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Short Communication

Nitric oxide synthase gene polymorphisms and diabetic nephropathy

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Abstract

Aims/hypothesis. Susceptibility to diabetic nephropathy in subjects with Type 1 diabetes is mainly genetically determined. Excess cardiovascular risk associated with diabetes is overwhelmingly concentrated in patients with nephropathy. Endothelial dysfunction is a feature of cardiovascular disease, hypertension, dyslipidaemia and smoking, all of which are associated with diabetic nephropathy. Nitric oxide regulates endothelial function and so genes encoding nitric oxide synthases could confer susceptibility to nephropathy. Recently positive associations have been reported. We examined polymorphisms within *NOS3* and *NOS2A*, the genes encoding endothelial- and inducible nitric oxide synthase, for association with nephropathy.
Methods. Large case-control studies of patients with Type 1 diabetes and overt nephropathy who had hypertension and diabetic retinopathy. The control group comprised Type 1 diabetic subjects who have been on insulin for 50 or more years and have an extremely low risk of nephropathy. Genotyping was by PCR and agarose- or automated polyacrylamide gel electrophoresis using fluorescence-labelled primers.
Results. NOS3 intron 4 genotype frequencies (n=860: 464 cases, 396 control subjects) were 2.6%, 23.3%, 74.1% and 2.3%, 22.7%, 75.0% for *aa*, *ab* and *bb* genotypes; p=0.935. *NOS2A* promoter genotype frequencies (n=715: 358 cases, 357 control subjects) were 0.3%, 16.8%, 83.0% and 0.3% 17.6% and 82.1% for +/+, +/– and –/– genotypes (p=0.952).
Conclusion/interpretation. In our cohort of Caucasian subjects with Type 1 diabetes there is no association between either of the polymorphisms studied and diabetic nephropathy. The previous suggestion from smaller studies that the intron 4 polymorphism in *NOS3* could play a role in susceptibility to the disease is not confirmed. [Diabetologia (2003) 46:426–428]

Keywords Kidney failure, chronic/*etiology, diabetic nephropathy/*genetics, diabetes mellitus, type 1/*genetics, polymorphism (Genetics), case-control studies, human, nitric oxide/*genetics, nitric-oxide synthase, endothelium, vascular/*physiopathology, coronary arteriosclerosis/*genetics.

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Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; VNTR, variable number tandem repeat; STZ, streptozotocin.

Background. Diabetic nephropathy is the leading indication for renal replacement therapy in the industrialised world. Subjects with diabetes have greatly increased cardiovascular morbidity and mortality and in Type 1 diabetes this excess risk is almost entirely confined to those with diabetic nephropathy [1].

Epidemiological evidence for genetic susceptibility to diabetic nephropathy is strong; even with very poor glycaemic control a maximum of about 35% of people with Type 1 diabetes develop diabetic nephropathy. In contrast to diabetic retinopathy, the complication al-

Table 1. Genotype/allele frequency

Genotype/allele frequency	Cases	(%)	Controls	(%)	
eNOS allele b	796	(85.8)	684	(86.4)	
eNOS allele a	132	(14.2)	108	(13.6)	$Chi^2=0.123$ $p=0.726$
eNOS bb	344	(74.1)	297	(75.0)	
eNOS ab	108	(23.3)	90	(22.7)	
eNOS aa	12	(2.6)	9	(2.3)	$Chi^2=0.135$, $p=0.935$
iNOS − allele	654	(91.3)	649	(90.9)	
iNOS + allele	62	(8.7)	65	(9.1)	$Chi^2=0.087$, $p=0.768$
iNOS −/−	297	(83.0)	293	(82.1)	
iNOS −/+	60	(16.8)	63	(17.6)	
iNOS +/+	1	(0.3)	1	(0.3)	$Chi^2=0.099$ $p=0.952$

most invariably develops within 20 years of diabetes onset or not at all. Family studies show strong concordance for nephropathy status amongst siblings with Type 1 diabetes and parents of subjects with diabetic nephropathy have reduced lifespans, higher average blood pressure, more cardiovascular disease, more dyslipidaemia and greater insulin resistance than parents of diabetic subjects without diabetic nephropathy.

Vascular dysfunction and nitric oxide synthesis. Cardiovascular disease and diabetic nephropathy [2] are associated with endothelial dysfunction, as are risk factors for diabetic nephropathy such as hypertension, smoking and dyslipidaemia. In subjects with these risk factors endothelial dysfunction precedes the establishment of atherosclerosis.

Polymorphisms of interest in endothelial and inducible nitric oxide synthase (NOS) genes. The polymorphisms we have investigated have been chosen because of plausible functional mechanisms of the genes concerned (or known specific effects of the polymorphism in the case of eNOS), and on the basis of previous smaller-scale work in this field.

The 27 bp variable number tandem repeat (VNTR) polymorphism in intron 4 of *NOS3*, the gene encoding constitutive endothelial NOS (eNOS), has been shown to affect plasma concentrations of nitric oxide in humans [3]. A possible association of this polymorphism with hypertension has been reported, but whether the polymorphism is associated with cardiovascular disease remains controversial. Six previous small to moderate-sized studies report differing results as to whether this polymorphism is associated with diabetic nephropathy.

Inducible nitric oxide synthase (iNOS) gene polymorphisms have not been studied as extensively as polymorphisms of eNOS, but functional work suggests iNOS plays an important role in disease states in a variety of cell types. It is expressed in glomerular mesangial cells of animals with streptozotocin (STZ)-induced diabetes, and iNOS-gene knockout animals show enhanced glomerular basement membrane deposition in response to STZ-induced diabetes. Association has been suggested with various diabetes-related complications, including nephropathy, in a study in Type 2 diabetes.

Subjects and methods

Both case- and control subjects were Caucasian from the United Kingdom. Cases had Type 1 diabetes with overt proteinuria (≥300 mg 24 h^{-1}), hypertension (BP>140/90) and diabetic retinopathy [4]. A cohort of Type 1 diabetic subjects who have been injecting themselves with insulin for 50 years or more were chosen as the control group. As diabetic nephropathy has such a high relative mortality, the very longevity of these subjects means that they are a particularly low-risk group for the development of diabetic nephropathy and are therefore an ideal control group for such studies; the nephropathy-free phenotype is greatly enhanced compared to unselected cohorts of subjects with Type 1 diabetes. Informed written consent was obtained from all subjects participating in the study and local and regional ethics committee approval was granted.

DNA was extracted by standard methods and genotype was determined using PCR and separation by agarose gel electrophoresis and automated gene scan analysis for the eNOS and iNOS polymorphisms respectively, as described elsewhere [4, 5, 6]. As one polymorphism was being investigated for each gene, a p value of 0.05 was defined as significant.

Results

The overall frequency of the eNOS intron 4 *a* allele was 14% (Table 1). The excess of *a* allele carriers in patients with nephropathy was not however reproduced in our study. There was no difference in genotype or allele frequencies.

The background frequency of the iNOS + allele was 9%. The excess frequency of + allele carriers was not reproduced. No difference in allele or genotype frequency was found. All results were in Hardy-Weinberg equilibrium.

Discussion

Genetic research in the field of diabetic nephropathy has two key goals. In the longer term such research can

make a major contribution to understanding the pathophysiology of the condition and aid the development of new therapeutic strategies. The more immediate goal is to improve on currently available screening strategies such as testing for microalbuminuria. If the genetic susceptibility factors are determined, people could be screened at diagnosis of diabetes so that available preventative measures (e.g. inhibition of the renin-angiotensin system and blood pressure reduction) could be most effectively and consistently targeted.

For these reasons the positive findings by several groups of an association of the *a*-allele of the intron 4 *a/b* VNTR polymorphism of eNOS with diabetic nephropathy has generated considerable interest. The epidemiological and functional evidence of endothelial dysfunction in conditions strongly associated with diabetic nephropathy suggest NOS gene polymorphisms as prime candidates for susceptibility to diabetic nephropathy. The roles that eNOS and iNOS play in regulating endothelial function in health and disease lend particular importance to establishing whether polymorphisms in these genes are involved in the development of diabetic nephropathy.

In our cohort of Caucasian subjects with Type 1 diabetes, allele frequencies of the VNTR polymorphism in intron 4 of eNOS and the tetranuclear repeat polymorphism in the promoter of iNOS were similar to those described by other groups. However, we found neither polymorphism to be associated with diabetic nephropathy. Our study has a power of 80% to detect a difference in allele frequency of just over 6.5% (eNOS) and 6% (iNOS). This makes a true association with diabetic nephropathy unlikely in this population. Moreover an association so small as not to be detectable by a study of this size would have little clinical utility. This means that polymorphisms in eNOS are not likely to be associated with diabetic nephropathy: two other polymorphisms of *NOS3* have been described to date. One of these is in linkage disequilibrium with the intron 4 *a/b* polymorphism (*-T786C*) and the other has failed to show association in previous studies (*G298T*). Association with diabetic nephropathy of other polymorphisms in the gene encoding iNOS does remain a possibility [7].

In the field of diabetic nephropathy, as in other fields of genetics, the large number of conflicting studies has lead to a confusing picture [8]. These discrepancies could represent differences between the populations studied, but are more likely caused by problems such as small sample size, multiple hypothesis testing and inadequate definition of phenotypes. Cases are often included on the basis of being positive for microalbuminuria despite its day-to-day variability and poor positive predictive value [9]. The requirement that cases should have features associated with nephropathy such as hypertension and retinopathy is also generally omitted in these subjects. Furthermore, control subjects are often taken from the background diabetic population rather than from defined low-risk groups, thereby weakening the power of studies. We chose to focus on Type 1 diabetes as a high proportion of Type 2 diabetic patients with microalbuminuria have glomerular lesions that are histologically atypical of diabetic nephropathy and decline in glomerular filtration rate correlates very poorly with the degree of albuminuria in Type 2 diabetes. The problem of conflicting results is further compounded by publication bias, with positive results receiving greater attention than negative findings. Even where editors are at pains to avoid this, researchers could themselves be less inclined to seek publication of their negative results.

In conclusion, we find no evidence of association between the intron 4 *a/b* polymorphism of *NOS3* or the promotor +/− polymorphism of *NOS2A* and diabetic nephropathy in this United Kingdom Caucasian population.

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