Brief Genetics Report

Association of the Nitric Oxide Synthase Gene Polymorphism With an Increased Risk for Progression to Diabetic Nephropathy in Type 2 Diabetes

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A mutation of endothelial nitric oxide synthase (ecNOS)—a key enzyme of the endogenous nitrovasodilator system that is essential for the regulation of blood flow and blood pressure—may aggravate the progression to diabetic nephropathy and/or retinopathy. To investigate the association of ecNOS tandem repeat polymorphism with diabetic nephropathy, the ecNOS genotype was assessed in 82 Japanese type 2 diabetic patients without nephropathy, 94 patients with microalbuminuria, 39 patients with nephropathy, and 155 healthy control subjects. The analysis revealed that type 2 diabetic patients with nephropathy (not with microalbuminuria) were significantly different from type 2 diabetic patients without nephropathy and healthy control subjects in genotype distribution (P = 0.0423) and frequency of the ecNOS4a allele (19.2% vs. 7.3 and 7.1%, respectively; P = 0.0078). The odds ratio of progression to diabetic nephropathy in diabetic patients who carry the mutated allele is about 2.87 compared with noncarriers. The stepwise multiple regression analysis in these patients showed that hypertension (F = 9.760) and ecNOS gene polymorphism (F = 5.298) are the relevant variables for nephropathy. However, no association was found between the ecNOS4a allele and hypertension or diabetic retinopathy. These results imply that the ecNOS gene polymorphism may be associated with progression to diabetic nephropathy in Japanese type 2 diabetic patients. *Diabetes* 49:500–503, 2000

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ecNOS, endothelial cellular nitric oxide synthase; NO, nitric oxide; NOx, NO metabolite; OR, odds ratio.

Nitroglycerin has been used for more than a century as a vasodilator for the treatment of coronary heart disease. Previous studies have confirmed that an endogenous nitrovasodilator system in humans maintains a physiological vasodilator tone. In response to stimuli, such as hypoxia, shear stress, and pulsatile flow, vascular endothelium cells synthesize picomoles of nitric oxide (NO) from the amino acid L-arginine by a constitutive, calcium/calmodulin dependent enzyme, endothelial cellular nitric oxide synthase (ecNOS) (1). The intraluminal release of NO mediates local vasodilatation (1,2) and antagonizes platelet aggregation (1–3) and inhibits vascular smooth muscle cell proliferation (2). The deletion of one of five nucleotide repeats (27 bp) in intron 4 (ecNOS4a allele) was suggested to be associated with coronary artery disease in Australian current and ex-smokers (4). The important role of NO release in the regulation of basal or stimulated vasodilatation suggests that an abnormal ecNOS activity due to a mutation could be implicated in different pathological conditions, such as hypertension and atherosclerosis, and may therefore aggravate retino- and/or renovascular injury in diabetic patients. Based on this rationale, we investigated the association of the ecNOS gene polymorphism with diabetic nephropathy and retinopathy in type 2 diabetic patients in a cross-sectional study. We demonstrated that this polymorphism is a risk factor for advanced diabetic nephropathy.

The ecNOS genotype was assessed in 287 (n = 136 with retinopathy; n = 151 without retinopathy) randomly selected diabetic patients from Tokyo University and its affiliated clinics and in 155 healthy control subjects. Diabetic patients who were >30 years of age and had a known diabetes duration of at least 5 years were further grouped according to the stage of progression to diabetic nephropathy as follows: type 2 diabetic patients without nephropathy (n = 82), with microalbuminuria (n = 94) (who are at risk to progress to diabetic nephropathy [5,6]), and with diabetic nephropathy (n = 39). Microalbuminuria was defined as the average of three measurements of the ratio of urinary albumin to urinary creatinine between 20 and 200 mg/g creatinine in proteinuria-negative patients. Patients with diabetic nephropathy had retinopathy and persistent dipstick-positive proteinuria (no end-stage

TABLE 1
Clinical characteristics of the study groups

	Diabetic patients					
	With nephropathy	With microalbuminuria	Without nephropathy	With retinopathy	Without retinopathy	Control subjects
n	39	94	82	136	151	155
Sex (F/M)	10/29	49/55	29/53	61/75	60/91	12/117
Age (years)	59 ± 8.6	59 ± 11.1	56 ± 8.6	59 ± 10*	55 ± 12	43 ± 8.6†
Known diabetes duration (years)	15.2 ± 4.5	13.8 ± 5.1	13.3 ± 4.5	17 ± 5 *	10 ± 6	—
Retinopathy (%)	100	38.3	18.3	100	—	—
Hypertension (%)	51.3‡	41.5	19.5	38*	24	—
Systolic blood pressure (mmHg)	139 ± 18	131 ± 12	130 ± 14	138 ± 18*	130 ± 14	119 ± 12
Diastolic blood pressure (mmHg)	80 ± 6	79 ± 6	79 ± 8	79 ± 7	79 ± 7	75 ± 8
HbA_{1c} (%)	8.0 ± 1.5	8.3 ± 1.1‡	7.8 ± 1.2	8.0 ± 1.4	7.6 ± 1.3	5.1 ± 0.3
Total cholesterol (mg/dl)	197 ± 42	214 ± 39	194 ± 32	200 ± 38	200 ± 32	203 ± 28
HDL cholesterol (mg/dl)	44 ± 14§	53 ± 11	51 ± 12	49 ± 14	48 ± 10	55 ± 12
Creatinine (μmol/l)	143 ± 69‖	78 ± 14	72 ± 11	201 ± 178¶	78 ± 14	78 ± 7
BMI (kg/m^2)	23.9 ± 2.3	23.4 ± 3.15	23.5 ± 2.85	22.9 ± 2.79	23.4 ± 2.88	22.7 ± 2.17

Data are means ± SD unless otherwise indicated. *P < 0.05 vs. diabetic patients without retionopathy; †P < 0.05 vs. diabetic patients; ‡P < 0.05 vs. diabetic patients without nephropathy; §P < 0.05 vs. diabetic patients with microalbuminuria; ‖P < 0.05 vs. diabetic patients with microalubuminuria and those without nephropathy; ¶P < 0.01 vs. diabetic patients without retionopathy.

renal disease or kidney transplantation), without any clinical or laboratory evidence of other kidney disease. The diagnosis of diabetic retinopathy was confirmed by ophthalmologists. Of the 287 diabetic patients, 72 were not included in the analyses because of lack of retinopathy (8 proteinuric patients), the association of nondiabetic kidney disease (5 patients), under hemodialysis treatment (13 patients), or insufficient clinical data (18 patients). The inclusion of the genotyping data from these excluded patients did not change the overall results. Hypertension was diagnosed according to World Health Organization criteria. The prevalence of hypertension was significantly higher in diabetic patients with nephropathy than in those without nephropathy. The subgroups of diabetic patients did not significantly differ in the lab data for age, blood pressures, total serum cholesterol, or BMI. Known duration of diabetes, serum HDL cholesterol, and creatinine level were slightly different among the three groups (Table 1).

The genotype analysis revealed that diabetic patients with nephropathy were significantly different in genotype distribution (P = 0.0423) and frequency of the ecNOS4a allele (P = 0.0078) compared with diabetic patients without nephropathy (allele frequency: 7.3%) and the control group (allele frequency: 7.1%) (Table 2). The number of carriers and frequency of the ecNOS4a allele in subjects with microalbuminuria were higher than in diabetic patients without nephropathy, but this difference did not reach the significant level (P = 0.1687 and P = 0.1204, respectively) (Table 2). About one-third of diabetic patients with nephropathy carried the ecNOS4a allele (homozygotes: 8.0%), whereas carriers of this mutation were rare in diabetic patients without nephropathy (homozygotes: 1.2%) and in the normal population (no homozygotes), accounting for only 13 and 14%, respectively (Table 2). The odds ratio (OR) of progression to diabetic nephropathy in diabetic patients who carry the mutated allele is ~2.87 (95% CI 1.13–7.28) compared with noncarriers. The stepwise multiple regression analysis in these patients showed that hypertension (F = 9.760) and ecNOS gene polymorphism (F = 5.298) are the relevant variables for nephropathy. Fifteen percent of the variability in

TABLE 2
Genotype distribution and allele frequencies in the groups with and without nephropathy

	Diabetic patients			
	With nephropathy	With microalbuminuria	Without nephropathy	Control subjects
n	39	94	82	155
Genotype				
ecNOS4b/4b	27 (69.2)	74 (78.7)	71 (86.6)	133 (85.8)
ecNOS4a/4b	9 (23.0)	17 (18.1)	10 (12.2)	22 (14.2)
ecNOS4a/4a	3 (8.0)†	3 (3.2)	1 (1.2)	0
Carrier of the ecNOS4a allele	12 (31.0)*	20 (21.3)	11 (13.4)	22 (14.2)
Allele				
ecNOS4b	63 (80.8)	165 (87.8)	152 (92.7)	288 (92.9)
ecNOS4a	15 (19.2)*	23 (12.2)	12 (7.3)	22 (7.1)

Data are *n* (%). ecNOS4b, wild-type allele; ecNOS4a, mutated allele. *P < 0.05 vs. diabetic patients without nephropathy and the control group; †P < 0.05 vs. the control group.

nephropathy in diabetic patients was explained by the variability in ecNOS genotype and hypertension (R^2 = 0.151, overall P = 0.0007). One may consider that ecNOS gene polymorphism might have contributed to nephropathy by promoting hypertension. However, no significant association was found between hypertension and the distribution of three genotypes (ecNOS4b/4b, 4a/4b, 4a/4a) (P = 0.4334) or the prevalence of genotypes carrying the ecNOS4a allele (ecNOS 4a/4b + 4a/4a) (P = 0.2000) when hypertensive diabetic patients were compared with normotensive patients. Similarly, the ecNOS4a allele showed no association with retinopathy (genotype distribution: P = 0.7060; genotypes carrying ecNOS4a allele: P = 0.2751).

Our data imply that the ecNOS4a allele may be one of the risk factors for diabetic nephropathy, associated with a 2.87 higher OR of progression to this complication than homozygotes of the wild-type allele. To reduce the influence of survival on the outcome of the study, diabetic patients with end-stage renal failure and consequently high mortality were not included. An additional risk for cardiac complications due to the ecNOS tandem repeat polymorphism could not be confirmed in Japanese type 2 diabetic patients (7). However, we cannot completely exclude an influence of survival on our study results. In addition, there remains the theoretical possibility that the mutated allele is rather protective against progression to end-stage renal disease and therefore becomes the source of the significant finding in this study. These speculative assumptions, however, need to be proven in well-designed future studies.

Although microalbuminuria is regarded as a risk factor for diabetic nephropathy (5,6), we could not find an association between the ecNOS gene polymorphism and microalbuminuria in type 2 diabetic patients. Therefore, we assume that this polymorphism could be an aggravating factor rather than an initiating factor for diabetic nephropathy. A recent preliminary observation, which also suggested an association of the ecNOS4a allele with increased risk of diabetic nephropathy in type 1 diabetic patients, also supports our results (8). These researchers found a preferential transmission of the ecNOS4a allele from heterozygous parents to men and women with end-stage renal failure and men with persistent proteinuria (8). Meanwhile, a discordant finding was reported in French type 1 diabetic patients (9), suggesting that further studies are obviously necessary to confirm the plausible role of ecNOS gene polymorphism in the progression of nephropathy. The susceptibility to diabetic retinopathy was not linked to ecNOS gene polymorphism in our study groups. There are marked differences in temporal patterns of occurrence and incidence rates of diabetic retinopathy and nephropathy, suggesting that these two diabetic complications may evolve as two distinct processes influenced in different ways by some genetic or metabolic determinants.

The present study further indicates that, if there is a causal relation between the ecNOS gene polymorphism and nephropathy, the ecNOS4a allele contributes to nephropathy by mechanisms other than the promotion of hypertension. Besides the tandem repeat polymorphism, only a few of several recently detected ecNOS mutations have been analyzed for association with vascular diseases. The only positive association found was between G298-T at exon 7 (Glu298Asp) and hypertension in French and Japanese study groups (10,11).

Evidence for a possible relation of ecNOS gene polymorphism and impaired function of ecNOS was revealed by a recent study of NO metabolite (NOx) levels in patients with different genotypes of the ecNOS gene (12). Homozygotes of the ecNOS4a allele had a nearly 20% lower mean plasma NOx level, and carriers of the ecNOS4a allele had a significantly lower NOx level than did noncarriers (12). Further studies on the molecular biological background of diabetic nephropathy provided some interesting insights on how changes in ecNOS activity in the hyperglycemic milieu may contribute to the pathogenesis of diabetic nephropathy. In response to hyperglycemia in the early stage of experimental diabetes, increased NO synthesis and/or sensitivity (13) in afferent arterioles and glomerular endothelial cells—suggested to be caused by enhanced expression of ecNOS (14)—may induce preferential afferent arteriolar dilatation, glomerular enlargement, and functionally glomerular hyperfiltration. If we accept the hypothesis of a functional impairment of ecNOS due to a mutation, a functional loss of this enzyme may be compensated by its overexpression during this stage. In advanced diabetic nephropathy, structural and functional changes like mesangium and matrix expansion (15), enhanced endothelial turnover, and endothelial dysfunction are dominant (16). Endothelial NO-mediated cGMP usually suppresses the protein kinase C activity in mesangium cells, and therefore the matrix protein synthesis. Studies on isolated glomeruli from diabetic rats have suggested that impaired NO-mediated cGMP generation in the glomeruli of diabetic individuals may amplify matrix-protein synthesis in response to hyperglycemia and other stimuli of protein kinase C (17). At this stage, a mutation of ecNOS may lead to aggravation of matrix expansion by enhanced reduction of NO-mediated cGMP generation. However, the hypothesis of a causal role of the ecNOS gene polymorphism for diabetic nephropathy deserves further evaluation.

The ecNOS gene is located on chromosome 7q35 (18). Interestingly, the gene coding for aldose reductase (19), a candidate gene for genetic susceptibility to diabetic nephropathy in type 1 diabetic patients (20), and the gene of a muscarinic acetylcholine receptor (21), which may also have influence on the vascular tone (21), have been localized on the same chromosomal region as the ecNOS gene. Therefore, it may be a candidate region for genetic susceptibility to diabetic microvascular complications. This highly speculative hypothesis needs to be clarified in future studies.

In conclusion, the present study with a relatively small number of patients suggested that the ecNOS4a allele may be associated with advanced diabetic nephropathy in Japanese type 2 diabetic patients. No association, however, was found between ecNOS4a allele and diabetic retinopathy or hypertension. These data should be viewed as a preliminary basis for future prospective trials with larger numbers of patients and different ethnic groups. The role of ecNOS gene polymorphism for the development of diabetic nephropathy, if it exists, needs to be evaluated in further studies.

RESEARCH DESIGN AND METHODS

Genotype analysis. For genotype analysis, genomic DNA was extracted from peripheral blood leukocytes of each individual (QIAmp blood kit, Funakoshi, Tokyo). With reference to a recent publication (4), the ecNOS gene fragment of interest was amplified by polymerase chain reaction. The presence of the ecNOS4a allele (deletion of one of five 27-bp tandem repeats) was identified by agarose gel (1.5% NuSieve 1:3 Agarose; FMC Bioproducts, Rockland, ME) electrophoresis and ethid-

ium bromide (0.016% vol/vol) fluorescence in reference to a molecular weight marker (Φ174 RF DNA-HAE III digest; Pharmacia LKB, Tokyo).

Statistical analysis. The statistical difference in genotype distribution and allele frequencies among the groups was assessed by the χ^2 test and Fisher's exact test, respectively. A two-sided P value <0.05 was regarded as significantly different. Where appropriate, the OR was calculated. The Kruskal-Wallis test was applied to compare the values among the three groups of diabetic patients with different stages of nephropathy. The Wilcoxon rank-sum test was used to compare the values between the two groups of diabetic patients with and without retinopathy. A backward stepwise multiple regression analysis was performed to assess the influence of independent variables (i.e., sex, age, diabetes duration, HbA_{1c}, serum total cholesterol, HDL cholesterol, BMI, ecNOS genotype, and presence of hypertension) on diabetic nephropathy. The F value was set at 4.0 at each step, and the variable(s) was deleted at each step of calculation if the relevance was not significant (i.e., $F < 4.0$). Retinopathy was not included as an independent variable for analysis because the diagnosis of nephropathy was based on the presence of retinopathy.

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