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Short Communication

Paraoxonase2 polymorphisms are associated with nephropathy in Type II diabetes

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Abstract

Aims/hypothesis. Paraoxonase is a member of a multigene family of three genes. *Paraoxonase2* gene polymorphisms have been associated with coronary heart disease in non-diabetic patients and with an increased fasting glycaemia in patients with Type II (non-insulin-dependent) diabetes mellitus. We tested the hypothesis of whether *paraoxonase1* and *paraoxonase2* polymorphisms were associated with diabetic nephropathy.
Methods. Our case-control study of 299 Swiss patients with Type II diabetes included 147 patients with confirmed diabetic nephropathy.
Results. In univariate analyses the two *paraoxonase2* polymorphisms were associated with diabetic nephropathy. When subjected to multivariate analyses, both *paraoxonase2* polymorphisms remained statistically associated with diabetic nephropathy independent of traditional risk factors (*paraoxonase2–148*: OR = 2.53, $p = 0.003$; *paraoxonase2–311*: OR = 2.67, $p = 0.002$). In addition, BMI interacted with *paraoxonase2* polymorphisms as a risk factor of nephropathy.
Conclusions/interpretation. The *paraoxonase2* gene polymorphisms were significantly associated with diabetic nephropathy independent of traditional risk factors in Type II diabetic patients. The susceptibility to diabetic nephropathy was intensified by the degree of obesity. Pathophysiological pathways should be investigated and could be involved in insulin resistance or lipids metabolism or both. [Diabetologia (2001) 44: 104–107]

Keywords Type 2 diabetes mellitus, nephropathy, microalbuminuria, paraoxonase, gene, polymorphism, association study, insulin resistance, dyslipidaemia, body mass index.

This study investigates the association between *PON1*, *PON2* polymorphisms and diabetic nephropathy (DN) in patients with Type II diabetes mellitus.

The prevalence of diabetic nephropathy is 30 to 40 % in patients with Type I (insulin-dependent) diabetes mellitus or with Type II (non-insulin-dependent) diabetes mellitus. Diabetic nephropathy is not fully explained by traditional risk factors; therefore, genetic factors are strongly suspected.

Paraoxonase genes comprise a multigene family in chromosome 7 [1]. Paraoxonase1 (PON1) is an enzyme bound to HDL which prevents the oxydation of LDL and HDL. Two *PON1* polymorphisms have been identified: *PON1 Arg/Gln 192* and *PON1 Met/Leu 55* [2] and these *PON1* polymorphisms have been associated with coronary heart disease (CHD) in diabetic and non-diabetic subjects.

Two *PON2* gene polymorphisms have been described recently: *PON2 Ala/Gly 148* and *PON2 Cys/Ser 311* [3]. The function of *PON2* is presently not at known. Association studies have shown that the *PON2–311* polymorphism is associated with CHD [4]. Moreover, other investigators found *PON2–148*

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Abbreviations: CVD, Cardiovascular disease; HBP, high blood pressure; PON, paraoxonase.

polymorphism to be associated with increased fasting plasma glucose in a Hutterite Indian cohort with Type II diabetes [5].

Subjects and methods

Subjects. We did a case-control study including 299 Swiss Caucasian patients with Type II diabetes, according to the World Health Organisation (WHO) criteria [9]. This cohort was recruited from specialised referral centres (University Hospital of Canton de Vaud and Geneva) and from private diabetes specialists in Switzerland.

Clinical data. Nephropathy was assessed either by a 24-h urine collection (defined as pathological if albuminuria was > 30 mg/24 h) or by a microalbumin to creatinin ratio calculated from first morning urine collections (defined as pathological if > 2.5 mg/mmol was found in men and > 3 mg/mmol in women from at least two separate samples).

Smokers were defined as subjects having smoked at least one cigarette a day, 3 years before their examination.

High blood pressure (HBP) was defined by a systolic blood pressure greater than 140 mmHg or a diastolic blood pressure greater than 85 mmHg (measured with a sphygomanometer after 10 min at rest) or by a pharmacological treatment for HBP.

Laboratory measurements. Total cholesterol, HDL-cholesterol and triglycerides were measured in serum on Hitachi automates using reagents from Boehringer, Mannheim/Roche, Basel, Switzerland. The HbA_{1c} value was determined by high performance liquid chromatography on a Diamad Analyser System (Bio-Rad, München, Germany).

Urinary creatinin was measured on a Hitachi automate using kits from Boehringer, Mannheim/Roche.

DNA analysis. The DNA was extracted from blood lymphocytes using the Nucleon BACC2 kit for blood and cell cultures (Amersham Life Science, Amersham, UK) according to the manufacturer's recommendations.

The *PON1–192*, *PON2–148* and *PON2–311* polymorphisms were analysed by PCR amplification of specific alleles [6].

PON2–148 sequence primers:
common primer: 5'-GGAAACACTTTCTTAAAGGTCAGG-3'
specific primer for the wild-type allele: 5'-TCAGATGCAACAGAGAATTTTCCG-3'
specific primer for the mutant allele: 5'-TCAGATGCAACAGAGAATTTTCCC-3'

PON2 311 sequence primers:
common primer sequence: 5'-CACATGCCATACATTTCTGGGTC-3'
specific primer for the wild-type allele: 5'-CTCGGCATCCAGAACATTCTAGG-3'
specific primer for the mutant allele: 5'-CTCGGCATCCAGAACATTCTAGC-3'

PON1 192 sequence primers:
Common primer: 5'-GTGAGCCCAGATATGTGAGCAC-3'
specific primer for the wild-type allele: 5'-CCCAAATACATCTCCCAGGACT-3'
specific primer for the mutant: 5'-CCCAAATACATCTCCCAGGACC-3'

PCR. The PCR reaction contained 100 ng DNA, 0.2 μmol/l of common primer, 0.2 mmol/l of allele specific primer, 0.1 mmol/l of each nucleotide and 0.5 U of *Taq* DNA polymerase from GibcoBRL, Cergy Pontoise, France in 1 × reaction buffer (20 mmol/l Tris (pH 8.4); 20 mmol/l KCl), 1.5 mmol/l MgCl2; 0.5 μl formamide (Fluka AG, Germany) for a total volume of 25 μl.

An initial denaturation at 95 °C was carried out for 5 min, followed by 30 cycles including: denaturation at 95 °C for 45 s, annealing at 50 °C (58 °C for *PON1–192*) for 45 s and elongation at 72 °C for 1 min, and the procedure was completed by an incubation at 72 °C for 5 min. After PCR 10 μl of the PCR reaction was loaded on a 2 % agarose gel and made visible by staining with ethidium bromide.

PON1 55 polymorphism was analysed by PCR – RFLP:
Primers sequence:
PON1–55 S: 5'-TTGAGGAATAAGCTCTAGTCCA-3'
PON1–55 AS: 5'-GAAAGACTTAAACTGCCAGTCC-3'

PCR. The PCR reaction was carried out under the same conditions as described for *PON2–148* and *311* genotyping (but without formamide). Fragments obtained by PCR were 384 bp long.

RFLP reaction. Mutation created the restriction site for Hsp92 II. If the analysed DNA was homozygote normal, we obtained a 384-bp long fragment; if homozygote mutated, we obtained 282 and 102-bp long fragments. After digestion, 15 μl of the PCR reaction was charged on a 2 % agarose gel and made visible by staining with ethidium bromide.

Statistical analysis. Because of non-parametric distributions we analysed clinical and biological variables by a non-parametric analysis (Mann-Whitney U test). We compared categorical variables between groups by using the chi-squared test. The Hardy-Weinberg equilibrium was tested by the chi-squared test. Interactions were assessed by the likelihood ratio test between the model including the interaction and the constraint model. Multivariate analyses were done by a logistic regression model adjusted for all significant variables.

We did these analyses using Stata software (College Station, Texas, USA) for a Windows 95 configuration and a $p < 0.05$ was regarded significant.

Results

In our Swiss cohort 147 patients had diabetic nephropathy and 152 did not. Allele frequency was 20.2 % for *PON2–148 G* allele and 20.6 % for *PON2–311 C* allele (Hardy-Weinberg disequilibrium test: $p > 0.05$). The two *PON2* polymorphisms were in a highly significant linkage disequilibrium ($p = 0.0001$), the *PON2–148* wild type being associated with the *PON2–311* mutant homozygote type and the *PON2–148* mutant homozygote type with the *PON2–311* wild type.

In univariate analysis, diabetic nephropathy was significantly associated with age ($p = 0.0001$), a longer duration of diabetes ($p = 0.0001$), the male sex ($p = 0.003$), higher blood pressure ($p = 0.001$), increased HbA_{1c} values ($p = 0.0001$), decreased HDL concentrations ($p = 0.002$) and increased triglyceride

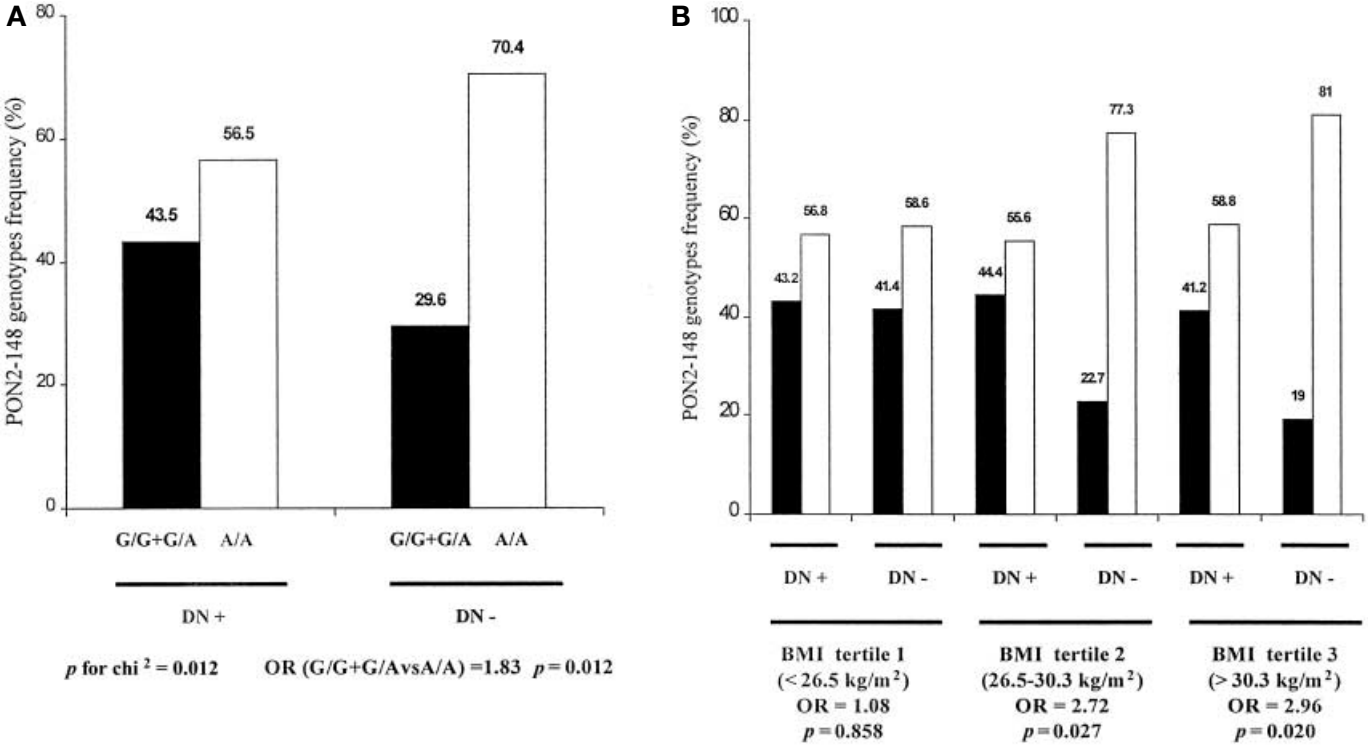

Fig. 1 A, B. PON2–148 genotype frequencies in Type II diabetic patients. **A** with and without diabetic nephropathy. **B** with and without diabetic nephropathy by BMI tertiles

Table 1 A, B. Multiple logistic regression analyses of diabetic nephropathy determinants **A** including PON2–148 polymorphisms **B** including PON2–148 polymorphisms and the interaction variable PON2–148 polymorphisms × BMI tertiles

	OR	OR 95 % CI	*p* value
A			
Age (years)	1.02	0.99–1.05	NS
Diabetes duration (years)	1.06	1.03–1.10	0.001
Sex (men, women)	2.92	1.57–5.46	0.001
HBP (yes, no)	2.31	1.22–4.36	0.010
HDL-cholesterol (mmol/l)	1.42	0.73–2.74	NS
Triglycerides tertiles (mmol/l)	1.68	1.16–2.45	0.006
HbA_{1c} (%)	1.29	1.05–1.38	0.006
BMI tertiles	1.27	0.89–1.83	NS
PON2–148 (*G/G* + *G/A* vs *A/A*)	2.53	1.37–4.64	0.003
B			
Age (years)	1.02	0.99–1.05	NS
Diabetes duration (years)	1.06	1.02–1.10	0.001
Sex (men, women)	2.77	1.48–5.20	0.002
HBP (yes, no)	2.38	1.25–4.50	0.008
HDL-cholesterol (mmol/l)	1.41	0.73–2.72	NS
Triglycerides tertiles (mmol/l)	1.69	1.16–2.48	0.006
HbA_{1c} (%)	1.22	1.07–1.39	0.004
BMI tertiles	0.96	0.61–1.50	NS
PON2–148 (*G/G* + *G/A* vs *A/A*)	0.52	0.10–2.62	NS
PON2–148 (*G/G* + *G/A* vs *A/A*) × BMI tertiles	2.21	1.04–4.73	0.040

NS: $p > 0.1$

concentrations in serum ($p = 0.0003$). We found a trend of increased BMI associated with diabetic nephropathy ($p = 0.083$).

Diabetic nephropathy was significantly associated with *PON2–148* (OR *A/G* + *G/G* vs *A/A* = 1.8, $p = 0.012$, 95 % CI: 1.14–2.95 Fig. 1) and with *PON2–311* (OR *C/C* + *C/S* vs *S/S* = 2.0, $p = 0.004$, 95 % CI: 1.24–3.22) polymorphisms but not with *PON1* polymorphisms. The power of the univariate analysis was 80 % for the *PON2–148* polymorphism and 91 % for the *PON2–311* polymorphism.

Furthermore, the association between diabetic nephropathy and the *PON2* polymorphisms was significant for the two superior BMI tertiles for *PON2–148* ($p = 0.027$ and $p = 0.02$) (Fig. 1). There was a trend towards an interaction between *PON2* polymorphisms and BMI (likelihood ratio test for models including *PON2–148* $p = 0.072$; for *PON2–311* $p = 0.087$).

We found no association between *PON1* and *PON2* gene polymorphisms and BMI, BP, lipids or HbA_{1c}.

In multivariate logistic regression analysis, *PON2–148* (Table 2) and *PON2–311* polymorphisms remained significantly associated with diabetic nephropathy independent of traditional risk factors (*PON2–148*: OR = 2.53 $p = 0.003$ 95 % CI: 1.37–4.64; *PON2–311*: OR = 2.67 $p = 0.002$ 95 % CI: 1.45–4.89).

Given the trend for the interaction between BMI, *PON2* polymorphisms and diabetic nephropathy, we introduced the interaction *PON2* polymorphisms × BMI tertiles in multivariate analysis as shown in Table 1 for *PON2–148*. For *PON2–148*, the

interaction remained significantly associated with diabetic nephropathy independent of traditional risk factors (OR for *PON2–148* BMI: 2.21 $p = 0.040$ 95 % CI: 1.04–4.73); for *PON2–311*, there was a trend towards an independent association between the interaction and diabetic nephropathy (OR for *PON2–311* BMI: 1.89 $p = 0.093$ 95 % CI: 0.90–3.98).

Discussion

Our study on Swiss Type II diabetic patients shows that *PON2* gene polymorphisms are strongly and significantly associated with diabetic nephropathy in Type II diabetes independent of traditional risk factors. In addition, the degree of obesity interacts with *PON2* polymorphisms affecting the risk of diabetic nephropathy.

The pathophysiology of *PON2* related to diabetic nephropathy is not known. Based on the *PON1* effect (*PON1* and *PON2* genes present 70 % of homology), we hypothesised that *PON2* could modulate the role of dyslipidaemia in generating or progressing diabetic nephropathy. Indeed, epidemiological and intervention studies suggest that dyslipidaemia is involved in the progression of diabetic nephropathy [7].

Because Type II diabetes and obesity are associated with insulin resistance and because, in our cohort, *PON2* polymorphisms interact with BMI contributing to the risk of diabetic nephropathy, *PON2* polymorphisms could relate to variables associated with insulin resistance as susceptible factors. This is supported by our data in that our study subjects with diabetic nephropathy tended to have a higher BMI and higher blood pressure, decreased HDL, increased glycated haemoglobin and increased triglycerides. All of these variables are components of the metabolic syndrome [9].

Furthermore, *PON2* gene polymorphisms could be associated with a more diffuse micro- and macrovascular disease, with microalbuminuria showing the presence of a widespread endothelial dysfunction [8]. Indeed, *PON2 148* polymorphism has already been associated with macrovascular disease in a previous study [4]. In our cohort, *PON2* polymorphisms were not associated with CHD (PON2–148: $p = 0.267$; PON2–311: $p = 0.678$).

The frequency of *PON2–148* G and *PON2–311* S alleles in our cohort did not differ from the frequencies published previously [4, 5]; this excludes a selection bias.

The *PON2* gene could also be in linkage disequilibrium with a functional unknown mutation in the *PON* gene cluster on chromosome 7.

There is a significant difference in the risk factors for nephropathy in Type II diabetes among different racial groups, the most frequent occurring among native Americans. It is not known whether these differences result from different frequencies of disease alleles at the same loci or whether different loci are responsible for the susceptibility in each of these racial groups.

Our study shows an association between *PON2* gene polymorphisms and diabetic nephropathy, independent of traditional risk factors. This susceptibility to diabetic nephropathy is enhanced by obesity. Pathophysiological pathways should be investigated and might involve insulin resistance or lipid metabolism or both.

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