Influence of Endothelial Nitric Oxide Synthase Gene Polymorphisms (-786T/C, 4a4b, 894G/T) on Iranian Kidney Transplant Recipients

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Abstract

Objectives: **Nitric oxide is a major mediator in vascular biology and regulator of regional blood flow. Its production is catalyzed by the enzyme endothelial nitric oxide synthase. Protective actions of nitric oxide in ischemia and reperfusion are due to its potential as an antioxidant and anti-inflammatory agent, along with its inhibitory effects on cell signaling pathways of nuclear proteins, such as NF-κB. The endothelial nitric oxide synthase gene polymorphisms affect endothelial nitric oxide synthase activity and are associated with endothelial dysfunction. This study sought to examine the association between single nucleotide polymorphisms in endothelial nitric oxide synthase gene (rs 2070744, 27VNTR, and rs1799983) and the development of acute rejection in renal transplant patients.**

Materials and Methods: **Sixty-six renal transplant recipients (33 patients with an episode of acute rejection and 33 recipients an episode of acute rejection), between June 2010 and March 2011, were included. The polymorphism was determined by simple polymerase chain reaction and polymerase chain reaction-restriction fragment-length polymorphism analysis.**

Results: **There was only a significant association of endothelial nitric oxide synthase -786T allele and acute rejection (*P* = .03). Recessive model of T-786C alleles (TT vs TC+CC) and acute rejection confirmed a significant association (odds ratio: 3.12; 95% CI: 0.01-9.83; *P* = .025). Haplotype CbG was higher in recipients without rejection as compared to rejection group (OR: 0.42, 95% CI: 0.16-1.13; *P* < .05). Respecting the endothelial nitric oxide synthase gene 894G/T single nucleotide polymorphisms and 27VNTR, no significant association between the allele/genotype and acute rejection was seen.**

Conclusion: **Recipient endothelial nitric oxide synthase gene polymorphisms do not alter the risk of acute rejection after a renal transplant. Rejection is a complex immunologic event. Therefore, finding associated genetic variants demands a multicentric larger sample size.**

Key words: *eNOS, Genetic, Haplotype, Renal, Allograft*

Introduction

Nitric oxide (NO) is synthesized from L-arginine, catalyzed by NO- synthase (NOS), and contributes to vessel homeostasis by inhibiting vascular smooth muscle contraction and growth, platelet aggregation, and leukocyte adhesion to the endothelium. There are 3 different isoforms of NOS; endothelial constitutive NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). Endothelial nitric oxide synthase diffuses from the endothelium to the vascular smooth muscle cells, increases the concentration of cGMP, and leads to vascular relaxation.[1] It has an important role in regulating vascular tone,[1,2] with protective effects by scavenging superoxide radicals. The nNOS produces NO in the central and peripheral nervous system and performs a role in cell communication.

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The iNOS (which is activated by interferon-gamma [IFN-γ]) is generated by monocytes, macrophages, and neutrophils, and affects the human immune system. In atherosclerosis, diabetes, or hypertension, the eNOS pathways are usually impaired. Ischemia and reperfusion (I/R) injury is a complex inflammatory phenomenon encountered during organ transplant; it induces delayed graft function, primary nonfunctioning graft, and organ loss. It also may cause induction of major histocompatability complex markers with an increase risk of acute and chronic rejection.[3]

Nitric oxide has protective effects in I/R injury by scavenging hydroxyl radicals and preventing accumulation of free radicals. Vascular smooth muscle relaxations with anti-apoptosis effect are other protective roles. It protects against I/R injury owing to its potential as an antioxidant.[3,4] Inhaled NO after an orthotopic liver transplant significantly lowered hepatocyte apoptosis.[5]

The gene encoding eNOS maps to chromosome 7q35-7q366. Two eNOS polymorphisms, (-786)T/C and the 894G/T (Glu298Asp), have been shown to be associated with altered NOS activity in experimental studies.[6,7]

The 894T and -786C alleles of the NOS3 gene are significantly associated with both hypertension and cardiovascular disease in renal allograft recipients.[8]

Yilmaz and associates analyzed the G894T mutation at exon 7 of the eNOS gene among children with chronic allograft nephropathy. They concluded that this polymorphism did not affect long-term renal allograft outcome.[9]

This study sought to examine the prevalence of the eNOS gene (T-786C, 894 G/T) and a repeat polymorphism (27VNTR) in a group of renal transplant recipients and determine whether these polymorphisms are linked to acute rejection (AR).

Materials and Methods

Sixty-six renal transplant recipients (33 patents with an episode of AR and 33 recipients without an episode of AR), between June 2010 and March 2011, were enrolled. The Ethics Committee of Shiraz University of Medical Sciences approved the protocol, and a written informed consent was obtained from all subjects in accordance with the Helsinki Declaration of 1975. Patients were followed-up for at least 2 months, and episodes of AR were recorded during this time. An AR episode was defined based on clinical or biopsy findings according to Banff criteria.[10,11] Clinical rejection was identified as an increase in the serum creatinine level that was ≥ 10% from the baseline value in the absence of infection, obstruction, or evidence of drug toxicity. The clinical characteristics were retrieved from our kidney transplant database. The routine immuno-suppression regimen consisted of cyclosporine or tacrolimus, with mycophenolate mofetil and prednisolone.

Single nucleotide polymorphisms selection

Single nucleotide polymorphisms in the eNOS gene selected for the present study, rs 2070744, 27VNTR, and rs1799983 located at the promoter, intron 4 and exon 7 of the eNOS gene (NOS3). The SNP ID numbers and detailed sequence information are recorded in the dbSNP database.[12]

Table 1. The PCR Conditions Used in Genotyping (-786)T/C, 27 VNTR and 894G/T SNPs of the eNOS Gene

SNP (rs)	Primers	Amplicon (bp)	PCR Condition	Restriction Enzyme/ Allele Size	Reference
(-786)T/C (rs 2070744)a	5′-GCA TGC ACT CTG GCC TGA AGT G-3′ 5′-CAG GAA GCT GCC TTC CAG TGC-3′	223	1 cycle 95°C 5 min 35 cycle: 94°C 20s, 65°C 50s, 72°C 40s	MspI (Fermentase) T = 162, 61 bp C = 116, 61, 46 bp	Ahluwalia et al. (13)
4a4b polymorphism	5′-AGG CCC TAT GGT AGT GCC TTT-3′ 5′-TCT CTT TAG TGC TGT GGT CAC-3′	393/420	1 cycle 95°C 5 min 35 cycle: 94°C 20s, 56°C 50s, 72°C 40s	a allele = 393 bp b allele = 420 bp	Kim et al. (15)
(-894) G/T rs1799983)	5′-CAT GAG GCT CAG CCC CAG AAC-3′ 5′- AGT CAA TCC CTT TGG TGC TCA C-3′	206	1 cycle 95°C 5 min 35 cycle: 94°C 20s, 65°C 50s, 72°C 40s	MboI (Fermentase) G= 206-bp T = 119, 87 bp.	Kim et al. (15)

Abbreviations: PCR, polymerase chain reaction; SNP, single nucleotide polymorphism

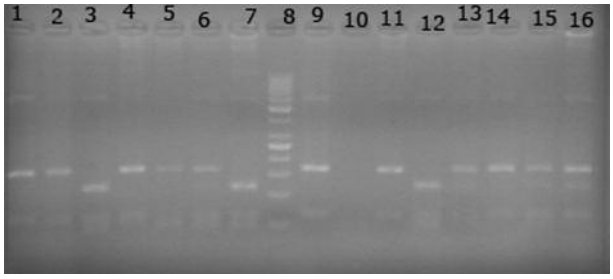
Genetic analyses

For genotype analysis, genomic DNA was extracted from buffy coat with the use of DNP DNA isolation kit (Cinagene, Tehran, Iran). Two eNOS SNPs, (-786)T/C and 894G/T, were genotyped using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), as previously used by Ahluwalia and associates and Wilcox and associates.[13,14] The 27VNTR is characterized by presence of either four 27-bp repeats (a allele) or five 27-bp repeats (b allele) and was genotyped using primers as previously used by Kim and associates. Details including primer sequences, PCR conditions, and restriction enzyme with product sizes are presented in Table 1 and Figures 1, 2 and 3.

Statistical analyses

Statistical analyses were performed with SPSS software (SPSS: An IBM Company, version 15.0, IBM Corporation, Armonk, NY, USA) and Epi Info Statcalc version 5. Discrete and continuous variables were compared between rejection and nonrejection using the Pearson chi-square test and *t* test as appropriate. Pearson chi-square test (3 × 2 contingency table) was used to assess the association of SNPs between rejection and nonrejection. The genotypes at their respective loci were coded as recessive (eg, eNOS-786CC vs -786TC + TT) and as dominant models (eg, eNOS -786CC + TC vs TT). Odds ratios and 95% confidence intervals (CIs) for relative risks were calculated. *P* values were subjected to the Bonferroni correction and considered significant when *P* value ≤ .05. Haplotype frequencies and Linkage disequilibria were estimated with Arlequin software (version 3.1). Fisher exact probability test was performed to determine significance and risk ratio of the haplotypes between the 2 groups.

Figure 1. Polymerase Chain Reaction Results for (-786) T/C Polymorphism

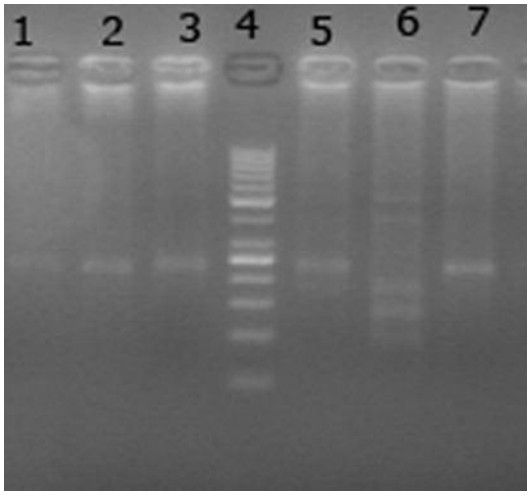


Lane 1: 2, 4, 5, 6, 9, 11, 13, 14: TT genotype (162 bp)
Lane 8: 50 bp DNA ladder
Lane 3: 7, 12: CC genotype (116, 61, 46 bp)
Lane 15, 16: CT genotype
Lane 10: Negative control

Figure 2. Polymerase Chain Reaction Results for 4a4b Polymorphism

Lane 1: 3, 4, 6, 7: bb genotype (420 bp)
Lane 2: 5, 8: ab genotype (393, 420)
Lane 9: 50 bp ladder

Figure 3. Polymerase Chain Reaction Results for (-894) G/T Polymorphism



Lane 1: 2, 3, 7: GG genotype (206 bp)
Lane 4: 50 bp ladder
Lade 5: GT genotype (206, 119, 87 bp)
Lane 6: TT genotype (119, 87 bp)

Results

In this study, we compared the eNOS T-786C polymorphism in a group of kidney allograft patients with and without AR. Patients included 46 men and 20 women (mean age, 31.35 + 10.2 y). The detailed data of patients' demographic characteristics and transplant status are shown in Table 2. Statistical analyses of recipient demographic characteristics including donor and recipient

age/sex, primary underlying kidney disease, and immunosuppressive regimen showed no differences between the ARs and non-ARs ($P > .05$). The majority of organs were donated from deceased donors.

Allele and genotype frequencies of the eNOS (-786) T/C and 894G/T SNPs and 27VNTR are given in Table 3. There is only a significant association of eNOS -786T allele and AR ($P = .03$). Further, recessive model of T-786C alleles and AR confirmed a significant association (odds ratio: 3.12; 95% CI: 0.01-9.83; $P = .025$) (Table 4).

Haplotype CbG was higher in recipients without rejection as compared to rejection group (OR: 0.42, 95% CI: 0.16-1.13; $P < .05$) (Table 5). No significant linkage disequilibria was observed among the polymorphisms (-786T/C, 27VNTR (a/b), 894G/T; $P > .05$).

Respecting genotyping and histologic grade, the distribution of different genotypes is present in Table 6. The major genotype in all groups was (-786T/T, 27VNTR (b/b), 894G/G). There was no association between histologic grade and any SNPs ($P > .05$).

Table 2. Demographics of Kidney Graft Recipients

Parameter	ARs (%)	Non-ARs (%)
Number of patients	33 (100)	33 (100)
Recipient sex		
Male	21 (63)	12 (37)
Female	25 (75)	8 (25)
Recipient age (y, mean ± SD)	32.42 ± 8.9	30.1 ± 10.1
Donor age (y, mean ± SD)	29.9 ± 11.3	27.44 ± 13.1
Donor sex		
Male	21 (63)	10 (53)
Female	20 (60)	13 (40)
Primary disease		
End-stage renal disease	27 (80)	28 (84)
Diabetic nephropathy	3 (10)	3 (10)
Glomerulonephritis	3 (10)	2 (6)
Living donor	13 (40)	9 (28)
Deceased donor	20 (60)	24 (72)
Immunosuppression		
CSA + mycophenolate mofetil + prednisone	13 (40)	18 (54)
Tacrolimus + mycophenolate mofetil + prednisone	20 (60)	15 (46)
Histologic grade of rejection		
Ia	21 (63)	
Ib	9 (27)	
IIa	2 (6)	
IIb	1 (4)	
III	0 (0)	

Abbreviations: ARs, acute rejection; CSA, cyclosporine; nonARs, nonacute rejection

Table 3. Allele and Genotype Frequencies of T(-786)C SNP of the eNOS Gene; 4a/4bVNTR of the eNOS Gene; (894G/T SNP of the eNOS Gene

	Acute Rejection n=33	Nonacute Rejection n=33	*P* Value
Genotype			
(%) TT	23	14	.08
TC	8	15	
CC	2	4	
Allele (%) T	54	43	.03*
C	12	23	
Genotype			
(%) aa	4	4	.74
ab	5	3	
bb	24	26	
Allele (%) a	13	11	.65
b	53	55	
Genotype			
(%) GG	24	26	.74
GT	5	3	
TT	4	4	
Allele (%) G	53	55	.65
T	13	11	

Abbreviations: eNOS, endothelial nitric oxide synthase; SNP, single nucleotide polymorphisms
*$P < .05$

Table 4. Dominant and Recessive Models of eNOS SNPs (-786T/C, 4a/4bVNTR, 894G/T) in Transplant Subjects —Comparison of Rejection and Nonrejection

	P Value	Odds Ratio	95% CI
T-786C			
CC vs TC+TT (Dominant model)	.39	0.47	0.05-3.33
TT vs TC+CC (Recessive model)	.025*	3.12	0.01-9.83
			4a/4a VNTR
aa vs ab+bb (Dominant model)	1.00	1.00	0.19-5.40
bb vs ab+aa (Recessive model)	.50	0.72	0.20-2.55
			G894T
TT vs GT+GG (Dominant model)	1.00	1.00	0.19-5.40
GG vs GT+TT (Recessive model)	.50	0.72	0.20-2.55

Abbreviations: eNOS, endothelial nitric oxide synthase; SNP, single nucleotide polymorphisms
*$P < .05$; CI: confidence interval

Table 5. Haplotype Frequency Distribution (Rejection and Nonrejection)

	Acute Rejection	Nonacute Rejection	*P* Value	Odds Ratio	95% CI
TbG	29	26	.76	1.12	0.51-2.42
CbG	9	17	.049	0.42	0.16-1.13
TaT	8	6	.63	1.31	0.38-4.61
TaG	5	3	.46	1.73	0.34-9.60
TBT	5	3	.46	1.73	0.34-9.60
CaT	3	3	.95	0.95	0.15-6.21
CaG	2	1	.58	1.93	0.13-10.11
CbT	2	1	.58	1.93	0.13-10.11

Order of SNPs in eNOS haplotypes: (PROMOTER) T/C (-786), 27VNTR (a/b), and EXON 7 Glu298Asp G/T(894).
*$P < .05$; CI: confidence interval

Table 6. eNOS SNPs (-786T/C, 4a/4bVNTR, 894G/T) in Transplant Subjects With Histological Grade of Rejection

Histologic Grade (n)	-786T/C (n)	4a/4bVNTR (n)	894G/T (n)
Ia (21)	TT (18); TC (3)	bb(20); ab(1)	GG(17); GT(2); TT(2)
Ib (9)	TT (6); TC (2); CC (1)	bb(7); ab(1), bb(1)	GG(7); GT(2)
IIa (2)	TT (2)	bb(2)	GG(2)
IIb (1)	TT (1)	bb(1)	GG(1)

Abbreviations: eNOS, endothelial nitric oxide synthase; SNP, single nucleotide polymorphisms

Discussion

Renal transplant is the treatment of choice for patients with end-stage renal disease. However, episodes of AR have a negative effect on long-term graft survival. The incidence of AR is higher especially the first month after surgery.[16] Human leukocyte antigen (HLA) mismatch and antigen-independent factors such as I/R injury, drug toxicity, and infections are factors the contribute to the development of this event. Endothelial cells play an important role in the regulation of vascular remodeling, and NO is critical for the health of vascular endothelium and blood vessels. It is rapidly degraded to the stable end products (eg, nitrite and nitrate) that can be measured in serum and urine.[17]

Three subtypes of NOS (endothelial, neuronal, and inducible) are expressed in the renal tissue. Endothelial nitric oxide synthase is expressed in the renal vascular endothelium, including afferent and efferent arterioles and thick ascending loop of Henle. Neuronal NOS has been secreted in the juxtaglomerular apparatus, and inducible NOS expression is seen in settings of inflammation and in the inner medullary collecting duct.[18-20]

Nitric oxide inhibits platelet aggregation and leukocyte adhesion to vascular endothelium and also has antiproliferative effects on vascular smooth muscle cells.[3, 4] There are reports about the role of NO in I/R and allograft rejection in liver, heart, kidney, and pancreatic islet cell transplant.[21-27] Inhaled NO or NO donor drugs are novel treatments that have been used clinically to diminish I/R.

Intraoperative liposome-mediated gene delivery of eNOS into donor hearts before transplant reduces I/R injury by inhibiting NF-kappaB pathway and the early infiltration of leukocytes, all of which improve graft survival.[26, 27] Ishimura and associates found that endothelial expression of eNOS after renal reperfusion is increased and recovery from renal ischemia with improved graft function is enhanced.[28]

Significant increase in serum NO levels also has been reported during episodes of rejection in renal transplant recipients.[27,19,29] Infections and surgical stress had an important role in stimulating NO production after transplant, while drugs such as glucocorticoids or calcineurin inhibitors such as tacrolimus inhibit its production.[25] Therefore, NO increases in response to various cytokines that are participate in rejection. The genetic variations in the endothelial nitric oxide synthase gene may influence NO levels and has effects on the inflammatory process. Nakayama and associates believed that the -786T to-C polymorphism is associated with significant variation in eNOS promoter activity.[30] The 894G/T polymorphism also is associated with an altered protein sequence and functional effect on the eNOS protein.[31] However, recent expression studies have demonstrated no functional difference between G894 (298Glu) and T894 (298Asp).[32]

Yilmaz and associates studied on G894T mutation at exon 7 of the eNOS gene and correlated it with chronic allograft nephropathy. This polymorphism did not influence long-term renal allograft outcome and is not consider a risk factor for chronic allograft failure.[9] Viklický and associates compared the eNOS (G894T) gene polymorphism in patients with preserved graft function with control group and found no differences in allele and genotype distributions between them. There were no links between genotypes, renal function and atherosclerosis risk factors.[33] Sezer and associates studied on angiotensin II type 1 receptor (ATR1) and eNOS gene polymorphism in renal transplant patients. They found that bb allele of the eNOS and nonAA allele of ATR1 1166 gene were associated with an anti-inflammatory state and may predict renal outcome in transplant patients.[34]

In present study, we found association of eNOS -786T allele and AR and higher prevalence of CbG haplotype in recipients without rejection. However, no significant association between each genotype and histologic grade of rejection was identified. Respecting other genotypes and keeping with published articles, we were unable to find any association between genetic findings and AR in kidney grafts. This finding may be due to small sample size that affects the strength of the data.

Therefore, identifying associations of genetic variants with complex immunologic event, such as the rejection demand a larger sample size with multicentric investigation.

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