Original Article

A Functional Polymorphism in the Manganese Superoxide Dismutase Gene and Diabetic Nephropathy

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Oxidative stress has been suggested to contribute to the development of diabetic nephropathy. Manganese superoxide dismutase (MnSOD) protects the cells from oxidative damage by scavenging free radicals. The demand for antioxidants is increased by smoking, which could disturb the balance between antioxidants and radicals. The present study aimed to determine whether a valine/alanine polymorphism in MnSOD (V16A, rs4880), alone or in combination with smoking, can contribute to development of diabetic nephropathy in 1,510 Finnish and Swedish patients with type 1 diabetes. Overt diabetic nephropathy (n = 619) was defined as having an albumin excretion rate (AER) >200 μg/min or renal replacement therapy; incipient diabetic nephropathy was defined as having an AER of 20–200 μg/min (n = 336). The control subjects had diabetes duration of ≥20 years, without albuminuria (AER <20 μg/min) and without antihypertensive treatment (n = 555). In addition to male sex and elevated A1C, smoking was significantly associated with diabetic nephropathy (overt plus incipient), odds ratio (OR) 2.00 (95% CI 1.60–2.50). When controlling for age at onset, diabetes duration, A1C, smoking, and sex, the Val/Val genotype was associated with an increase in risk of diabetic nephropathy (1.32 [1.00–1.74], P = 0.049). When evaluating the combined effect of genotype and smoking, we used logistic regression with stratification according to smoking status and genotype. The high-risk group (ever smoking plus Val/Val genotype) had 2.52 times increased risk of diabetic nephropathy (95% CI 1.73–3.69) compared with the low-risk group, but no departure from additivity was found. Our results indicate that smoking and homozygosity for the MnSOD Val allele is associated with an increased risk of diabetic nephropathy, which supports the hypothesis that oxidative stress contributes to the development of diabetic nephropathy. *Diabetes* 56:265–269, 2007

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AER, albumin excretion rate; MnSOD, manganese superoxide dismutase; ROS, reactive oxygen species.

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The increased mortality in patients with type 1 diabetes is predominantly accounted for by a poor prognosis for patients with diabetic nephropathy, the major causes of death being cardiovascular disease and renal failure (1). Diabetic nephropathy is a complex disease depending on interplay between many different mechanisms. A combination of both genetic and nongenetic risk determinants is clearly indicated from clinical and epidemiological studies (2), the most important being hyperglycemia (3). There is strong evidence that hyperglycemia causes oxidative stress that can influence multiple systems linked to diabetes complications (4–6). Both animal and in vitro studies have associated reactive oxygen species (ROS) with increased glomerular albumin permeability (7,8), which in turn can lead to mesangial growth, and glomerular sclerosis (9). The major source of intracellular ROS is the mitochondrial respiratory chain, which produces large amounts of superoxide radicals (10). Manganese superoxide dismutase (MnSOD), encoded by the *SOD2* gene, is translocated into the mitochondrial matrix where it scavenges superoxide radicals (11). Homozygous *SOD2* knockout mice die within a few days of birth (12), and even heterozygous knockout mice show altered mitochondrial function (13,14). A valine/alanine-polymorphism (rs4880 or V16A) has been identified in the targeting sequence of *SOD2* (15), and an in vitro study shows that valine (Val) instead of alanine (Ala) results in less efficient transport of MnSOD into the mitochondrial matrix (16), which can compromise the ability to neutralize superoxide radicals in the cell. This polymorphism has also been associated with diabetic nephropathy in Japanese and Korean patients with type 2 diabetes (17,18).

Smoking is associated with an increased risk of diabetic nephropathy (19–21). Cigarette smoke contains free radicals (22) and is known to increase the demand for antioxidants (23). The balance between hyperglycemia- and smoking-induced ROS production and genetically determined antioxidant systems can be important in the development of diabetes complications. The aims of this study were to investigate whether the *SOD2* rs4880 polymorphism influences the risk of diabetic nephropathy and to study whether the effect of this polymorphism is more pronounced in combination with smoking.

RESEARCH DESIGN AND METHODS

In Sweden, we invited type 1 diabetic patients from the Department of Medicine, Umeå University Hospital (Umeå, Sweden) and the Department of Endocrinology and Diabetology, Karolinska Hospital (Stockholm). In Finland,

TABLE 1
Clinical characteristics of the participants

	Control subjects			Case subjects			
	Finnish	Swedish	Finnish + Swedish	Finnish	Swedish	Finnish + Swedish	*P* value*
n	358	197	555	805	150	955	
Age (years)	41.3 ± 9.4	43.8 ± 11.4	42.2 ± 10.2	38.7 ± 9.2	48.7 ± 10.2	40.3 ± 10.0	0.001
Age at diabetes onset (years)	12.9 ± 7.5	12.9 ± 7.8	12.9 ± 7.6	11.5 ± 6.5	12.7 ± 7.7	11.7 ± 6.7	0.003
Diabetes duration (years)	27.4 (20–51)	30.0 (20–57)	28.0 (20–57)	27.3 (5–47)	35.0 (11–65)	28.0 (5–65)	0.048
Duration to diabetic nephropathy onset (years)				17 (5–39)	19 (5–59)	18 (5–59)	
A1C (% of normal limit)	134.5 ± 19.8	141.9 ± 22.1	136.8 ± 20.9	148.0 ± 25.3	146.7 ± 26.7	147.8 ± 25.5	<0.001
Systolic blood pressure (mmHg)	130.3 ± 15.2	127.0 ± 15.3	129.3 ± 15.3	144.2 ± 20.8	141.9 ± 18.6	143.9 ± 20.5	<0.001
Diastolic blood pressure (mmHg)	77.4 ± 8.5	73.1 ± 7.0	76.2 ± 8.3	84.1 ± 10.9	79.3 ± 10.6	83.4 ± 11.0	<0.001
Smoking, never/ever (%)	61.6/38.4	51.1/48.9	58.6/41.4	41.4/58.6	42.0/58.0	41.5/58.5	<0.001
Male/female (%)	39.1/60.9	44.7/55.3	41.1/58.9	58.9/41.1	50.7/49.3	57.6/42.4	<0.001

Data are given as means ± SD or median (range). **P* values for differences between Finnish + Swedish control subjects and Finnish + Swedish case subjects.

type 1 diabetic patients from the Finnish Diabetic Nephropathy Study (FinnDiane) were included. The study was approved by the ethics committees in both countries. All of the 1,510 patients had type 1 diabetes onset before 30 years of age, were insulin dependent from onset, and were of Caucasian origin. Microalbuminuria (incipient nephropathy) was defined as an albumin excretion rate (AER) of 20–200 μg/min in at least two consecutive overnight samples. This was present in 264 Finnish and 72 Swedish participants. Macroalbuminuria (overt nephropathy) was defined as AER >200 μg/min in at least two consecutive overnight samples, and this was present in 360 Finnish and 32 Swedish participants. Patients with type 1 diabetes–derived end-stage renal disease (receiving dialysis treatment or kidney transplantation) were also included as overt nephropathy case subjects (Finland, $n = 181$; Sweden, $n = 46$). Patients with at least 20-year duration of type 1 diabetes, without albuminuria (<20 μg/min) and without antihypertensive treatment, were considered as control subjects (Finland, $n = 358$; Sweden, $n = 197$). In both countries, AER is routinely measured once a year, using either nephelometric or immunoturbidometric methods of comparable sensitivity. Smoking habits (current or previous smoking) were obtained from questionnaires. Patients stating that they were current smokers (smoking at least one cigarette per day for at least 1 year) or had been smokers were combined into one ever smoking group. Patients answering that they were not current or ex-smokers were considered as never smoking. Participants that did not answer the question on smoking habits in the questionnaire, 9.5%, were excluded from all smoking-related calculations. The latest known A1C and blood pressure values were obtained from hospital records or from measurements at the inclusion visit. A1C was measured with high-pressure liquid chromatography. The method protocols and standardizations were a little different in the two countries, resulting in different reference values for healthy individuals. In Finland, the reference limits were 4.0–6.0%, and in Sweden, <5.2%. To compensate for the different normal values, the A1C measures were transformed to percentage of upper normal limit in both countries, i.e., an A1C value of 7.0 equals 116.7% of reference in the Finnish sample set and 134.6% of reference in the Swedish sample set.

DNA was isolated from peripheral blood samples preserved in EDTA. The rs4880 polymorphism was analyzed with the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) and allele-specific fluorescent probes. PCRs were performed according to the manufacturer's recommendations.

Statistical methods. Analyses were performed with the Finnish and Swedish sample sets combined because the genotype frequencies did not differ. Genotype distribution was tested for Hardy-Weinberg equilibrium. Allele and genotype distributions between case and control subjects were compared using standard χ^2 analyses. Pearson χ^2 or Mann-Whitney *U* tests were used as appropriate when comparing case and control subjects. Significance level was chosen to $P < 0.05$. In most of the calculations, we analyzed type 1 diabetic patients with diabetic nephropathy (incipient and overt diabetic nephropathy grouped together) and type 1 diabetic patients without diabetic nephropathy as control subjects. When overt and incipient diabetic nephropathy case subjects were separated, this is stated in the text. Power calculations were performed using a program developed by Purcell et al. (24) available at http://statgen.iop.kcl.ac.uk/gpc/cc2.html. We calculated the power of our sample size to replicate the association of the rs4880 polymorphism with diabetic nephropathy. The power calculations were performed for Val/Val genotype versus Ala/X genotype, with the assumptions that the prevalence of diabetic nephropathy was 63% (as observed in this case-control setting) and the allele frequency was 0.50. When assuming that the heterozygous genotype did not add to the relative risk, the power was 92% ($\alpha = 0.05$) to find a relative risk of 1.2. When assuming that heterozygous genotype gives a relative risk of 1.1, the study had 90% power ($\alpha = 0.05$) to find a relative risk of 1.2 associated with Val/Val genotype.

As an estimate of the unadjusted risk of diabetic nephropathy associated with genotype, smoking, and metabolic control, we calculated the odds ratios (ORs) and 95% CIs. A recessive model of the Val allele was used, rs4880 Val/Val versus Ala/Val plus Ala/Ala. To assess the independent contribution of the genetic polymorphism to the risk of diabetic nephropathy, we used logistic regression analysis, which included the possible confounders age at diabetes onset, diabetes duration, A1C (transformed value), sex, and smoking. To study possible combined effects between genotype and smoking, we applied a test of additive interaction using a logistic regression analysis with stratification according to smoking status (never or ever smoked) and genotype (Val/Val or Ala/Val plus Ala/Ala). Never smoking and having low-risk genotype was considered as the reference group. There is no sign of interaction if the combined effect is 0, thus there is no departure from additivity. This is calculated as follows; $1 + OR_{A+B+} - OR_{A+B-} - OR_{A-B+}$, where 1 is the effect of the reference (background) (25). All statistical analyses were performed with the statistical program SPSS 13.0 for Windows (SPSS, Chicago, IL).

RESULTS

Clinical characteristics and smoking. The clinical characteristics of participating patients are shown in Table 1. The Finnish and Swedish sample sets were similar regarding the clinical characteristics, but the Swedish patients were older than the Finnish patients (mean 46.6 compared with 39.6 years, $P < 0.001$), had longer duration of diabetes (median 31.5 compared with 27.4 years, $P < 0.001$), and had lower blood pressure (mean 134/76 compared with 140/82 mmHg, $P < 0.001$). Because, however, there were no significant differences in genotype distributions (Table 2), the two sample sets were combined in the main analyses. The crude risk of having diabetic nephropathy was significantly increased by ever smoking, OR 2.00 (95% CI 1.60–2.50), $P < 0.001$, and having A1C values above median, 2.54 (2.03–3.18), $P < 0.001$.

SOD2 polymorphism and combined effect with smoking. In logistic regression analysis controlling for age at diabetes onset, OR 0.96 (95% CI 0.95–0.98), $P < 0.001$; diabetes duration, 0.98 (0.97–1.00), $P = 0.022$; A1C, 1.02 (1.02–1.03), $P < 0.001$; male sex, 1.97 (1.55–2.51), $P < 0.001$; and smoking, 1.86 (1.46–2.36), $P < 0.001$, the Val/Val genotype significantly increased the risk of diabetic nephropathy, 1.32 (1.00–1.74), $P = 0.049$. When separately

TABLE 2
Genotype and allele frequencies of the *SOD2* rs4880 polymorphism

		Control subjects				Case subjects					
rs4880		Finnish	Swedish	*P* value*	Finnish + Swedish	Finnish	Swedish	*P* value*	Finnish + Swedish	OR (95% CI)†	*P* value‡
Genotype	Val/Val	25.4 (91)	19.9 (39)		23.5 (130)	26.5 (212)	28.7 (43)		26.8 (255)	1.15 (0.85–1.55)	
	Val/Ala	51.1 (183)	51.5 (101)		51.3 (284)	48.9 (392)	43.3 (65)		48.1 (457)	0.94 (0.73–1.22)	
	Ala/Ala	23.5 (84)	28.6 (56)	0.23	25.3 (140)	24.6 (197)	28.0 (42)	0.44	25.1 (239)	1.00 (Ref.)	0.32
Allele	Val	51.0	45.7		49.1	49.3	51.3		50.8		
	Ala	49.0	54.3	0.09	50.9	50.7	48.7	0.85	49.2		0.36

Data are % (*n*). Control subjects, no diabetic nephropathy; case subjects, overt + incipient diabetic nephropathy. *Overall *P* values for differences in genotype and allele frequencies between Finnish and Swedish patients. †ORs and 95% CIs for each genotype using Ala/Ala as reference (Ref.), Finnish + Swedish control subjects versus Finnish + Swedish case subjects. ‡*P* values for differences between Finnish + Swedish control subjects and Finnish + Swedish case subjects.

analyzing the risk of overt and incipient diabetic nephropathy in similar regression analyses, the Val/Val genotype was significantly associated with incipient diabetic nephropathy, 1.55 (1.10–2.18), $P = 0.012$. There was a tendency for association with overt diabetic nephropathy, however, this was not statistically significant, 1.23 (0.91–1.68), $P = 0.185$. In unadjusted analyses, the Val/Val genotype was not significantly associated with diabetic nephropathy.

To study possible interaction between the gene and smoking, we used a variable that stratified the participants according to smoking status and genotype in a logistic regression adjusted for age at diabetes onset, duration of diabetes, A1C, and sex. The high-risk group (ever smoking and having Val/Val genotype) had 2.58 times increased risk of diabetic nephropathy (95% CI 1.73–3.84) compared with the low-risk group (never smoking and having Val/Ala or Ala/Ala genotype) (Table 3), however, there was no clear indication of departure from additivity (0.60), and the interaction hypothesis was not supported. In the Swedish patients separately, the high-risk group had OR 2.95 (95% CI 1.26–6.91) compared with the low-risk group, and departure from additivity was 1.76, thus indicating a possible synergistic interaction in the Swedish sample set.

DISCUSSION

In the present study, including patients from two different populations, there was a weak but significant risk increase for diabetic nephropathy associated with homozygosity for the Val allele of the rs4880 polymorphism in *SOD2*, when adjustments were made for other risk factors. Smoking was also an independent associated factor, supporting the oxidative stress hypothesis (4–6). The association with the Val/Val genotype seemed stronger in patients with incipient diabetic nephropathy than in patients with overt diabetic nephropathy. Despite a very large sample, stratifying the cases into overt and incipient diabetic nephropathy will ultimately lead to greater risk of statistical instability, but we cannot exclude that what we see is an association to incipient diabetic nephropathy and that additional factors, stronger than the polymorphism, are more important for the progression from incipient to overt diabetic nephropathy. Our results are in agreement with results by Nomiyama et al. (17) and Lee et al. (18), who found lower frequency of the Ala allele in Japanese and Korean type 2 diabetic patients with diabetic nephropathy than in patients without diabetic nephropathy. The Val allele was more common in the Japanese and Korean populations than in this northern Caucasian population (85–90% compared with 50%), and they found stronger associations with diabetic nephropathy.

TABLE 3
Combined analysis of the effects of smoking and rs4880 genotype

Val/Val genotype	Ever smoked	*P* value	OR (95% CI)
−	−		1.00 (Ref.)
−	+	<0.001	1.77 (1.35–2.33)
+	−	0.334	1.21 (0.82–1.77)
+	+	<0.001	2.58 (1.73–3.84)

No diabetic nephropathy control subjects versus diabetic nephropathy case subjects in a multivariate logistic regression analysis with patients stratified according to smoking status and genotype, adjusted for age at diabetes onset, diabetes duration, A1C, and sex. Patients that never smoked, without the rs4880 Val/Val genotype, were considered as referent (Ref.) group. The combined effect was 0.60, which does not indicate departure from additivity.

We were unable to detect an association in crude analyses, but when adjusting for age at diabetes onset, diabetes duration, A1C, male sex, and smoking in the logistic regression analysis, the polymorphism was significantly associated with diabetic nephropathy. The most likely explanation for this is that the effect of the gene can be detected only when we reduce the variability of the outcome by adjusting for the other associated factors.

Cigarette smoking increases the demand for antioxidants (23), and an impaired translocation of MnSOD into the mitochondria may lead to increased oxidative stress in smokers with the Val/Val genotype. In our large case-control sample of type 1 diabetic patients, we confirmed that smoking is a risk factor for diabetic nephropathy, irrespective of genotype, and we found that the Val/Val genotype of rs4880 may be involved in diabetic nephropathy development. In the combined Finnish-Swedish group, the results show that smoking will add to the effect of the gene, but we found no statistically significant interaction between smoking and the rs4880 polymorphism. When looking separately in the Swedish population, however, the association of the Val/Val genotype and diabetic nephropathy was clearly stronger among smokers, and a tendency toward positive interaction (synergism) was found. The different results in the two groups of patients could reflect possible differences in modifying genes or lifestyle-related risk factors in the two populations but could also reflect statistical fluctuation due to the smaller size of the Swedish sample. Another possibility is that of differential misclassification of smoking in Finland and Sweden, despite similar questions and questionnaires.

Thus the possibility of an interaction between smoking and the *SOD2* gene is interesting, but the findings are preliminary and need to be confirmed.

The present study is relatively large compared with previous studies, it is statistically well powered, and it involves two well-defined groups of patients. Unfortunately, we lack measurements of ROS and antioxidant status from the patients, so we cannot analyze this as a possible link between genotype and disease. The A1C values were adjusted to be comparable between the two populations. Because our data were based on single A1C values, we did not analyze possible interactions between the gene and metabolic control. Because age at diabetes onset has been suggested as a contributing factor in the development of diabetic renal complications (26–28) and the diabetes duration in the control group was ≥20 years, these factors were also controlled for in the logistic regression analyses.

In conclusion, this case-control study indicates that smoking and homozygosity for the *SOD2* rs4880 Val allele are associated with increased risk of diabetic nephropathy and thus supports the hypothesis that oxidative stress contributes to this complex and severe long-term complication in diabetic patients. Different effects are indicated in the Swedish and Finnish patients, and further explorations of the interaction between smoking and the *SOD2* gene in different populations are needed.

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