

To Analyze the Modifier Effect of NOS3 Gene in Hypertensive Patients of ADPKD

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Abstract

Background: Nitric oxide is important for the functional integrity of the vascular endothelium and is produced in endothelial cells by the enzyme endothelial nitric oxide synthase (eNOS). Hypertension has a strong genetic component, and the NOS3 gene, which encodes eNOS, represents an interesting candidate for contribution to the phenotype. The most clinically relevant polymorphisms in the NOS3 gene are a variable number tandem repeat (VNTR) in intron 4, and rs2070744 (T-786C) in the promoter region. **Subjects and Methods:** This study aims to investigate the association between these three polymorphisms in the NOS3 gene and modifier effect on age, progression of disease and hypertension in patients of ADPKD. **Results:** The present study carryout in the ADPKD patients admitted in NRI Medical College & Hospital. Univariate and multivariate logistic regression analyses were performed to assess the effect of genotypes and hypertension on the progress of chronic kidney disease (CKD). A stratified analysis was also performed to assess the evidence of the modification of hypertension-CKD relationship among VNTR genotypes. **Conclusion:** The results of this study indicated that the polymorphism in NOS3 may be a genetic susceptibility factor for hypertension in the Indian population.

Keywords: VNTR genotypes, NOS3 gene, ADPKD, Hypertension.

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Introduction

Polycystic kidney disease (PKD) is a multisystem disorder characterized by the formation of cysts within the kidneys. PKD is mainly caused by the unfolding of its immunoglobulin-like PKD domains. There are two major forms of PKD; autosomal dominant (ADPKD) that caused by mutations of PKD1 and PKD2^[1] and autosomal recessive (ARPKD) caused by mutations in PKHD1 gene.^[2,3] Although, cellular changes and mechanisms involved in initial stages of cyst formation might differ between ADPKD and ARPKD, both are important causes of kidney failure, morbidity and mortality in children and adults of all racial groups worldwide.^[4] Further, it is clear that the disease progression in the ADPKD and ARPKD involves almost similar pathogenetic mechanisms.^[5,6] Hypertension is an important factor in the progression of kidney failure ADPKD patients, 60% of ADPKD patients exhibit high blood pressure even before the impairment of kidney function.^[7] The prevalence of Hypertension continues to rise despite recent advances in diagnosis and treatment. Approximately 40% of the global adult population aged 25 and above suffer from hypertension.^[8] Another argument for modifier genes influencing renal disease progression in ADPKD is provided by the excess

variability in the age at ESRD observed among siblings or dizygotic twin's vs genetically identical monozygotic twins.

Modifier effect of ENOS in ADPKD

The effect of ENOS on renal disease progression in ADPKD has been assessed recently in a large series of unrelated patients from a restricted geographic area.^[9] The frequent Glu298Asp (E/D298) polymorphism of exon 7 of ENOS was associated with a significant, 5 year lower age at ESRD in the whole group of ADPKD males, as well as a lower renal survival (Kaplan–Meier analysis) in males from PKD1-linked families. In contrast, no effect was found in females with ADPKD. The more critical influence of ENOS in ADPKD males may be explained by the fact that oestrogens stimulate eNOS expression and the release of NO in endothelial cells.^[10]

A molecular basis for the effect of the Glu298Asp polymorphism was provided by the demonstration of decreased enzymatic activity and modified expression of eNOS in renal arteries from patients harbouring the Asp allele.^[9] The functionality of this polymorphism has been confirmed independently in vitro.^[11] The mechanism by which this conservative amino acid substitution leads to degradation and decreased activity of eNOS remains to be elucidated. The Glu298 of eNOS is conserved among species, and located within the oxygenase domain of

eNOS.^[12] An increased degradation of the Asp298 eNOS may yield a cleaved protein that could contribute to endothelial dysfunction.^[13] Alternatively, the Glu298Asp polymorphism may influence the complex post-translational regulation of eNOS.^[14] Thus, lower enzymatic activity and/or partial cleavage of eNOS could be responsible for increased endothelial dysfunction, and possibly increased systemic blood pressure and/or alteration of intra-renal microcirculation, in ADPKD patients harbouring the Asp298 allele.

Finally, insights derived from genetic modifiers may lead to therapeutic opportunities. From the data summarized above, a logical approach would be to address specifically the endothelial dysfunction in ADPKD. Nitrates come first to mind, but their systemic use is hampered by tolerance, activation of the sympathetic nervous system and production of superoxide and endothelin.^[15] Similarly, the effect of other types of NO donors may be limited by the redox status of the tissue and the potential formation of peroxynitrite. These limitations may be resolved by using drugs that stimulate eNOS expression in endothelial cells, such as statins or ACE inhibitors.^[16] In any case, the possibility of pharmacomodulation by the ENOS genotype should be kept in mind.

Hypertension in Autosomal Dominant Polycystic Kidney Disease

Hypertension is common in ADPKD and is diagnosed early, at a mean age of 29 years. Several mechanisms play a role in the development of hypertension in ADPKD, including hyperactivity of the renin-angiotensin-aldosterone system (RAAS), activation of the sympathetic nervous system and abnormalities of the endothelium.

Hypertension is responsible for the diagnosis of ADPKD in approximately 30% of patients. Hypertension is a common and early manifestation of ADPKD and occurs in more than 60% of affected individuals before decline or any substantial reduction in glomerular filtration rate (GFR) has been observed (GFR >75ml/minute/1.73m²).^[17]

The observation of the high incidence of hypertension in ADPKD patients was first noted in the early 20th century. Studies from 1916, 1931, and 1949 reported an incidence of hypertension of 72, 75, and 73%, respectively, but did not account for the presence of renal insufficiency.^[18]

Schact et al. reported a much lower incidence of hypertension in age- and gender-matched patients with another non-glomerular renal disease characterized by tubulo-interstitial abnormalities and low-grade proteinuria compared with patients with ADPKD (chronic pyelonephritis 26% versus ADPKD 73%).

In chronic kidney disease (CKD), hypertension is in large part due to renal dysfunction and its associated increased sodium retention, as well as the underlying renal disorder. Thus, ADPKD patients are unique where hypertension appears early and independently of renal dysfunction.^[19]

Additional knowledge of NOS3 gene polymorphisms and their role in hypertension will improve understanding of hypertension, its common predisposing factors, and potential treatment options. The potential contribution of NOS3 polymorphisms to the development of hypertension in

Indian population has received no attention to date, and no previous studies have addressed this subject. The objective of the present study is to identify the modifier effect of NOS3 gene with hypertensive patients of ADPKD.

Subjects and Methods

Subjects

Patients visiting Department of Nephrology, NRI Medical College & Hospital, Guntur, Andhra Pradesh during from June 2017-June 2019 for treatment with the complications of cysts in the kidney, will be the main source of the samples in the present study.

The following inclusion criteria will be used in the present study

- i. Presence of at least two renal cysts in any one of the kidney, if the individual is aged less than 30 yrs.
- ii. Presence of at least two renal cysts in each kidney if the individual is aged between 30-60 yrs
- iii. Presence of at least four renal cysts in each kidney if the individual aged greater than 60 yrs.
- iv. Abdominal ultrasonography will be conducted on each subject
- v. Biochemical studies such as serum urea, creatinine and uric acid will be estimated
- vi. Urine microscopy and a renal ultrasound also will be performed.
- vii. Urine studies for fractional extraction of sodium, fractional excretion of uric acid, and protein/ creatinine ratio will be performed for twenty-four hour basis.
- viii. Following their clinical assessments, the blood samples from 50 unrelated subjects with the history of hereditary renal cysts were collected. 90 age and sex matched controls were also be recruited from the same geographical location. Patients with other end stage renal disorders were excluded from the study. Written informed consent was collected from the study participants before collecting the blood samples. DNA from the above samples will be isolated using phenol and chloroform method.

DNA sample preparation

Venous blood (5 mL) was drawn from each subject. DNA extraction and genotyping were performed at the Institute. DNA extraction was performed using a Qiagen Genra Puregene Blood Kit (Qiagen, Germany).

Genotyping

DNA, extracted from blood samples of the 53 ADPKD and 94 control subjects, was genotyped for **intron 4 VNTR of endothelial nitric oxide synthase gene (NOS3)** using the forward 5'- AGGCCCTATGGTAGTGCCCTT- 3' and the reverse 5'- TCTCTTAGTGCTGTGGTCAC-3' that flank the region of the 27 bp repeat in intron 4 of eNOS gene.

Materials Required For Polymerase Chain Reaction (PCR)

All materials required for PCR except high molecular weight DNA were commercially obtained.

- PCR buffer
 - dNTPs
 - Taq Polymerase
 - Milli Q water
 - Primers (Forward and reverse)
- Primer for NOS3-VNTR

27 bp intron 4 VNTR	Forward primer	5'- AGGCCCTATGGTAGTGCCTTT- 3'
	Reverse Primer	5'- TCTCTTAGTGCCTGTGGTAC-3'

The specificity of the primers was also checked by using the electronic PCR.

PCR Program for NOS3-VNTR:		
STEPS	TEMPERATURE	TIME
Initial Denaturation	95°C	5 min
Denaturation	95°C	1 min
Annealing	60°C	1 min
Extension	72°C	1 min
Final Extension	72°C	7 min

} 35 cycles

PCR products were resolved and then visualized under UV light.

The PCR products were run on 2% agarose gel electrophoresis, and the fragments separated were visualized by ethidium bromide staining under UV trans-illumination. PCR analysis of genomic DNA generated fragments of 393 bps corresponding to 4 a/a homozygotes, 420bps to 4b/b homozygotes and 393 and 420 bp heterozygote.

Statistical analysis
HWSIM Software's

To indicate statistical significance, p values, were considered at 0.05 level. Distribution of biochemical variables between cases and controls was assessed using t-Test among genotypes using ANOVA. The association was tested in three different genetic models. All the analysis was done using the SPSS software.

Results

Table 3: Distribution of different biochemical variables among different NOS3 Genotypes.

	Wild	Het	Deletion	P value
Age at renal failure (Yrs)	47.10±11.38	48.10±13.24	44.20±10.91	0.762
Blood Urea Nitrogen (mg/dl)	48.67±25.65	47.52±17.79	40.17±13.16	0.434
Creatine (mg/dl)	7.20±4.02	7.48±2.63	7.27±3.75	0.163
Sodium (mg/dl)	136.48±11.72	136.48±11.39	140±6.29	0.757
Potassium (mg/dl)	4.81±0.999	4.85±0.95	4.28±0.89	0.219
Chloride (mg/dl)	97.18±7.86	99.43±8.31	94.50±8.89	0.326

Bicarbonates (mg/dl)	20.42±4.84	20.95±4.22	23.5±2.58	0.274
Calcium (mg/dl)	7.83±0.874	7.70±0.89	7.70±0.89	0.842
Glomerular filtration rate (ml/hr)	16.68±20.51	9.10±5.34	11.85±8.29	0.016

ADPKD can lead to ESRD most commonly in middle age or later in life. In the present study the age at renal failure is not significantly associated with NOS3 genotypes of ADPKD [Table 5].

Table 4: Distribution of CKD stage among different genotypes of ADPKD

CKD Stage	Deletion	Het	Wild
Satge 1	0	0	2
Stage 2	0	0	5
Stage 3	0	0	8
Stage 4	1	5	9
Stage 5	1	5	17
Chi-square(8 d.f)	P=0.523		

The ADPKD gets worse slowly; eventually resulting in end-stage kidney failure, the distribution of VNTR genotypes in different stages of CKD in ADPKD patients was not significant in the present study [Table 4].

Table 5: Changes in the age at renal failure among different genotypes of ADPKD

Genotype (n)	Age at renal failure
Deletion (1)	51.00
Het (09)	51.10+10.45
Wild (30)	44.95+9.237
ANOVA (2 d.f)	p=0.196

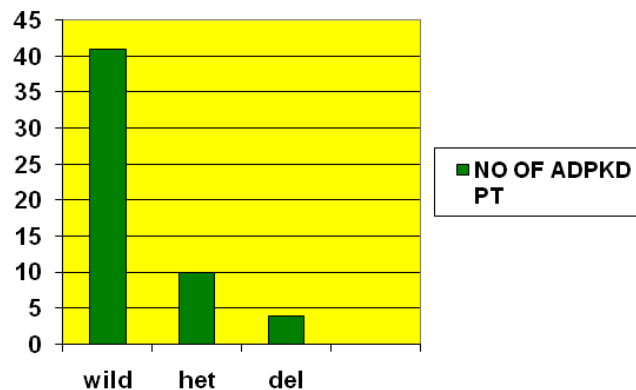


Figure1: Distribution of NOS3 Gene VNTR in control and ADPKD.

Figure 2 depicts the distribution of patients with htn in our study with respect ive polymorphism.

Discussion

The univariate analysis revealed that the NOS3 VNTR, age and hypertension contributed to the advancement of CKD. After controlling other variables in multivariate analysis, only VNTR genotypes and age were found to increase the risk of CKD advancement in ADPKD. Stratified analysis

further showed that the NOS3 VNTR was an effect modifier of the relationship between hypertension and CKD advancement among the ADPKD patients. The results were also consistent with the existing reports in this regard. The first study linking this VNTR and hypertension was published by Miyamoto and co-workers.^[20] Though these findings were not substantiated in the Australian population,^[21] a positive nitrate levels and blood pressure in normotensive African Americans.^[22] Further, the a/a genotype of the NOS3 VNTR was shown to significantly increase the risk of developing hypertension in Indian men.^[23] Following the conflicting results generated from these association studies, Intron 4 VNTR and haplotype analysis of several other SNPs of NOS3 showed significant association with daytime systolic blood pressure.^[24] In the present study 4a allele was significantly associated with CKD advancement in ADPKD. Higher incidence of 4a allele was observed among Czech patients with end-stage renal disease (ESRD) caused by ADPKD.^[25] On the contrary, NOS3 VNTR polymorphism showed no effect on the age at ESRD in unrelated ADPKD patients from Belgium and the north of France (26). Several studies have demonstrated that a decrease of NO synthesis and release may be important in the progression of renal disease.^[27]

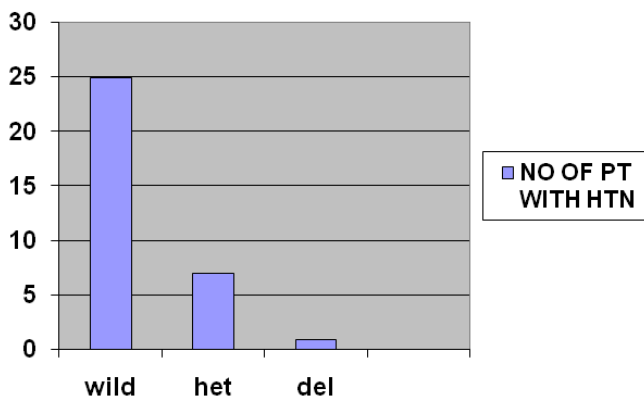


Figure 2: Number of Patient with Hypertension.

This study has number of limitations that are worth mentioning. The small samples size with potential lack of power to the study is a limitation. Furthermore, cases and controls were significantly different in their age, gender, BMI and smoking status. However, statistical analyses showed that none of these variables had an effect on any of the genotypes distribution.

There was also weaker linkage between (rs1799983 and rs2070744), and (rs2070744 and the VNTR). It is unlikely that the VNTR itself has a functional role, as it is in an intronic region. However, it has been suggested to act as a marker for other functional variants elsewhere in the gene. Nakayama et al. first reported that the VNTR is in linkage disequilibrium with rs2070744; hence, the effect of VNTR on NOS3 mRNA expression, eNOS protein concentration, and enzyme activity is likely to be mediated by the differences in transcriptional efficiency associated with the rs2070744 polymorphism.^[28-31] The degree of linkage disequilibrium between the three polymorphisms may help in

understanding of the evolutionary divergence of the NOS3 gene.

Conclusion

In conclusion, in this study we demonstrate that the polymorphism in the NOS3 gene promoters associated with hypertension in the Indian population. We identified the significant modifier effect of NOS3 27-bp VNTR and CKD progression in hypertensive ADPKD patients. However, more focused research in this regard is required to further validate our findings. Further studies with larger sample sizes and family-based analyses are required to confirm this association.

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