbA1c, body mass index, presence of hypertension, presence of dyslipidemia, and the number of -436GG genotype were included as independent variables.

NS, not significant

(Supplementary Table 4). In addition, multiple logistic regression analyses showed that the number of G allele (OR 1.82; 95%CI 1.05–3.16, $p=0.034$) as well as GG genotype (OR 1.81; 95%CI 1.03–3.16, $p=0.038$) were independently associated with low eGFR ($< 60 \text{ mL/min/1.73 m}^2$) after adjustment for conventional risk factors (Supplementary Table 5).

These findings suggest that G allele of the *MPO* G-463A polymorphism is a susceptibility allele for kidney damage, especially for the development of macroalbuminuria and reduction of GFR in Japanese type 2 diabetic patients.

Increase of MPO expression in the advanced stage of renal damage in HFD-induced diabetic mice

The findings of our human genomic study suggest that the *MPO* G-463A polymorphism is associated with the presence of macroalbuminuria and negatively correlated with eGFR (Tables 3 and 4), but not with the presence of microalbuminuria (Table 2). To understand the association of *MPO* G-463A polymorphism with each stage of diabetic nephropathy, we examined the relationship between *MPO* expression and the severity of renal damage, by using two kinds of diabetes-related renal injury mouse models: HFD-induced glomerular injury to mimic the early stage of diabetic nephropathy; and an intraperitoneal protein overload model in HFD-induced obese mice as a model of proteinuria-induced tubulointerstitial damage in obese type 2 diabetes.

First, to elucidate the involvement of *MPO* in the pathogenesis of microalbuminuria in diabetes, we examined renal *MPO* expression in diabetic mice fed with a HFD for 12 weeks [19]. Mice fed a HFD developed an obese diabetic phenotype coupled with a renal phenotype of early stage diabetic nephropathy characterized by albuminuria and glomerular hypertrophy, but not with tubulointerstitial lesions or renal dysfunction (Fig. 1A–F). In real-time PCR, *MPO* expression was not detected at all in the kidneys of HFD-fed mice as well as ND-fed mice (Fig. 1F).

The progressive nature of proteinuric kidney disease, including diabetic nephropathy, is dependent on the degree of proteinuria-induced tubulointerstitial damage [20]. Therefore, to elucidate the involvement of *MPO* in the advanced stage of diabetic nephropathy, we examined *MPO* expression in the proteinuria-induced tubulointerstitial lesions in the HFD-induced diabetic mice. Although mice maintained on the HFD for 6 weeks had significant weight gain in addition to a significant increase in fasting blood glucose and glycated hemoglobin levels, there was no difference in serum cystatin C levels (Fig. 2A–D). Next, to examine the effect of HFD on overt proteinuria-induced tubulointerstitial lesions, tubulointerstitial lesions were induced by equal volumes of protein overload in mice maintained on a ND or HFD for 6 weeks (Fig. 2E) [19]. In mice fed a ND, protein overload led to mild proximal tubular cell damage (Fig. 2F) with significant increases

Fig. 1 (A-E) Body weight (A), blood glucose (B), glycated hemoglobin levels (C), urinary albumin excretion levels (D), and serum cystatin C levels (E) in each group of mice fed with a normal diet (ND) or high-fat diet (HFD) for 12 weeks. (F) Representative pictures of hematoxylin eosin (HE) staining in kidney sections from each group of mice. (H) Renal mRNA expression levels of MPO determined by real-time PCR. mRNA of β actin was used as an internal control. Data are mean $\pm$ SEM ($n=8$). * $p<0.01$. † $p<0.05$. NS: no significant. MPO: myeloperoxidase.

in mRNA expression of Ngal, MCP-1 and fibronectin, markers of proximal tubular cell damage, inflammation and fibrosis (Fig. 2G–I). No increases in serum cystatin C levels were observed (Fig. 2D). In contrast, in mice fed a HFD, protein overload caused more severe tubulointerstitial lesions with significant increases in renal mRNA expression of Ngal, MCP-1, fibronectin and serum cystatin C levels (Fig. 2E–I). These results suggest that the diabetic state markedly exacerbates proteinuria-induced tubulointerstitial damage.

In ND-fed mice, protein overload also caused an increase in MPO mRNA expression (Fig. 2J). Interestingly, this increase in MPO expression was further facilitated in the kidneys of HFD-induced diabetic mice (Fig. 2J), which was similar to changes observed in the expression of Ngal, MCP-1 and fibronectin (Fig. 2G–I). Furthermore, MPO expression was positively correlated with the serum cystatin C levels and mRNA expression levels of Ngal, MCP-1 and fibronectin (Fig. 2K–N). Also, FFA-non-depleted BSA increased MPO expression in the whole kidney samples of mice. This change was not observed in all the samples from cultured podocytes, mesangial cells and proximal tubules (Fig. 2O). These results suggest that MPO is likely to be involved in the relatively advanced stage, rather than

the early stage, of renal damage found in HFD-induced diabetic mice, and the infiltration of inflammatory cells is a principal reason for the increase in MPO expression in kidneys of mice with protein overload.

Discussion

This is the first report to examine the relationship of *MPO* gene G-463A polymorphism and renal damage in a large cohort of type 2 diabetic patients, although there have been several studies showing that *MPO* gene G-463A polymorphism is associated with coronary heart disease [21, 22]. We revealed that the G allele or GG genotype of this polymorphism could be a risk factor for both macroalbuminuria and the reduction of GFR in Japanese type 2 diabetic patients. The increased risk of renal damage associated with the presence of the G allele observed in the present study is in accordance with the previous studies showing that the G allele is associated with an increased expression of MPO [14, 15] and that MPO plays roles in the pathogenesis of several renal diseases such as acute immune complex crescentic glomerulonephritis [7], membranous glomerulonephritis [8], and ischemia/reperfusion injury [9].

In our human genomic study, the G-463A polymor-

Fig. 2 (A-E) Body weight (A), blood glucose (B), glycated hemoglobin levels (C), serum cystatin C levels (D) and urinary protein excretion levels (E) in each group of mice at the end of the experimental period (n=8). (F) Representative pictures of hematoxylin eosin (HE) staining in kidney sections from each group of mice. (G-J) Renal mRNA expression levels of Ngai (G), MCP-1 (H), fibronectin (I) and MPO (J) determined by real-time PCR. β actin was used as an internal control. (K-N) Correlation between mRNA expression levels of MPO and serum cystatin C (K), Ngai (L), MCP-1 (M) or fibronectin (N). Data are mean ± SEM. * $p < 0.01$. PBS: phosphate-buffered saline; BSA: bovine serum albumin, protein overload model; ND: normal diet group; HFD: high-fat diet group; Ngai: Neutrophilgelatinase-associated lipocalin; MCP-1: monocyte chemoattractant protein-1; MPO: myeloperoxidase; FFA-BSA: free fatty acid-non-depleted BSA. (O) A representative picture showing the PCR products to detect MPO expressions in the samples from the cultured podocytes, mesangian cells and proximal tubular cells stimulated with/without FFA-non-depleted albumin, and in the whole kidney samples from mice fed with ND or HFD with/without FFA-non-depleted albumin-overload.

phism, which is known to positively regulate *MPO* gene expression, was associated with the development of macroalbuminuria and the reduction of GFR, but not the appearance of microalbuminuria. In the animal study, MPO expression was significantly increased in the kidneys of HFD-induced diabetic mice with increased serum cystatin C levels and renal Ngai, MCP-1 and fibronectin expressions induced by protein load, but not in the kidneys of mice with microalbuminuria alone. Furthermore, MPO expression was significantly and positively correlated with the severity of protein load-induced proximal tubular damage, inflammatory and fibrotic responses, and renal dysfunction. Also, MPO expression was not found in the cultured renal component cells exposed to FFA-non-depleted BSA, although it was detected in the whole kidney samples from protein-overloaded mice. Taken together, these results suggest that increase of MPO-positive inflammatory cells (i.e., neutrophils) and subsequent production of reactive oxygen species (ROS) may be involved in the advanced stage, rather than the early stage, of renal damage in HFD-induced diabetic mice. These experimental data were consistent with the results of our human genomic study. However, additional research using MPO knockout mice are needed to ascertain whether the diabetes-related increases in MPO expression represent a marker for the severity of kidney damage or whether these changes play a pathological role in the development of diabetic nephropathy.

Although it is still speculative to regard MPO as a relevant pathogenetic factor for renal complications in diabetes, it is possible that MPO plays roles in the pathogenesis of diabetic nephropathy through the production of ROS and HOCl/OCl⁻ [1, 10-12], since increased production of ROS as well as HOCl/OCl⁻ have been shown to promote renal injury [10-13]. In diabetes, MPO-derived ROS can accelerate the generation of advanced glycation end products (AGEs) and subsequent interaction with receptor for AGE (RAGE), which lead to renal injury [23, 24].

There are several limitations in the current study. First, the number of the study subjects was relatively small and the statistical power was not enough in the present study. Therefore, a replication study with a larger size would be necessary to confirm the hypothesis that this polymorphism is associated with the renal damage in type 2 diabetic patients.

Second, since all the subjects were recruited through hospitals, the observed association is not completely

unrelated to population stratification and pleiotropic effects of this polymorphism. Furthermore, it might not be appropriate to apply our findings to other race or ethnic groups, since all the subjects of this study were Japanese.

Third, it is possible we are not studying the actual disease-associated polymorphism. Polymorphisms tend to be linkage disequilibrium and thus the *MPO* G-463A polymorphism may not be pathogenic but in linkage disequilibrium with another SNP that is pathogenic.

Fourth, the results of the mouse study did not allow concluding that increased MPO expression in diabetes is a causal factor in renal damage. Renal injury may be a cause of increased activity of neutrophils and macrophages, and higher MPO expression could be merely downstream effect. Additional research using MPO knockout mice are needed to make conclusions of a cause-effect relationship between MPO gene expression and renal damage. Similarly, our findings should be confirmed by experiments using other animal models for diabetes. In addition, animal models of renal injury are significantly different from kidney pathology in humans. The pattern of MPO expression and activity in human diabetic nephropathy is still unresolved. Although enhanced MPO activity has been detected in epiretinal membranes of patients with proliferative diabetic retinopathy [25], no reports exist on renal MPO expression in patients with diabetic nephropathy. The exact role of MPO in human diabetic nephropathy must be explored more extensively.

Finally, discovering a polymorphism-disease association does not directly lead to the improvement of risk prediction and clinical medicine. However, discovering genetic markers that are associated with disease risk would be an essential first step in applying genetic profiles to clinical medicine. Although approaches to predict a high-risk group using genetic profiles have not been established yet, the combined information about a number of susceptible variants including *MPO* G-463A polymorphism could lead to the identification of subjects with high risk of diabetic nephropathy.

In conclusion, it is likely that the G allele of the *MPO* G-463A polymorphism is a susceptibility allele for renal injury in Japanese type 2 diabetic patients.

Declaration of Competing Interests

Nothing to declare.

Author contributions

N.K. and S.K. conceived and designed the experiments, researched data, and wrote the manuscript. H.K. and T.U. researched data, reviewed and edited the manuscript. Y.Y. designed the research and analyzed the data. A.K., H.M., and I.S. contributed to the interpretation of the data and reviewed the manuscript. All authors reviewed and approved the report.

Acknowledgements

Co-investigators, in addition to the authors of

this study, included Dr. Ikki Shimizu, who directed Department of Diabetology, The Sakakibara Heart Institute of Okayama,; Dr. Keizo Ohno, who directed Ehime Prefectural Imabari Hospital; Dr. Takeshi Osonoi, who directed Naka Memorial Clinic; Dr. Koichi Kawai, who directed Kawai Clinic; Dr. Fukashi Ishibashi, who directed Ishibashi Clinic; Dr. Kenichi Imamura, who directed Imamura Clinic; Prof. Ryuzo Kawamori (Sportology Center, Juntendo University Graduate School of Medicine) and Prof. Munehide Matsuhisa (Diabetes Therapeutics and Research Center, Tokushima University), who contributed to the discussion.

Supplementary Table 1 Clinical characteristics of the study subjects with at least 10 years' duration of diabetes (G/G vs. G/A or A/A)

<i>G-463A</i> polymorphism of the <i>myeloperoxidase</i> (MPO) gene	Total (n = 715)	G/G (n = 598)	G/A or A/A (n = 117)	<i>p</i> value
Gender (male / female)	461 / 254	391 / 207	70 / 47	NS
Age (years)	63.3 ± 8.3	63.6 ± 8.1	61.6 ± 9.4	0.021
Smoking status (B.I.<200 / ≥200)	360 / 346	293 / 297	67 / 49	NS
Presence of obesity (%)	240 (33.6%)	199 (33.3%)	41 (35.0%)	NS
Body mass index (kg/m ²)	24.0 ± 3.4	24.0 ± 3.4	24.2 ± 3.8	NS
HbA _{1C} (%)	7.8 ± 1.1	7.8 ± 1.2	7.9 ± 1.1	NS
Duration of diabetes (years)	17.4 ± 6.9	17.3 ± 6.8	17.6 ± 7.2	NS
Presence of hypertension (%)	538 (75.2%)	450 (75.3%)	88 (75.2%)	NS
Systolic blood pressure (mmHg)	131 ± 14	132 ± 14	131 ± 14	NS
Diastolic blood pressure (mmHg)	75 ± 10	74 ± 10	75 ± 9	NS
Presence of dyslipidemia (%)	513 (71.7%)	424 (70.9%)	89 (76.1%)	NS
Total-cholesterol (mg/dL)	200 ± 36	198 ± 35	208 ± 39	0.008
HDL-cholesterol (mg/dL)	57 ± 18	57 ± 18	58 ± 18	NS
Triglyceride (mg/dL)	104 (26 – 2233)	104 (26 – 798)	103 (29 – 2233)	NS
Ln-Triglyceride (mg/dL)	4.67 ± 0.57	4.67 ± 0.56	4.68 ± 0.66	NS
Cr (mg/dL)	0.91 ± 2.64	0.95 ± 2.89	0.75 ± 0.27	NS
eGFR (mL/min/1.73m ²)	73.6 ± 21.5	72.3 ± 20.5	80.2 ± 25.1	<0.001
eGFR < 60 mL/min/1.73m ² (%)	173 (24.2%)	154 (25.8%)	19 (16.2%)	0.028
Urinary albumin excretion index (mg/gCr)	20.6 (0 – 4023)	20.3 (0 – 4023)	20.9 (0.5 – 3573)	NS
Ln-UAE (mg/gCr)	3.32 ± 1.61	3.33 ± 1.62	3.28 ± 1.57	NS
Proteinuria (normo/micro/macro)	416 / 231 / 68	350 / 186 / 62	66 / 45 / 6	–
Albuminuria >30mg/gCr (%)	299 (41.8%)	248 (41.5%)	51 (43.6%)	NS
Albuminuria >300mg/gCr (%)	68 (9.5%)	62 (10.4%)	5 (4.3%)	0.037
Antidiabetic drugs (yes / no)	541 (75.7%)	445 (74.4%)	96 (82.1%)	NS
Antihypertensive drugs (yes / no)	308 (43.1%)	259 (43.3%)	49 (41.9%)	NS
Antihyperlipidemic drugs (yes / no)	164 (22.9%)	142 (23.7%)	22 (18.8%)	NS
Serum MPO (ng/mL)	311 (12 – 2480) (n=122)	335 (12 – 2480) (n=111)	223 (47 – 743) (n=11)	NS
Ln-serum MPO (ng/mL)	5.79 ± 0.99	5.82 ± 1.00	5.45 ± 0.83	NS

Data are shown as numbers or means ± SD. All the subjects were divided into 2 groups based on the major allele's recessive genetic models (*GG* genotype vs. *GA* + *AA* genotype) and the parameters were compared among the groups. Quantitative data between the groups were compared by the 2-tailed unpaired *t* test and categorical data were analyzed with the χ^2 test. Nephropathy was graded as follows: normoalbuminuria, urinary albumin excretion less than 30mg/g Cr; microalbuminuria, 30 to 300mg/g Cr; or macroalbuminuria, more than 300mg/g Cr. *p*-values over 0.05 were displayed as NS (not significant).

B. I., Brinkman's Index; Ln-, log-transformed; UAE, Urinary albumin excretion index; MPO, myeloperoxidase

Supplementary Table 2 Clinical characteristics of the study subjects with at least 10 years' duration of diabetes (proteinuria (-) vs. proteinuria (+))

	Total (n = 715)	proteinuria (-) (n = 648)	proteinuria (+) (n = 67)	<i>p</i>
Gender (male / female)	461 / 254	417 / 231	44 / 23	NS ^a
Age (yr)	63.3 ± 8.3	63.2 ± 8.3	64.2 ± 8.0	NS
Smoking (B.I. < 200 / ≥200)	346 / 360	311 / 328	35 / 32	NS ^a
Body mass index (kg/m ²)	24.0 ± 3.4	24.0 ± 3.4	24.5 ± 3.2	NS
HbA _{1c} (%)	7.8 ± 1.1	7.8 ± 1.1	8.0 ± 1.6	NS
Duration of diabetes (yr)	17.4 ± 6.9	17.4 ± 6.9	16.9 ± 7.0	NS
Presence of hypertension (%)	538 (75.2%)	480 (74.1%)	58 (86.6%)	0.024 ^a
Systolic blood pressure (mmHg)	131 ± 14	131 ± 13	138 ± 17	0.001
Diastolic blood pressure (mmHg)	75 ± 10	74 ± 9	75 ± 11	NS
Presence of dyslipidemia (%)	513 (71.7%)	462 (71.3%)	51 (76.1%)	NS
Presence of obesity (%)	240 (33.6%)	215 (33.2%)	25 (37.3%)	NS
Total-cholesterol (mg/dL)	200 ± 36	199 ± 35	206 ± 42	NS
HDL-cholesterol (mg/dL)	57 ± 18	57 ± 18	55 ± 15	NS
Triglyceride (mg/dL)	104 (26 - 2233)	103 (26 - 2233)	124 (38 - 516)	NS
Ln-triglyceride (mg/dL)	4.67 ± 0.57	4.65 ± 0.57	4.76 ± 0.56	NS
Administration of				
Antidiabetic drugs (%)	542 (75.8%)	494 (76.2%)	48 (71.6%)	NS ^a
Antihypertensive drugs (%)	308 (43.1%)	263 (40.6%)	45 (67.2%)	<0.0001 ^a
Anti-hyperlipidemic drugs (%)	164 (22.9%)	143 (22.1%)	21 (31.3%)	NS ^a
Allele frequency (%)				
G	91.7	91.2	96.3	0.047 ^a
A	8.3	8.8	4.7	0.047 ^a
Genotype frequency (%)				NS (MDM)
GG, GA, AA	83.6 / 16.1 / 0.3	82.7 / 17.0 / 0.3	92.5 / 7.5 / 0.0	0.037 (MRM)
	598 / 115 / 2	536 / 110 / 2	62 / 5 / 0	0.038 (MAM)

Data are shown as numbers (%) or means ± SD. In the major allele's dominant and the recessive genetic models, the associations between the polymorphism and variables were evaluated with the χ^2 test (^a). In the major allele's additive genetic model, the associations between the polymorphism and variables were evaluated with the Cochran-Armitage test.

p-values over 0.05 were displayed as NS (not significant).

B. I., Brinkman's Index; Ln-, log-transformed; MDM, major allele's dominant model; MRM, major allele's recessive model; MAM, major allele's additive model.

Supplementary Table 3 Multivariate logistic regression analysis to identify independent determinants for proteinuria in type 2 diabetic subjects with at least 10 years' duration of diabetes

Parameters	Model 1		Model 2	
	Odds ratio (95%CI)	<i>p</i> value	Odds ratio (95%CI)	<i>p</i> value
Gender (male)	1.12 (0.65 – 1.93)	NS	1.12 (0.65 – 1.93)	NS
Age (years)	1.02 (0.99 – 1.06)	NS	1.02 (0.99 – 1.06)	NS
Duration of diabetes (years)	0.99 (0.95 – 1.03)	NS	0.99 (0.95 – 1.03)	NS
HbA _{1c} (%)	1.21 (0.98 – 1.49)	NS	1.21 (0.98 – 1.49)	NS
Body mass index (kg/m ²)	1.03 (0.95 – 1.11)	NS	1.03 (0.95 – 1.11)	NS
Presence of hypertension	2.18 (1.04 – 4.59)	0.039	2.18 (1.04 – 4.58)	0.040
Presence of dyslipidemia	1.21 (0.65 – 2.21)	NS	1.20 (0.66 – 2.21)	NS
-463G allele	2.63 (1.63 – 6.70)	0.043	—	—
-436GG genotype	—	—	2.64 (1.03 – 6.76)	0.044

Multivariate logistic regression analysis was done for 715 patients to select variables significantly associated with an increase in the risk of proteinuria. The threshold of statistical significance was defined as *p* < 0.05.

Model 1; gender, age, duration of diabetes, HbA_{1c}, body mass index, presence of hypertension, presence of dyslipidemia, and the number of -436G allele were included as independent variables.

Model 2; gender, age, duration of diabetes, HbA_{1c}, body mass index, presence of hypertension, presence of dyslipidemia, and the number of -436GG genotype were included as independent variables.

Abbreviations: NS, not significant

Supplementary Table 4 Clinical characteristics of the study subjects with at least 10 years' duration of diabetes (high eGFR vs. low eGFR)

	Total (n = 715)	preserved eGFR ($\geq 60\text{mL/min/1.73m}^2$) (n = 542)	low eGFR ($< 60\text{mL/min/1.73m}^2$) (n = 173)	<i>p</i>
Gender (male / female)	461 / 254	348 / 194	113 / 60	NS ^a
Age (yr)	63.3 $\pm$ 8.3	62.3 $\pm$ 8.2	66.3 $\pm$ 8.0	<0.001
Smoking (B.I. < 200 / ≥ 200)	346 / 360	261 / 276	99 / 70	0.024 ^a
Body mass index (kg/m ²)	24.0 $\pm$ 3.4	24.0 $\pm$ 3.5	24.0 $\pm$ 3.3	NS
HbA _{1c} (%)	7.8 $\pm$ 1.1	7.9 $\pm$ 1.2	7.5 $\pm$ 1.0	<0.001
Duration of diabetes (yr)	17.4 $\pm$ 6.9	17.1 $\pm$ 6.7	18.2 $\pm$ 7.3	NS
Presence of hypertension (%)	538 (75.2%)	383 (70.7%)	155 (89.6%)	<0.001 ^a
Systolic blood pressure (mmHg)	131 $\pm$ 14	130 $\pm$ 13	135 $\pm$ 15	<0.001
Diastolic blood pressure (mmHg)	75 $\pm$ 10	74 $\pm$ 9	76 $\pm$ 12	0.027
Presence of dyslipidemia (%)	513 (71.7%)	385 (71.0%)	128 (74.0%)	NS
Presence of obesity (%)	240 (33.6%)	184 (33.9%)	56 (32.4%)	NS
Total-cholesterol (mg/dL)	200 $\pm$ 36	201 $\pm$ 35	198 $\pm$ 37	NS
HDL-cholesterol (mg/dL)	57 $\pm$ 18	58 $\pm$ 18	54 $\pm$ 15	0.038
Triglyceride (mg/dL)	104 (26 - 2233)	105 (26 - 2233)	115.5 (35 - 798)	NS
Ln-triglyceride (mg/dL)	4.67 $\pm$ 0.57	4.65 $\pm$ 0.58	4.73 $\pm$ 0.56	NS
Administration of				
Antidiabetic drugs (%)	542 (75.8%)	422 (77.9%)	120 (69.4%)	0.023 ^a
Antihypertensive drugs (%)	308 (43.1%)	210 (38.7%)	98 (56.4%)	<0.001 ^a
Anti-hyperlipidemic drugs (%)	164 (22.9%)	115 (21.2%)	50 (28.9%)	0.037 ^a
Allele frequency (%)				
G	91.7	90.8	94.5	0.029 ^a
A	8.3	9.2	5.5	0.029 ^a
Genotype frequency (%)				NS (MDM)
GG, GA, AA	83.6 / 16.1 / 0.3	81.9 / 17.7 / 0.4	89.0 / 11.0 / 0	0.028 (MRM)
	598 / 115 / 2	444 / 96 / 2	154 / 19 / 0	0.024 (MAM)

Data are shown as numbers (%) or means $\pm$ SD. In the major allele's dominant and the recessive genetic models, the associations between the polymorphism and variables were evaluated with the χ^2 test^(a). In the major allele's additive genetic model, the associations between the polymorphism and variables were evaluated with the Cochran-Armitage test.

p-values over 0.05 were displayed as NS (not significant). B. I., Brinkman's Index; Ln-, log-transformed; MDM, major allele's dominant model; MRM, major allele's recessive model; MAM, major allele's additive model.

Supplementary Table 5 Multivariate logistic regression analysis to identify independent determinants for low eGFR ($< 60\text{mL/min/1.73m}^2$) in type 2 diabetic subjects with at least 10 years' duration of diabetes

Parameters	Model 1		Model 2	
	Odds ratio (95%CI)	<i>p</i> value	Odds ratio (95%CI)	<i>p</i> value
Gender (male)	1.60 (1.00 – 2.58)	NS	1.61 (1.00 – 2.58)	NS
Age (years)	1.05 (1.03 – 1.08)	<0.001	1.05 (1.03 – 1.08)	<0.001
Smoking (B.I. ≥ 200)	0.53 (0.34 – 0.84)	0.006	0.53 (0.34 – 0.84)	0.006
HbA _{1c} (%)	0.74 (0.61 – 0.90)	0.002	0.74 (0.61 – 0.90)	0.002
Body mass index (kg/m ²)	1.00 (0.95 – 1.06)	NS	1.00 (0.95 – 1.06)	NS
Presence of hypertension	3.16 (1.84 – 5.43)	<0.001	3.16 (1.84 – 5.42)	<0.001
Presence of dyslipidemia	1.17 (0.76 – 1.79)	NS	1.17 (0.77 – 1.80)	NS
-463G allele	1.82 (1.05 – 3.16)	0.034	—	—
-436GG genotype	—	—	1.81 (1.03 – 3.16)	0.038

Multivariate logistic regression analysis was done for 715 type 2 diabetic patients to select variables significantly associated with an increase in the risk of proteinuria. The threshold of statistical significance was defined as $p < 0.05$.

Model 1; gender, age, smoking status, HbA_{1c}, body mass index, presence of hypertension, presence of dyslipidemia, and the number of -436G allele were included as independent variables. Model 2; gender, age, smoking status, HbA_{1c}, body mass index, presence of hypertension, presence of dyslipidemia, and the number of -436GG genotype were included as independent variables. NS, not significant

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