*Short communications***Polymorphisms of human paraoxonase 1 gene (*PON1*) and susceptibility to diabetic nephropathy in Type I diabetes mellitus****S. Araki, Y. Makita, L. Canani, D. Ng, J. H. Warram, A. S. Krolewski**

Section on Genetics and Epidemiology, Research Division, Joslin Diabetes Center and Department of Medicine, Harvard Medical School, Boston, Massachusetts, USA

Abstract

Aims/hypothesis. Oxidative stress is a putative mechanism in the development of diabetic nephropathy. Paraoxonase gene 1 is an HDL-bound enzyme that protects tissues against oxidative damage. Three common polymorphisms of paraoxonase gene 1, T-107C in the promoter, Leu54Met and Gln192Arg, that modify paraoxonase activity have been associated with cardiovascular disease. This study aimed to find whether these polymorphisms also contribute to the development of diabetic nephropathy.

Methods. The association between diabetic nephropathy and these three polymorphisms was examined in a case-control study. For this purpose, genomic DNA was collected from 188 patients with Type I (insulin-dependent) diabetes mellitus and diabetic nephropathy and from 179 unrelated patients with

Type I diabetes but without diabetic nephropathy despite the duration of diabetes of 15 or more years.

Results. The genotype and allele frequencies for each of the three polymorphisms (T-107C, Leu54Met and Gln192Arg) were similar in cases and control subjects.

Conclusion/interpretation. The three polymorphisms in paraoxonase gene 1 that have been associated with serum levels of paraoxonase are not associated with diabetic nephropathy. We show that this genetically determined component of the antioxidant capacity of HDL does not play a critical part in the development of diabetic nephropathy. [Diabetologia (2000) 43: 1540–1543]

Keywords Diabetic nephropathy, *PON1*, polymorphism, case-control study, Type I diabetes.

Diabetic nephropathy is the main cause of end-stage renal disease (ESRD) and premature mortality in Type I (insulin-dependent) diabetes mellitus. Whereas prolonged hyperglycaemia is an important risk factor of diabetic nephropathy, epidemiologic and family studies suggest the existence of genetic susceptibility to diabetic nephropathy [1]. Despite intensive re-

search, the genes responsible for this susceptibility are not known. Genes for proteins involved in mitigating oxidative stress have been proposed as candidates because increased oxidative stress is a putative mechanism for the development of diabetic nephropathy [2].

There is increasing evidence that paraoxonase (PON), which is an enzyme exclusively bound to high-density lipoprotein (HDL), protects tissues from oxidative damage [3]. In vitro studies indicate that PON isolated from HDL prevents low-density lipoprotein (LDL) from excess oxidation, which could partly explain the protective effect of HDL against oxidative stress [3]. Clinical studies support this hypothesis in that serum activity and concentration of PON are lower in patients with cardiovascular disease than those without.

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Corresponding author: A.S. Krolewski, MD, PhD, Section on Genetics and Epidemiology, Research Division, Joslin Diabetes Center, One Joslin Place, Boston, MA 02215, USA

Abbreviations: ACR, Albumin to creatinine ratio; ASO, allele-specific oligonucleotide; ESRD, end-stage renal disease; PCR, polymerase chain reaction; PON, paraoxonase; *PON1*, paraoxonase gene 1.

Two polymorphisms in the coding region of the paraoxonase gene 1 (*PON1*), Leu54Met and Gln192Arg, influence serum levels of PON [3–5]. Association of these polymorphisms with the risk of cardiovascular disease has been reported in two studies [4, 5] but not in others [3], suggesting that they are only genetic markers of another polymorphism that is directly responsible for the association with cardiovascular disease. Three polymorphisms in the promoter region of *PON1* have been found recently [6]. In reporter gene assays, the C variant of the T-107C polymorphism had twice the activity of the T variant. In studies of healthy control subjects, C/C homozygotes had higher PON enzyme activity than T/T homozygotes [6].

This study aimed to find whether polymorphisms in *PON1* are associated with the development of diabetic nephropathy in caucasian Type I diabetic patients. For this purpose, we examined three polymorphisms Gln192Arg, Leu54Met and T-107C in a case-control study.

Subjects and methods

Subjects. Caucasian patients with Type I diabetes attending the Joslin Clinic in the early 1990's were recruited for the study [7]. Patients were classified as control subjects ($n = 179$) if they had diabetes for 15 years or more and their urinary albumin to creatinine ratio measured in $\mu\text{g}/\text{mg}$ (ACR) was less than 17 for men or less than 25 for women in at least two out of the last three urine specimens. Patients with microalbuminuria or intermittent proteinuria were excluded. Patients were classified as study patients ($n = 188$) if they had diabetes, persistent proteinuria or ESRD and no clinical evidence of another cause for their renal disease. Persistent proteinuria was diagnosed as two out of three successive positive urinalyses by reagent strip (≥ 2 + on Multistix, Bayer, Diagnostics Division, Elkhart, Ind., USA) or by an ACR of 250 or more for men or of 355 or more for women.

The Committee on Human Subjects of the Joslin Diabetes Center approved the protocol.

Genetic markers and genotyping. Genomic DNA was extracted from peripheral lymphocytes using a Phenol and Chloroform method. To determine the genotypes for Leu54Met and Gln192Arg, fragments containing each polymorphic site were amplified by polymerase chain reaction (PCR), as reported previously [4, 5]. For the T-107C, the target fragment was amplified by PCR using forward 5'-AACTGGGACCAAAA-GCCTTG-3' and reverse 5'-GATAGACAAAGGGATCGA-TG-3' primers. To genotype these polymorphisms, we developed allele-specific oligonucleotide (ASO) hybridization protocols [7]. As ASO probes, we used 5'-GGGGTGGGGC-GGGCCAA-3' and 5'-GGGGCGGGGCGGGCCAA-3' (for T or C allele detection) to recognize the T-107C polymorphism, 5'-AGACTTGGAGATACTGC-3' (for Leu) and 5'-AGACATGGAGATACTGC-3' (for Met) to recognize the Leu54Met polymorphism, and 5'-TTACAATCCTGGGAGAT-3' (for Gln) and 5'-TTACGATCCTGGGAGAT-3' (for Arg) to recognize the Gln192Arg polymorphism.

The distributions of genotypes at these polymorphisms did not differ significantly from Hardy-Weinberg law expectations in the total study group including patients and control subjects, separately ($p > 0.25$ in all cases).

Table 1. Selected clinical characteristics of the subjects with Type I diabetes according to the status of nephropathy

Clinical characteristics at the time of examination	Control subjects	Study patients
Number (n)	179	188
Sex (male/female)	90/89	95/93
Age at diabetes diagnosis (years)	13 ± 7	12 ± 6
Age at examination (years)	36 ± 7	35 ± 6
Duration of diabetes (years)	23 ± 6	24 ± 7
Hemoglobin A _{1c} (%)	8.2 ± 1.2	$9.2 \pm 1.5^*$
Total Cholesterol (mmol/l)	5.20 ± 1.22	$6.36 \pm 2.04^*$
Triglycerides (mmol/l)	1.28 ± 0.67	$2.12 \pm 1.58^*$
ESRD (%)	–	50

Data are means $\pm$ SD

* $p < 0.01$ Control subjects vs Study patients

Analytic methods. The study groups were compared by chi-squared tests for frequencies and student's t test for continuous variables (The SAS system for Windows version 6.12, SAS Institute, Cary, N. C., USA). Multiple logistic regression analysis was also used to assess the association of three polymorphisms with diabetic nephropathy. Indicator variables were used for the male sex and risk-associated genotypes: T/T genotype for T-107C, Arg carrier (Arg/Arg + Gln/Arg) for Gln191Arg, and Met carrier (Met/Met + Leu/Met) for Leu54Met. Continuous variables were coded as follows: duration of diabetes in years, and HbA_{1c} in per cent, and serum cholesterol and triglycerides in mmol/l. Pairwise linkage disequilibrium coefficients between polymorphisms were estimated as outlined previously [8].

With 188 patients and 179 control subjects and a p value of less than 0.05 for statistical significance, this study had a 95% power to detect a difference of 7% or more in the frequency of T/T for the T-106C polymorphism (relative odds ≥ 1.4), a difference of 9% or more for the frequency of Met allele carriers at the Leu54Met polymorphism (relative odds ≥ 1.5) or a difference of 9% or more for the frequency of Arg allele carriers at the Gln191Arg polymorphism (relative odds ≥ 1.5).

Results

Selected clinical characteristics of the study groups are summarized in Table 1. Age at examination, age at diagnosis of diabetes, and duration of diabetes were similar in the two groups. Values of HbA_{1c}, serum total cholesterol and triglycerides were significantly higher in study patients than in control subjects at the examination ($p < 0.01$ for each).

Pairwise linkage disequilibrium coefficients between the polymorphisms were calculated for the entire cohort. In agreement with other studies the T-107C and Gln191Arg polymorphisms were in linkage disequilibrium with the Leu54Met polymorphism ($p < 0.0001$ for each) but not with each other ($p = 0.4$) [5, 6].

The genotype and allele frequencies of these three polymorphisms in *PON1* are summarized in Table 2. The genotype and allele frequencies of T-107C polymorphism did not differ between patients and control subjects. The distribution of genotypes of the T-107C

Table 2. Frequencies of genotypes and alleles of three *PON1* polymorphisms according to the status of nephropathy

Polymorphisms		Genotypes			Alleles		
		Control subjects (n = 179)	Study patients (n = 188)		Control subjects (n = 358)	Study patients (n = 376)	
T-107C	C/C	0.25	0.27	$p = 0.5$	C	0.49	$p = 0.4$
	C/T	0.48	0.51		T	0.51	
	T/T	0.27	0.22			0.48	
Leu54Met	Leu/Leu	0.38	0.43	$p = 0.6$	Leu	0.63	$p = 0.5$
	Leu/Met	0.50	0.45		Met	0.37	
	Met/Met	0.12	0.12			0.34	
Gln191Arg	Gln/Gln	0.48	0.45	$p = 0.5$	Gln	0.70	$p = 0.3$
	Gln/Arg	0.44	0.43		Arg	0.30	
	Arg/Arg	0.08	0.12			0.34	

polymorphism was compatible with that in a healthy Caucasian cohort [6] when tested by chi-squared ($p = 0.25$ and $p = 0.21$ in control subjects and study patients, respectively). The genotype and allele frequencies of the Gln192Arg and Leu54Met polymorphisms were similar in study patients and control subjects (Table 2). Both genotype distributions also agree with those observed in healthy Caucasians [6].

Stratified analyses according to sex, duration of diabetes, value of HbA_{1c} or severity of diabetic nephropathy (proteinuria vs ESRD) did not reveal any association between diabetic nephropathy and the three polymorphisms in any subgroup (data not shown). In multiple logistic regression models, these three polymorphisms were not associated with diabetic nephropathy after adjustment for HbA_{1c} values, total cholesterol and triglycerides (data not shown).

Discussion

Oxidative stress is hypothesized to play an important part in the development of late diabetic complications [2]. Chronic hyperglycaemia increases oxidative stress and modifies the structure and function of proteins and lipids considerably due to glycoxidation and peroxidation [2]. These modified products could contribute to the morphological and functional abnormalities seen in the kidneys or eyes of patients with diabetes. Paraoxonase, encoded by *PON1*, is an important component of the mechanisms protecting against tissue damage caused by oxidative stress. Therefore, *PON1* can be considered a candidate gene for susceptibility to diabetic nephropathy. We aimed to examine the association of diabetic nephropathy with three known polymorphisms in *PON1* that have been associated with different concentrations and activities of PON [4–6]. Unfortunately, we did not find any of these polymorphisms to be associated with diabetic nephropathy in Type I diabetes.

An association between the Gln191Arg polymorphism of *PON1* and diabetic nephropathy in Type I

diabetes has been reported [9]. The Gln191Gln genotype was associated with low PON activity and increased urinary albumin excretion (≥ 40 mg for 24 h) in a multiple logistic analysis [9]. The risk allele in that report is, however, the opposite of that for cardiovascular disease [4, 5]. At present, there is no clear explanation for the discrepancy in these results. The effect of the *PON1* on the risk of diabetic nephropathy has also been examined in Japanese patients with Type II (non-insulin-dependent) diabetes mellitus [10]. Similar to our results, that study did not find any difference in the genotype distributions for Leu54Met or Gln191Arg between patients with nephropathy and those without.

The influence of oxidative stress on the development of diabetic nephropathy could vary according to different conditions. We therefore compared the genotype and allele frequencies for the three *PON1* polymorphisms between patients and control subjects, stratifying the entire study group according to sex, duration of diabetes and HbA_{1c} values. No difference was found in any of the strata between patients and control subjects for the frequencies of genotypes or alleles of any of the three *PON1* polymorphisms. Also, the multiple logistic analysis did not show any effect of these three polymorphisms on the risks of diabetic nephropathy.

The shortcomings of our study should be acknowledged. First, although our study is large, we did not have statistical power to detect very weak associations between the examined polymorphisms and the risk of diabetic nephropathy. Second, whereas excluding *PON1* as a candidate gene for diabetic nephropathy, we did not completely exclude the hypothesis that oxidative stress is a pathway involved in the development of late diabetic complications. Of note, the human genome contains two similar genes, *PON2* and *PON3*, on chromosome 7 whose physiological functions are still not known. One can hypothesize that their products could play a part similar to PON and they should be considered as candidate genes for the susceptibility of diabetic nephropathy.

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