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Original Article

Nitric oxide synthase gene polymorphisms in children with minimal change nephrotic syndrome

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Abstract ***Aims***: Nitric oxide (NO) attenuates many functions within the kidney, and all NO synthase (NOS) isoforms are constitutively expressed in the kidney. But the exact role of NO in renal diseases is still debatable. The aim of the present study was to investigate *endothelial* (*eNOS*), and *neuronal* (*nNOS*) *NOS* gene polymorphisms in children with minimal change nephrotic syndrome (MCNS).

Materials and methods: Eighty-six Turkish children with clinical MCNS, ranging in age from 2 to 10 years, were compared with 114 healthy age- and sex-matched controls. The glu 298 Asp (G/T) polymorphism of the eNOS, and C276T (C/T) polymorphism of *nNOS* genes were genotyped using polymerase chain reaction.

Results: The distribution of GG, TG, and TT genotypes for eNOS was 52%, 33% and 15% in MCNS compared with 61%, 26% and 13% in the controls ($P > 0.05$). The distribution of CC, TC, and TT genotypes for nNOS was 16%, 66% and 18% in MCNS compared with 10%, 43% and 47% in the controls. TT genotype distribution of nNOS was found to be lower in patients ($P = 0.003$). The *eNOS* and *nNOS* gene polymorphisms were not associated with gender, positive family history, frequency of relapses, or response to steroid.

Conclusions: The present study is the first to investigate eNOS and nNOS gene polymorphisms in children with MCNS. The *nNOS* gene polymorphism may be associated with MCNS in children, but further studies in a larger population with different glomerular diseases are needed to confirm the results.

Key words endothelial nitric oxide synthase, minimal change nephrotic syndrome, neuronal nitric oxide synthase, nitric oxide, nitric oxide synthase.

The mediators of glomerular hyperpermeability are not well understood in minimal change nephrotic syndrome (MCNS).[1] Some investigators have studied the role of new molecules, such as nitric oxide (NO), in glomerular diseases.[1,2]

Nitric oxide is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS). It is a highly reactive, free radical gas that serves many functions within the kidney, including regulation of afferent arteriolar tone and proliferation of mesangial cells.[3,4] But the exact role of NO in renal diseases is still debatable. It mediates immunologic injury to kidney mesangium in experimental glomerulonephritis.[5] In contrast, administration of L-arginine decreased infiltration of the kidney by macrophages in acute puromycin aminonucleoside nephropathy, an analog of MCNS.[6]

Three NOS isoforms have been identified: endothelial (eNOS or NOS III); inducible (iNOS or NOS II); and neuronal (nNOS or NOS I). All are constitutively expressed in the kidney.[7] Endothelial NOS is abundantly expressed in the endothelial lining of the renal arterial tree and capillaries, as well as in collecting duct epithelial cells, while nNOS is heavily expressed in the macula densa and to a lesser extent in the efferent arterioles and epithelial cells of the thick ascending limb, mesangial cells and intrarenal neurons. Inducible NOS is expressed in the epithelial cells of the thick ascending limb, proximal tubule and collecting duct, as well as in vascular smooth muscle, mesangial and medullary interstitial cells.[7]

Intrarenal NO serves as a major regulator of renal sodium and water excretion, like an endogenous diuretic.[8] NO derived from nNOS in the macula densa attenuates tubuloglomerular feedback-mediated afferent arteriolar vasoconstriction in response to luminal Na+ content, and facilitates Na+ excretion and glomerular filtration.[7,9,10] This isoform of NOS has attracted the attention of nephrologists because of its renal expression and the possible role(s) in the control of glomerular hemodynamics. We determined the NOS gene polymorphisms in children with primary nocturnal enuresis (PNE), and found that *nNOS* gene polymorphism, predominantly the CC genotype, may be associated with PNE in Turkish children.[11] Yamamoto *et al.* had found significantly increased expression of nNOS gene in the paraventricular (PVN) and supraoptic nuclei (SON) in homozygous (di/di) rats in comparison to normal Wistar and heterozygous rats, and they suggested that the expression of *nNOS* gene was upregulated in the magnocellular neurons in the PVN and SON of inherited diabetes insipidus rats.[12]

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Recent studies have also reported that increased NO production may contribute to hyperfiltration and microalbuminuria that characterizes early diabetic nephropathy.[13] In an experimental study it was demonstrated that intrarenal production of nNOS increased in the rats with chronic diabetes, and this overproduction of nNOS in the kidneys suppresses the tubulo-glomerular feedback system, and this suppression might be a critical mechanism for the development of diabetic renal hyperfiltration.[14]

Considering the pivotal role of NO in regulation of renal and systemic hemodynamics and renal sodium handling, which are markedly deranged in nephrotic syndrome, the aim of the present study was to investigate whether any of the known polymorphisms in eNOS and nNOS were associated with any clinical or laboratory findings of MCNS in children.

Methods

Patients

Eighty-six Turkish children with clinical MCNS, ranging in age from 2 to 10 years, were investigated in comparison with 114 healthy age- and sex-matched controls. The mean age was 5.8 ± 2.9 years.

Nephrotic syndrome was defined as heavy proteinuria >40 mg/m^2/h, or 4+ proteinuria measured on dipsticks, hypoalbuminemia <2.5 g/dL, hypercholesterolemia >250 mg/dL, and edema. MCNS was diagnosed from the clinical findings. Patients who had onset of nephrosis between 2 and 5 years of age, with normal renal function, absence of hypertension, absence of hematuria in urine microscopy, and complete response to corticosteroids were presumed to have minimal change disease on clinical grounds. Control subjects who were free of kidney and/or systemic disease were enrolled from the general pediatric clinic. All had complete physical examination, tested negative for urinary protein on dipstick, had normal blood urea nitrogen, creatinine level, and were normotensive at the time of study. The study was approved by the local ethical committee.

DNA isolation and genotyping for eNOS and nNOS

Each child provided a blood sample for biochemical analysis and DNA extraction in the remission period, after the termination of steroid therapy. Blood samples with disodium ethylenediamine tetra-acetic acid were used for the analysis of genetic polymorphism, Genomic DNA was extracted from whole peripheral blood samples using the salting-out method and stored at –20°C.[15]

Analysis of the polymorphism of the eNOS gene was performed as described previously.[16,17] The Glu298Asp polymorphism in the *eNOS* gene was analyzed on polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP). Oligonucleotide primers for PCR were designed as follows: sense, 5′-AAGGCAGGAGACAGTGGATGGA-3′; antisense, 5′-CCCAGTCAATCCCTTTGGTGCTCA-3′. PCR reaction was performed in a total volume of 50 μL containing 100 ng genomic DNA, 2.5 μL thermophilic DNA polymerase buffer, 1.5 mM $MgCl_2$, 250 μM dNTPs, 0.4 μM primer, and 2.5 U Taq polymerase (MBI Fermentas Epo402). PCR conditions were as follows: after denaturation at 94°C for 5 min, 40 cycles were performed (94°C for 1 min, 61°C annealing temperature for 1 min, 72°C for 1 min), followed by extension at 72°C for 5 min to amplify the target DNA. The amplified product was digested with 5 U restriction enzyme BanII.[16,17] The 248 bp PCR product is cleaved into 163 bp and 85 bp fragments in the presence of the G allele at position 894, but not in the presence of the T allele. In the case of the heterozygous mutant, digestion with BanII resulted in three fragments 248 bp, 163 bp, and 85 bp in size. DNA fragments were separated by electrophoresis on 3% agarose gel and visualized with ethidium bromide (Fig. 1).

Genotyping of nNOS 3′-UTRC276T polymorphism was performed according to recent reports.[18] The region of interest, as detailed here, was amplified on PCR in a total volume of 20 μL solution containing 100 ng genomic DNA, 100 mM Tris-HCL, 500 mM KCL, 15 mM $MgCl_2$, 200 μM dNTPs, 20 μM of each primer, and 1 U Taq DNA polymerase for 40 cycles (94°C for 1 min, 50.5°C for 1 min, and 72°C for 1 min 30 s). The primers used were 5′ ACTCCTTGAGTTTTCCTGCTGCGA 3′ and 5′ CCATGTTCCAGTGGTTTCATGCACAC 3′. The PCR products (128 bp) were digested with Eco*72*I at 37°C and then electrophoresed on 3% agarose gels stained with ethidium bromide. The C allele was determined by the presence of 100 bp and 28 bp fragments, while the T allele remained uncut and presented as a 128 bp band (Fig. 2).

Determination of other clinical parameters

Blood and 24 h urine samples were collected from all patients to determine clinical parameters. Serum creatinine and blood urea nitrogen as well as urinary creatinine and protein were determined by routine methods. Excretion of urinary protein and creatinine clearance were calculated from urine collected during the 24 h period.

Statistical analysis

Results are provided as percentages. Differences between patients and controls were compared using χ^2 test. $P < 0.05$ was considered to be statistically significant. Statistical analysis was performed using SPSS version 10.0 (SPSS, Chicago, IL, USA).

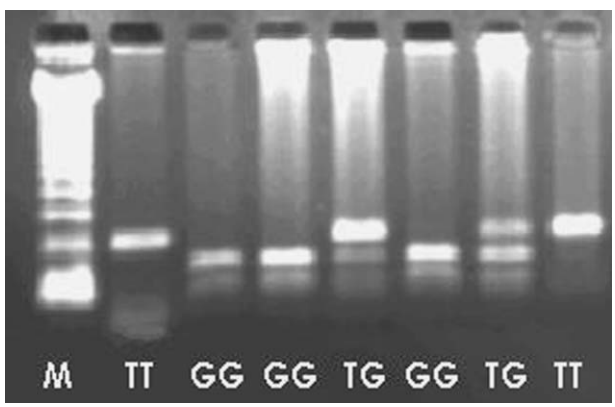

Fig. 1 *Endothelial nitric oxide* synthase Glu298Asp (G/T) polymorphism. (M, 100 bp DNA ladder; T, 248 bp; G, 163 bp, 85 bp).

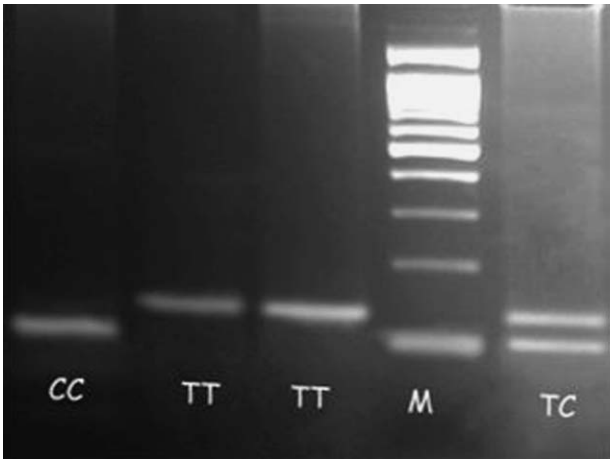

Fig. 2 *Neuronal nitric oxide synthase* 3′-UTRC276T (C/T) polymorphism. (M, 100 bp DNA ladder; T, 128 bp; C, 100 bp, 28 bp).

Results

The serum creatinine concentration and blood pressure were within the normal range, and renal function was normal (glomerular filtration rate >90 mL/min/1.73 m^2) in all subjects. The *eNOS* gene polymorphism was determined in 200 Turkish children (MCNS, $n = 86$; healthy controls, $n = 114$), and *nNOS* gene polymorphism was determined in 161 Turkish children (MCNS, $n = 86$; controls, $n = 75$; we could not obtain nNOS genotypes in other 39 controls). Mean age was 5.8 ± 2.9 years.

The distribution of GG, TG, and TT genotypes of eNOS was 52%, 33% and 15% in MCNS patients compared with 61%, 26%, and 13% in the controls. No statistically significant differences were found between groups in allele frequency and genotype distribution (Table 1).

The distribution of CC, TC, and TT genotypes of nNOS was 16%, 66%, and 18% in MCNS patients compared with 10%, 43% and 47% in the controls (Table 2). TT genotype distribution was lower in patients ($P = 0.003$). The *eNOS* and *nNOS* gene polymorphisms were not associated with gender, positive family history, frequency of relapses, or early or late responses to steroid.

Discussion

The exact role of NO in renal diseases is still debatable. Urinary nitrite excretion, a stable product of NO, was found to be higher in urinary specimens of children with MCNS.[1,2] But the role of increased urinary nitrite excretion in mediating the manifestations of clinical disease in those children is unknown. Trachtman *et al.* showed that increased urinary nitrite excretion in MCNS cannot be attributed to the nephrotic syndrome itself because they did not find any difference between control subjects and patients with focal segmental glomerular sclerosis in their study.[1] As in that study,[1] the persistently elevated nitrite excretion in relapse and remission in our previous study[2] also suggests that renal NO synthesis probably does not modulate proteinuria, but somehow involved in MCNS.

Table 1 *eNOS* gene polymorphism in Turkish children

	GG (%)	TG (%)	TT (%)
MCNS ($n = 86$)	52	33	15
Controls ($n = 114$)	61	26	13
P	>0.05	>0.05	>0.05

eNOS, endothelial nitric oxide synthase; MCNS, minimal change nephrotic syndrome.

Table 2 *nNOS* gene polymorphism in Turkish children

	CC (%)	TC (%)	TT (%)
MCNS ($n = 86$)	16	66	18
Controls ($n = 75$)	10	43	47
P	>0.05	>0.05	0.003

MCNS, minimal change nephrotic syndrome; nNOS, neuronal nitric oxide synthase.

Considering the important role of NO in kidney, we chose to examine whether any of the known polymorphisms in *eNOS* and *nNOS* were associated with any clinical or laboratory findings of MCNS.

It has been indicated that both nNOS and eNOS produce NO in the kidney, and eNOS is the predominant isoform involved in the regulation of renal blood flow under basal conditions, and nNOS produces a significant amount of NO normally found in the renal interstitial space.[19] Recently it has been shown that mRNA for eNOS is heterogeneously expressed in renal structures, preferentially in the renal interlobular artery/afferent arterioles and glomerulus, where it can modulate renal blood flow and glomerular filtration rate.[20] It has been shown that eNOS abundance in the kidney, aorta and heart was unaffected by either nephrotic syndrome or inherited hypoalbuminemia despite moderate hypertension that should have upregulated eNOS abundance in the nephrotic animals by raising shear stress and cyclic strain.[21]

Recently, Komers *et al.* reported that selective local inhibition of nNOS normalizes renal hemodynamics in hyperfiltering diabetic rats without increasing systemic blood pressure,[22] and stressed the nephroprotective effects of nNOS inhibition during the early stages of nephropathy.[23] There are no data, however, on the role of nNOS in nephrotic syndrome.

In the present study we failed to detect associations between these polymorphisms and clinical laboratory findings of MCNS. The only positive finding was that TT genotype of *nNOS* was lower in MCNS. But three limitations of the present study should be discussed. First, several studies have described *eNOS genotype* distributions that are different in healthy individuals with various ethnic backgrounds.[24,25] Second, the total sample size involved in statistical testing is also important for detecting genotype-disease associations,[26] and third, we could not measure the plasma/urinary total nitrite levels and investigate the relationship between the levels of those and the genotypes of these NOS isoforms. Therefore, we cannot determine whether TT genotype of nNOS is related to plasma and/or urinary NO levels from the present data.

Despite these limitations, the present study is the first to investigate *eNOS* and *nNOS* gene polymorphisms in children with MCNS. Because the present patients had MCNS, a most

benign form of childhood NS, the lack of difference in *eNOS* gene polymorphism may be an expected result, or the present findings may be a false negative due to small sample size.

Considering the lower TT genotype distribution of nNOS gene in the present patients, we conclude that *nNOS* gene polymorphism may be associated with MCNS in children. It would be very interesting to investigate other forms of glomerular diseases but the small sample size precluded this in the present study. Further studies exploring the relationship between other variants of the *NOS* gene in a larger population with different glomerular diseases are therefore needed.

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