*Original Article*

# Functional Polymorphism of the Myeloperoxidase Gene in Hypertensive Nephrosclerosis Dialysis Patients

Kent DOI[1),2)], Eisei NOIRI[1),3)], Rui MAEDA[1),3)], Akihide NAKAO[1)], Shuzo KOBAYASHI[4)], Katsushi TOKUNAGA[2)], and Toshiro FUJITA[1)]

**Myeloperoxidase (MPO) may play an important role not only in host defense reactions but also in local inflammations, especially in atherosclerotic diseases such as hypertensive nephrosclerosis (HN). Paradoxically, MPO-deficient mice have been reported to show increased atherosclerosis compared with wild mice, although higher MPO levels are thought to exacerbate atherosclerotic disease. To clarify the genetic role of MPO in HN, we examined the function and distribution of the −463G/A polymorphism located in the promoter region of the MPO gene with *ex vivo* flow cytometry analysis and a study in end-stage renal disease patients, respectively. This polymorphism has been reported to have a functional significance *in vitro*, with the A allele being associated with lower MPO expression. In the present study, we also found significantly higher reactive oxygen species (ROS) production with peripheral neutrophils isolated from subjects with the GG genotype compared with those from subjects with other genotypes by flow cytometry assay with 2-[6-(4′-amino) phenoxy-3H-xanthen-3-on-9-yl] benzoic acid (APF), which shows higher sensitivity with hypochlorite ($OCl^-$). Genotyping the −463G/A polymorphism in HN, chronic glomerulonephritis (CGN) and diabetic nephropathy (DM) patients who were under hemodialysis treatment demonstrated that the GG genotype was more frequent in the HN group than in the CGN and DM groups. However, the distribution of the GG genotype in the HN group was similar to that in healthy individuals. Although the −463G/A polymorphism is associated with ROS production, careful interpretation may be required to conclude that the −463G/A polymorphism can serve as a useful marker of atherosclerosis and cardiovascular events in dialysis patients. (*Hypertens Res* 2007; 30: 1193–1198)**

***Key Words*: hypertensive nephrosclerosis, dialysis, myeloperoxidase, polymorphism, flow cytometry**

## Introduction

Myeloperoxidase (MPO) is a heme protein derived from leukocytes and generates specific oxidants such as hypochlorous acid. It may play an important role not only in host defense reactions but also in local inflammations, especially in atherosclerotic lesions (*1*). Zhang *et al*. (*2*) demonstrated that elevated MPO levels were associated with the presence of coronary artery disease. Given the presence of MPO in ath-

From the [1)]Department of Nephrology and Endocrinology, [2)]Department of Human Genetics, and [3)]Center for Nanobiology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; and [4)]Department of Nephrology and Kidney and Dialysis Center, Shonan Kamakura General Hospital, Kamakura, Japan.

This study was supported by a Grant-in-Aid for Scientific Research on Priority Areas (C) "Medical Genome Science" (to K.T.), a grant from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan (14370315 to E.N.), the BioBank Japan Project on the Implementation of Personalized Medicine, MEXT, Japan (3023168 to E.N.), and a grant from the Cell Science Research Foundation (to E.N.).

Address for Reprints: Eisei Noiri, M.D, Ph.D., Department of Nephrology and Endocrinology, Graduate School of Medicine, University of Tokyo, 7–3–1 Hongo, Bunkyo-ku, Tokyo 113–8655, Japan. E-mail: noiri-tky@umin.ac.jp

Received March 13, 2007; Accepted in revised form July 11, 2007.

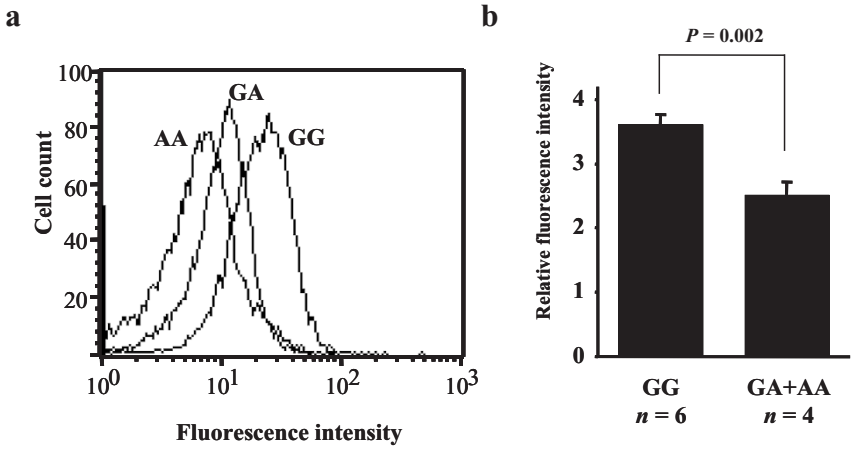


**Fig. 1.** *Association of the −463G/A polymorphism with ROS production in neutrophils. A representative histogram of the fluorescence intensity measured by APF (a) and results of the quantitative analysis of ROS production (b) are shown. ROS, reactive oxygen species; APF, 2-[6-(4′-amino) phenoxy-3H-xanthen-3-on-9-yl] benzoic acid.*

erosclerotic plaques, higher MPO levels may exacerbate atherosclerotic disease. However, the role of MPO in atherosclerosis is still unclear because MPO-deficient mice unexpectedly show increased atherosclerosis (*3*) and increased cerebral infarction volume (*4*) compared with wild mice.

The −463G/A polymorphism in the promoter region of the MPO gene has been detected in patients with leukemia (*5*). This polymorphism is in an Alu element in the promoter region, and the transition from G to A results in the loss of an SP1-binding site. SP1 is a strong transcription factor, and therefore the A allele is associated with lower MPO expression *in vitro* (*6*). In the present study, we investigated the association of the −463G/A polymorphism with *ex vivo* reactive oxygen species (ROS) production in peripheral neutrophils. The −463G/A polymorphism has also been associated with diseases such as acute leukemia (*7*), Alzheimer's disease (*8*) and lung cancer (*9*). Recently, Asselbergs *et al*. (*10*) showed that the GG genotype, which might be associated with higher MPO levels, was related to an increased risk of cardiovascular events. Pecoits-Filho *et al*. (*11*) reported that the GG genotype is associated with higher prevalence of cardiovascular complications in predialysis end-stage renal disease (ESRD) patients.

Hypertensive nephrosclerosis (HN) has been recognized as a clinical syndrome characterized by long-term essential hypertension, hypertensive retinopathy, left ventricular hypertrophy, proteinuria, and progressive renal insufficiency. Long-term severe hypertension causes atherosclerotic changes in the renal vessels and subsequent renal hypoxia, which plays an important role in the progression of kidney disease (*12*). Mesangial α-smooth muscle actin and collagen type III expression have also been reported in renal biopsy specimens of HN patients (*13*). HN is the third most frequent diagnosis in patients starting dialysis and its incidence is increasing in Japan (Annual Report of the Japanese Society of Dialysis Therapy 2005). In the present study, we hypothesized that the −463G/A polymorphism might be associated with HN and examined the association of the −463G/A polymorphism with HN and non-HN dialysis patients and healthy controls in order to clarify the effects of MPO genetic polymorphism on ESRD caused by HN.

## Methods

### Measurement of ROS Production of Peripheral Neutrophils

We examined ROS production of neutrophils by flow cytometry in 10 healthy individuals (−463GG: $n=6$; −463GA: $n=3$; −463AA: $n=1$). Peripheral polymorphemic neutrophils (PMNs) were isolated using Polymorphprep (Axis-Shield PoC AS, Oslo, Norway). After incubation with 2-[6-(4′-amino) phenoxy-3H-xanthen-3-on-9-yl] benzoic acid (APF; Dai-ichi Chem, Tokyo, Japan) at a concentration of 10 μmol/L for 30 min at room temperature, dye-loaded PMNs were stimulated with 4β-phorbol-12-myristate-13-acetate (PMA; Sigma-Aldrich Corp., St. Louis, USA) at the concentration of 2 ng/mL for 10 min. Then, PMNs were run on a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, USA) flow cytometer. Neutrophils were gated according to forward and side scatters and the fluorescence emissions at 510 to 550 nm were recorded. The data analysis was performed by BD CELLQuest Pro software (Becton Dickinson). The relative fluorescence intensity of PMNs was calculated as the ratio of the fluorescence of neutrophils activated by PMA to that of unstimulated PMNs.

**Table 1. Multiple Logistic Regression Analysis for HN Group**

| | HN ($n$=72) | CGN ($n$=224) | DM ($n$=135) | HN *vs*. CGN+DM | |
|---|---|---|---|---|---|
| | | | | Odds ratio | $p$ value |
| MPO −463 GG (%) | 86.1 | 74.6 | 71.9 | 2.53 | 0.013 |
| Age (years) | 70.4±11.3 | 60.9±12.8 | 65.3±10.8 | 1.06 | <0.0001 |
| Males (%) | 83.3 | 60.7 | 77.8 | 2.50 | 0.009 |
| Duration of dialysis (month) | 66.1±42.1 | 126.2±83.7 | 53.8±43.0 | 0.99 | 0.016 |

HN, hypertensive nephrosclerosis; CGN, chronic glomerulonephritis; DM, diabetic nephropathy; MPO, myeloperoxidase.

**Table 2. Allele Carrier Frequencies of the −463G/A Polymorphisms**

| | CGN ($n$=224) | DM ($n$=135) | HN ($n$=72) | Control ($n$=490) |
|---|---|---|---|---|
| MPO −463 GG | 167 (74.6%) | 97 (71.9%) | 62 (86.1%) | 404 (82.4%) |
| GA | 56 (25.0%) | 37 (27.4%) | 9 (12.5%) | 83 (16.9%) |
| AA | 1 (0.4%) | 1 (0.7%) | 1 (1.4%) | 3 (0.6%) |

HN, hypertensive nephrosclerosis; CGN, chronic glomerulonephritis; DM, diabetic nephropathy; MPO, myeloperoxidase.

### Study Population

This study enrolled 431 patients with ESRD who were treated with hemodialysis at five dialysis centers in the Tokyo area. Dialysis patients consisted of patients with chronic glomerulonephritis ($n$=224), diabetic nephropathy ($n$=135), and hypertensive nephrosclerosis ($n$=72). Patients with miscellaneous other conditions such as polycystic kidney disease were excluded. Diagnoses were determined at the initiation of dialysis based on clinical records. The control group consisted of 490 unrelated healthy individuals who visited the urban health center (Tokyo, Japan) for routine health check-ups. All individuals in the control group were healthy. They showed no urinary abnormality, renal dysfunction, or hyperglycemia; and they reported no use of medication. This study was approved by the Ethics Committee for Human Genome Study of the University of Tokyo and informed consent was obtained from each individual at the time of recruitment.

### Genotyping of Polymorphisms

Genomic DNAs were extracted from peripheral blood leukocytes using a FlexiGene DNA kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Genotyping of the −463G/A polymorphism (rs 2333227) was performed as described previously by using a high-throughput and accurate single nucleotide polymorphism (SNP) typing system (*14*).

### Statistical Analyses

Conformity of the genotype proportion to Hardy-Weinberg equilibrium was examined in the patient group and the control group, and the $\chi^2$ test was used to compare the frequencies of the SNP alleles in the study groups. Logistic regression analysis for the distribution of the −463G/A polymorphism was used considering confounding factors. These calculations were performed using the program StatView-J5.0 (SAS Institute Inc., Cary, USA) and a $p$ value of $<0.05$ was considered significant.

## Results

### The −463G/A Polymorphism and ROS Production in Peripheral Neutrophils

Flow cytometry analysis using APF showed significantly lower ROS production of peripheral neutrophils in the A allele carriers compared with non-carriers (GG [$n$=6], 3.62±0.14 *vs*. GA+AA [$n$=4], 2.52±0.20 relative fluorescence units, $p$=0.002) (Fig. 1). This result was in agreement with a previous *in vitro* study that demonstrated lower MPO expression in an A allele group (*6*).

### Distribution of the −463G/A Polymorphism in Dialysis Patients and Healthy Controls

The frequencies of genotypes in the ESRD patients and the healthy controls did not differ significantly from those expected under Hardy-Weinberg equilibrium. The frequency of the GG genotype of the −463G/A polymorphism was higher in the HN group compared with the chronic glomerulonephritis (CGN) and diabetic nephropathy (DM) groups. Logistic regression analysis incorporating the confounding factors of as age, gender, and duration of dialysis showed a significant difference of the GG genotype distribution between the HN group and the non-HN dialysis patient groups (CGN and DM) (Table 1). However, the frequency of the GG genotype in the HN group turned out to be virtually

the same as that in the healthy controls (Table 2). The frequency of the GG genotype in healthy controls in this study was approximately consistent with previous reports from Japan (*15–17*).

The GG genotype was less frequent in the non-HN groups than in healthy controls ($p=0.0033$). Because the average age of healthy controls was much younger than dialysis patients (33.1±4.6 [mean±SD] years in the control group), no significant difference was found after adjusting age with logistic regression analysis.

We also performed a subclass analysis in terms of gender. There was no significant difference in the genotype distribution between males and females in each group (data not shown). The GG genotype frequencies in the HN group were higher both in males and females compared with the non-HN groups (males: HN 85.0% *vs*. non-HN 72.6%, $p<0.05$; females: HN 91.7% *vs*. non-HN 75.4%, not significant), although the difference in females was not statistically significant possibly due to the small number of samples.

## Discussion

The present study aimed to elucidate the genetic role of MPO in HN dialysis patients. We demonstrated the functional significance of the −463G/A polymorphism located in the promoter region of the MPO gene and evaluated the association of this polymorphism with HN dialysis patients.

First, we demonstrated the functional significance of the −463G/A polymorphism in ROS *ex vivo* production of neutrophils by using flow cytometry with APF (Fig. 1). APF is a novel fluorescence probe which shows much more specific reactivity to an MPO product, hypochlorite ($HClO^-$), compared with other fluorescence probes such as 2′,7′-dicholorodihidrofluorescein (DCFH) (*18*). Moreover, MPO products, particularly $HClO^-$, play an important role in the subsequent ROS generation in terms of interaction with NADPH oxidase (*19*). Hydroxyl radicals ($OH^{\bullet}$), which have the highest oxidation potential among ROS, are formed by reaction between the neutrophil-derived superoxide ($O_2^{\bullet -}$) and $HClO^-$ (*20*). Interaction of MPO with vascular NADPH oxidase has also been reported (*21*). Therefore, the flow cytometry analysis with APF performed in this study was considered an adequate representation of MPO activity, and allowed the detection of not only $HClO^-$ but also other ROS.

Second, we performed genotyping on the −463G/A polymorphism with 431 dialysis patients and 490 healthy individuals. A logistic regression analysis showed a significant difference of the GG genotype distribution between the HN group and non-HN dialysis patient groups (Table 1). The HN group was characterized by long histories of hypertension and progressive renal sufficiency. The age of initiation of dialysis was older and the 5-year survival rate was worse compared with the CGN and DM groups because of the higher incidences of cardiovascular complications. Pecoits-Filho *et al*. (*11*) studied the −463G/A polymorphism in pre-dialysis ESRD patients and showed that the GG genotype is associated with higher prevalence of cardiovascular complications. From these data, the −463G/A polymorphism seemed to be a useful marker for cardiovascular events in ESRD patients. However, the distributions of the GG genotype were similar in the HN group and healthy controls in the present study (Table 2). Pecoits-Filho *et al*. did not examine the polymorphism in healthy controls and drew their conclusion by comparison between ESRD patients with and without cardiovascular disease (*11*). Therefore, it would be difficult to conclude that the −463G/A polymorphism is a useful marker of atherosclerosis and cardiovascular events in dialysis patients.

Why was the distribution of the GG genotype similar between HN patients and healthy controls? ROS are generally thought to have an exacerbating effect on various diseases, and the GG genotype that showed higher ROS production was expected to be less frequent in healthy controls than dialysis patients. Surprisingly, however, our genotyping data showed that the frequency of the GG genotype was lower in both the CGN and DM groups compared with healthy individuals, although statistical significance was not found after adjusting for age (Table 2). This result may suggest that MPO has some protective effects on kidney disease. There are several reports demonstrating the role of MPO in antiinflammatory responses, such as inactivation of chemotactic proteins (*22*) and alterations of lymphocyte functions (*23*). Hypochlorous acid donor tetrachlorodecaoxide is currently reported as a monocyte modulator (*24*) and a post-transplantation immunosuppressant (*25*). Taking these results together, MPO might possess immunomodulatory properties as an antiinflammation factor and could show a protective effect on the progression of kidney diseases, including HN, because chronic inflammation plays a central role in the common pathway to ESRD (*26*).

The unexpected results in the present study may suggest that MPO has dual effects. The GG genotype that is associated with higher MPO activity can be a risk factor for atherosclerosis and a protective factor for the progression of kidney disease simultaneously. Compared with the general population, hypertensive patients without kidney disease may show a higher frequency of the GG genotype and non-HN kidney disease patients may show a lower frequency, as suggested in this study. Therefore it may be speculated that HN patients possess factors of both atherosclerosis and kidney disease, and that the frequency of the GG genotype in these patients is not different from that in healthy individuals because of the dual effect of MPO. Further evaluation with hypertensive patients without kidney disease and HN patients should be performed to confirm this speculation.

A potential limitation of this study was the small number of patients with HN. Although HN is the third most commonly diagnosed disease requiring dialysis in Japan, HN patients still make up less than 10% of all dialysis patients in this country, and the distribution of CGN, DM, and HN in our

samples was similar to that in the annual report of the Japanese Society of Dialysis Therapy. We calculated the statistical power (1-β) for a case-control association study as described previously (*27*). Considering that the frequency of the disease-susceptible allele (G), which showed a recessive mode of inheritance because the GG genotype is a risk factor, was approximately 85% and prevalence of HN in dialysis patients (CGN, DM and HN) in Japan is 7%, we estimated the penetrances for genotypes GG, GA, and AA as 8.5%, 3.5%, and 3.5%, respectively. With these parameters, the power of 1-β with 72 cases (HN) and 459 controls (non-HN) was calculated as 0.78, although generally the power of 1-β must be greater than 0.8 to be considered sufficient. Another limitation of this study was the relatively young age of the healthy controls. The dialysis patients in this study represent advanced phenotypes of kidney disease. Iseki *et al*. (*28*) reported that only 0.2% of 86,253 Japanese individuals who participated in healthy check-ups and showed no proteinuria developed ESRD during 17 years of follow-up. Therefore, the estimated probability of false-positive association might be minimal in this particular disease group. Further evaluation with large and age-matched samples will be needed to confirm our results and clarify the genetic role of MPO in atherosclerosis and kidney diseases.

In conclusion, the −463G/A polymorphism was associated with ROS production of neutrophils *ex vivo*; however, careful interpretation may be required to determine that the −463G/A polymorphism can serve as a useful marker for atherosclerosis in dialysis patients, because this polymorphism was similarly distributed in hypertensive nephrosclerosis dialysis patients and healthy individuals.

## Acknowledgements

The authors are grateful to Drs. Yasushi Yukiyama, Hitoshi Miyake (Fujitsu Kawasaki Hospital), Tadahiro Nishi (Nishi Clinic), Takashi Ozawa (Kodaira Kitaguchi Clinic), Kyosuke Nishio, Hisakazu Degawa (Sinkoiwa Clinic Funabori), and Hiroshi Nonaka (Nonaka Clinic) for providing samples.

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