

To Analyze the Association between the Polymorphism of NOS3 Gene VNTR in ADPKD

Shiva Nagendra Reddy Annareddy¹, Pratap Duggirala²

¹Assistant Professor, Department of Nephrology, NRI Medical College & Hospital, Chinakakani, Guntur, Andhra Pradesh, India, ²Assistant Professor, Department of Plastic Surgery, NRI Medical College & Hospital, Chinakakani, Guntur, Andhra Pradesh, India.

Abstract

Background: Autosomal dominant polycystic kidney disease (ADPKD) is an inherited disorder, and it is mainly associated with renal cyst formation. Endothelial nitric oxide plays a crucial role in the control of local hemodynamics and systemic blood pressure. Thus, it has been proposed that gene coding for endothelial nitric oxide synthase (eNOS) could have a modifying effect on hypertension and related complications in autosomal dominant polycystic kidney disease (ADPKD). As endothelial dysfunction and oxidative stress are evident early in ADPKD patients, eNOS holds therapeutic promise in the treatment of ADPKD. **Objectives:** The aim of the present investigation is to determine the association between NOS3 27-bp VNTR in ADPKD patients and also investigate the role of progression of renal disease in ADPKD. **Subjects and Methods:** In the present study, we investigated by studying the rural population of 50 ADPKD patients and 90 unrelated healthy controls admitted in Dept. of Nephrology, NRI Medical College & Hospital, Andhra Pradesh, India. Genetic analysis was mainly performed by PCR and agarose gel electrophoresis. Genotype comparison was observed between control and ADPKD. **Results:** Dyspnea was the most common symptom at presentation, being present in 35 (100 %) of the patients. Cough with or without expectoration was All three genotypes of NOS3 were observed in both cases and controls of the present study. In both cases and controls wild type genotype is more followed by heterozygote and minor genotypes. Blood Urea Nitrogen and creatinine is more in the controls when compared to the ADPKD patients. The other core electrolytes are shown to be in the normal range in both case and controls of the present study. Glomerular filtration rate is also exhibited differences between cases and controls. None of the biochemical variable has shown significant differences among NOS3 genotypes. But the difference in glomerular filtration rate is significant among different genotypes. **Conclusion:** The present study confirms the significant association between the 27-bp VNTR and CKD advancement among the ADPKD patients in the rural population.

Keywords: Polymerase chain reaction, Autosomal dominant polycystic kidney disease, VNTR, CKD.

Corresponding Author: Dr. Pratap Duggirala, Assistant Professor, Department of Plastic Surgery, NRI Medical College & Hospital, Chinakakani, Guntur, Andhra Pradesh, India.

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Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is a common disorder, occurring in approximately 1 in every 400 to 1000 live births.^[1-3] It is estimated that less than one-half of these cases will be diagnosed during the patient's lifetime, as the disease is often clinically silent.^[1]

Approximately 85 percent of families with ADPKD have an abnormality on chromosome 16 (PKD1 locus) that is tightly linked to the alpha-globin gene locus.^[4] The remaining patients have a different defect that involves a gene on chromosome 4 (the PKD2 locus).

Patients with PKD2 have a less severe phenotype than those with PKD1, but neither disorder is benign.^[5] Cysts occur later in PKD2 disease, as does end-stage renal disease (mean age 74.0 versus 54.3 years in PKD1).^[6]

Genetics

A variety of genetic defects have been described, including

frameshift, deletion and missense mutations in patients with ADPKD. Most families have an abnormality on chromosome 16 in the PKD1 locus that is tightly linked to the alpha-globin gene locus.^[7-10] Most of the remaining patients have a defect in the PKD2 locus on chromosome 4.^[11-13] A few families have a defect unrelated to either locus.^[14-16]

Initial studies suggested that PKD1 was responsible for 96 percent of cases. Subsequent data suggest that, at least in Europe, the incidence is lower at about 86 percent. Macroscopic cysts occur later in PKD2 disease as does end-stage renal disease (mean age 74 versus 54 years in PKD1).^[17] As a result, false negative results are more likely when screening young subjects with this disorder.

A similar impairment of the endothelium-dependent vasorelaxation, contrasting with an intact response to exogenous nitric oxide (NO), has also been documented in normotensive ADPKD patients.^[18] These data suggest that endothelial dysfunction, secondary to an impaired release of NO, exists in ADPKD.

NO and endothelial NO synthase

NO is the molecular counterpart of the endothelium-derived relaxing factor.^[19] In endothelial cells, NO is synthesized from arginine by the endothelial NO synthase (eNOS), a constitutively expressed enzyme that is encoded by the *ENOS* (*NOS3*) gene located on 7q36.^[20] The enzymatic activity of eNOS is regulated by intracellular Ca²⁺ levels, as well as by Ca²⁺-independent mechanisms such as the phosphatidylinositol-3-OH kinase (PI3k)/Akt pathway.^[21] Once released, NO diffuses rapidly through cell membranes and relaxes smooth muscle cells through the production of guanosine 3',5'-cyclic monophosphate (cGMP). Furthermore, NO inhibits platelet activation, regulates angiogenesis and controls microvascular permeability.^[19] The influence of *ENOS* on hypertension, coronary vasospasm, atherosclerosis and, most importantly, progression of diabetic nephropathy.^[22] led to the hypothesis that it could be a modifier gene in ADPKD.

Hence, the objective of the present study, to determine the association between the polymorphism of NOS3 gene in autosomal dominant polycystic kidney disease.

Subjects and Methods

Subjects

Patients visiting Department of Nephrology, NRI Medical College & Hospital, Guntur, Andhra Pradesh during from 2017 June – 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.

Methodology for DNA isolation from buccal samples

DNA was isolated from all samples using the phenol chloroform method.

1. Blood samples were re-suspended in Cell Lysis Solution (0.01M Tris HCl; 320mM sucrose; 5mM MgCl₂; 1% Triton X 100) and incubated with Proteinase K to digest the proteins.
2. The supernatant was discarded and the pellet was disturbed. Then 5ml of reagent B (*Lysis Buffer II*: 0.4M Tris HCl; 150mM NaCl; 0.06M EDTA; 1% SDS) and 1.25ml of reagent C (5M Sodium per chlorate) were added and shaken thoroughly. Then 3ml of Tris saturated Phenol (Proteins and restriction enzymes are removed by phenol) and 3ml Chloroform-isoamyl alcohol (24:1)(chloroform-isoamyl alcohol helps in disrupting protein secondary structure causing proteins to denature and precipitate from solution) were added, mixed gently and centrifuged at 3000rpm for 10min.
3. The three layers were formed. The bottom layer was phenol, middle chloroform and the upper layer was aqueous. The aqueous phase was taken in another tube using blunt tips.
4. The precipitated proteins collect at an inter phase between the aqueous and organic layers. It is very important to understand the effect of pH on the performance of phenol.
5. To the aqueous aliquot 3ml chloroform-isoamyl alcohol was added, mixed well and centrifuged at 3000rpm for 5min.
6. The aqueous phase was separated into fresh tube and 2 volumes of ice-cold absolute alcohol were added to precipitate DNA.
7. The DNA was taken in the sterile eppendorf tube carefully and 80% ethanol was added.
8. The DNA was given a short spin and the ethanol was removed by inverting on tissue paper. Then it was air-dried.
9. 80–100µl TE solution was added to the eppendorf containing DNA (Stock).
10. For dissolving DNA, the eppendorf were kept in water bath at 55°C for 1-2 hours and then stored at 4°C in the fridge.

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'- AGGCCCTATGGTAGTGCCCTTT- 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'- TCTCTTAGTGCTGTGGTCAC- 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 test the departure of allele frequency spectrum from the Hardy-Weinberg equilibrium, we employed χ^2 test with one degree of freedom, and Monte Carlo simulation test using the HWSIM program. To indicate statistical significance, p values, were considered at 0.05 levels. 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

The association of the VNTR polymorphism in intron 4 of the endothelial nitric acid gene (NOS3) gene susceptible to Hypertension and have been studied over the years in different populations. There is no data available in Indian populations with reference to the NOS3 gene allele frequencies and its association with ADPKD or ESRD. In the present study we have analyzed for the first time the association between the polymorphism of NOS3 gene VNTR and ADPKD in rural Population.

The 27-base pair variable-number-of-tandem-repeats (VNTR) region located in the intron 4 of NOS3 gene are postulate to be associated with impaired NO synthesis (1). The primers used in the present study generated fragments of 393 bps corresponding to 4 a/a homozygotes, 420bps to 4b/b homozygotes and 393 and 420 bp heterozygote [Figure 1].

Table 1: Distribution of NOS3 Gene VNTR in control and ADPKD

	Control	ADPKD
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Deletion	4 (4.26)	2 (3.77)
Het	32 (34.04)	10 (18.87)
Wild	58 (61.70)	41 (77.36)
Maf	22.3	14.2
HWE p	0.875	0.197



Figure 1: Gel picture showing 100bp ladder, heterozygote for the NOS3 VNTR, 4 a/a homozygotes, 420bps to 4b/b homozygotes (from Right to Left).

All three genotypes of NOS3 was observed in both cases and controls of the present study. In both cases and controls wild type genotype is more followed by heterozygote and minor genotypes. The distributions of these genotypes are following the Hardy Weinberg equilibrium in control as well as cases. The minor allele frequency in controls and cases is 22.3% and 14.2% respectively [Table 1].

Baseline data on biochemical variables between cases and controls in the present study is documented in table 2. Blood Urea Nitrogen and creatinine is more in the controls when compared to the ADPKD patients. The other core electrolytes are shown to be in the normal range in both case and controls of the present study.

Glomerular filtration rate is also exhibited differences between cases and controls. None of the biochemical variable has shown significant differences among NOS3 genotypes. But the difference in glomerular filtration rate is significant among different genotypes [Table 3].

Table 2: Distribution of different biochemical variables between cases and controls

Variable	Control	ADPKD	P value
Blood Urea Nitrogen (mg/dl)	54.69± 21.66	35.30±20.25	<0.001
Creatinine (mg/dl)	8.83±3.029	4.28±2.74	<0.001
Sodium (mg/dl)	134.76±13.13	140.0±6.82	0.004

Potassium (mg/dl)	4.96±1.095	4.45±0.636	0.001
Chloride (mg/dl)	98.51±6.94	96.25±9.37	0.09
Bicarbonates (mg/dl)	20.20±4.75	21.77±4.26	0.042
Calcium (mg/dl)	7.89±0.939	7.60±0.672	0.042
Glomerular filtration rate (ml/hr)	6.89±2.807	28.84±26.60	<0.001

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].

Several previous studies have established typical rates of decline of glomerular filtration rate (GFR) in ADPKD patients. Variations in the GFR among different genotypes of ADPKD did not demonstrate significant differences [Table 3].

Table 3: Variations in the GFR among different genotypes of ADPKD

Genotype	GFR
Deletion (2)	20.55±10.39
Het (10)	14.25±7.75
Wild (41)	31.03±25.68
Anova (2 d.f)	P=0.123

Table 6: Variations in the GFR among different genotypes of ADPKD

Association between NOS3 Gene VNTR and ADPKD revealed significant association in the dominant model [Table 4].

Table 4: Association between NOS3 Gene VNTR and ADPKD.

Association model	Odds Ratio	P value
Dominant	0.47	0.05
Recessive	0.88	0.887
Additive	0.56	0.086

Chart 2 depicts the distribution of patients with htn in our study with respect five polymorphism
The minor allele frequency of the NOS3 VNTR locus in different world populations has shown differences in the global populations [Figure 2].

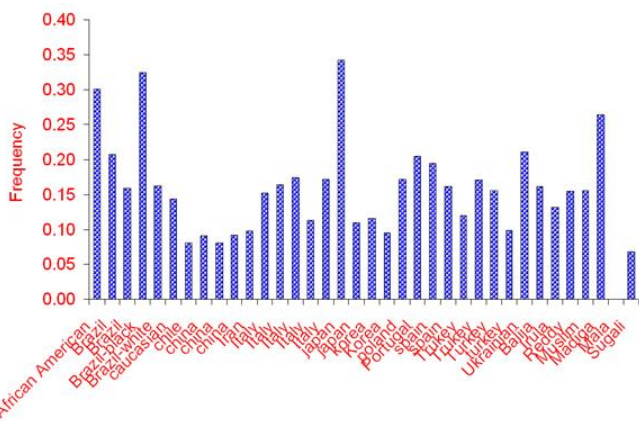


Figure 2: NOS3 VNTR polymorphism minor allele frequency in world populations

Discussion

In view of the strong association of ADPKD with hypertension, the eNOS enzyme responsible for nitric oxide production, variation in its expression and activity can be linked to hypertension. Hence eNOS/NOS3 gene has long been thought to be a candidate gene for ADPKD. In this study, after investigating 90 controls and 50 ADPKD we demonstrate that there is a positive association between NOS3 VNTR polymorphism and ADPKD in the study. The results were consistent to those previously reported studies. The first study linking this SNP and hypertension was published by Miyamoto and co workers (Miyamoto 1998). They examined the association of several SNPs of the NOS gene and hypertension including the VNTR Intron 4 in 2 different geographic sites in Japan, comparing hypertensives and normotensive controls. Though these findings in an were not substantiated in Australian or a Scandinavian populations further evidence of an association of the NOS3 allele, and with essential hypertension resistant to conventional treatment, came from a Czech population. Following the conflicting results generated from these association studies Intron 4 VNTR and several other SNPs of NOS3, was recently published by Persu.^[25] Although sib-pair analysis failed to show any significant association of any specific SNP with blood pressure, haplotype analysis disclosed a significant association between NOS3 haplotypes and daytime systolic blood pressure. Ultimately as this approach is more informative further studies and specifically functional studies within a given phenotype would be most informative.

A recent study using India samples did not find any correlation between the presence of the eNOS 4 a/b variant and hypertension (Arora 2009).^[23] Previous reports indicate that the carriers of this variant have lower plasma nitric oxide levels and decreased protein expression (Wang et al.2000), but this finding is not supported by all studies (Yoon et al. 2000).^[24] It is possible that the variant is in linkage disequilibrium with other functional variants in regulatory regions of the NOS3 gene.

In the present study the age at renal failure is not significantly associated with NOS3 genotypes of ADPKD. An independent study using the same intron 4 polymorphism in unrelated ADPKD patients from Belgium and the north of France, has also reported similar findings (Persu et al. 2002).^[25]

Variations in the GFR among different genotypes of ADPKD did not demonstrate significant differences are indicating no association between GFR and NOS3 gene VNTR. Persu and colleagues also failed to find the association between intron 4 VNTR renal phenotype of ADPKD (Persu et al. 2002).^[25]

Several studies have demonstrated that a decrease of nitric oxide (NO) synthesis and release may be important in the progression of renal disease (Zatz and de Nucci 1991; Baylis et al. 1992),^[26,27] and a significant endothelial dysfunction has been documented in ADPKD patients (Wang et al. 2000).^[28] Direct analysis of NOS3 gene polymorphisms in ADPKD patients also revealed inconclusive results from

many populations. The -786T>C SNP, in the promoter of the NOS3 gene, is a functional polymorphism, C allele being associated with higher gene expression (Nakayama et al. 1999).^[29]

Another functional polymorphism in the NOS3 is E298D, the D allele is known to lower the enzyme activity by post-translational modifications (Persu et al. 2002).^[25] NOS3 gene polymorphisms in ADPKD disease progression has been noticed in few studies (Baboolal et al. 1997).^[30]

In a study the association was confirmed in a subgroup of male PKD1 patients and it was reflected by a molecular counterpart in terms of eNOS activity and processing (Persu et al. 2002; Reiterova et al.2002).^[25& 31, 32] Only a slight correlation but not significant association between the promoter polymorphism of NOS3 and renal survival was found in a multi-centric PKD1 population.

Furthermore, the association was not detected in another series (Walker et al. 2003).^[33] These inconsistent results might have originated from different inclusion criteria, sex and/or population heterogeneity and sample ascertainment (Devuyst et al. 2003).^[34] In addition, the allelic frequency variations in NOS3 gene are different among the world populations, making the association studies as in conclusive or population specific.

Several studies have demonstrated that a decrease of NO synthesis and release may be important in the progression of renal disease^[35] and a significant endothelial dysfunction has also been documented in ADPKD patients.^[36] Direct analysis of NOS3 gene polymorphisms in ADPKD patients have also revealed inconclusive results from many populations. Although no direct association between NOS3 VNTR and ADPKD has been observed, patients who carried 4a allele showed faster ESRD progression in the group of ADPKD.^[37] Higher frequency of the NOS3 4a allele carriers among CKD children suggests that the NOS3 VNTR may be associated with an increased risk of chronic renal failure.^[38]

Conclusion

We concluded that, analysis of ADPKD cases and controls revealed three genotypes of NOS3 in the present study. In both groups wild type allele is more than the minor allele. Analysis of ADPKD patients did not demonstrate significant differences in the GFR among different genotypes of NOS₃ VNTR.

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