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## Analysis of Aldosterone Synthase Gene Promoter (-344 C >T) Polymorphism in Indian Diabetic Nephropathy Patients

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### Abstract

**Aim:** the aim of the present study is to evaluate the association between *CYP11B2* gene and type 2 diabetic nephropathy patients in Indian population.

**Result:** There was a significant difference in age, cholesterol, triglycerides, HDL, creatinine, BUN, Uric acid, total protein, Albumin, SBP, DBP variables except LDL Cholesterol among the study groups. The frequencies of *CYP11B2* gene CC, CT, TT genotypes among the T2DNH are 16.67 %, 51.19 %, 32.14 %; in T2DM patient 10.66 %, 52.46 %, 36.88 % and 16.95%, 38.14%, 44.91% in controls respectively and we could not observe significant differences in both genotype ( $\chi^2=7.289$ ,  $p=0.121$ ) and allele frequency ( $\chi^2=1.82$ ,  $p=0.403$ ) of the *CYP11B2* gene (-344 T>C) polymorphism between T2DNH, T2DM patients and Controls subjects.

**Conclusion:** Our findings do not support the hypothesis that *CYP11B2* polymorphism is associated with prevalence of Type 2 diabetic nephropathy patients in Indian populations.

### Introduction

Nephropathy is a chronic micro vascular complication of diabetes. Uncontrolled blood sugar level and high blood pressure are risk factor for the development of nephropathy or End Stage Renal Disease (ESRD) [1-4]. The Renin-Angiotensin-Aldosterone System (RAAS) is a regulator of both blood pressure and kidney functions and is suggested to play an important role in the development of nephropathy in Type 2 Diabetes Mellitus and genes encoding components of the RAAS can be candidate genes for evaluating predisposition for the development of hypertension, cardiovascular disease, ESRD or progression of renal disease in type 2 diabetes [5-12]. Aldosterone is an important component of RAAS, and plays an important role in controlling blood pressure, water and electrolyte homeostasis in the body [13]. Aldosterone is synthesized from deoxycorticosterone by a mitochondrial cytochrome P450 enzyme, aldosterone synthase (*CYP11B2*) [14] catalyze the final steps of the glucocorticoid and aldosterone biosynthesis pathways [15]. The *CYP11B2* gene encodes a steroid 11/18-beta-hydroxylase that functions in mitochondria in the zona glomerulosa of the adrenal cortex to synthesize the mineralocorticoid aldosterone and its expression is regulated by angiotensin II and potassium [16]. The *CYP11B2* gene located on chromosome 8q22 [17-19] and contains 9 exons and 8 introns [20].

The -344 (C>T; rs1799998) variant is a commonly reported polymorphism of the *CYP11B2* gene, which is located at a putative binding site for the steroidogenic transcription factor (SF-1) of the promoter region and involves a cytosine to thymidine substitution [21]. The -344 (C>T) polymorphism is associated with serum aldosterone level and production [22-24], blood pressure [19,25-27], left ventricular size and mass [18,28], Ischaemic Stroke [7]. Its association was reported with progression of renal function [29-31] and ESRD [6]. However, there were few studies from India have been found on the association of *CYP11B2* -344C/T polymorphism and CRI [5], T2DM [9] healthy volunteers [32]; hypertension [33,34], high-altitude adaptation [35].

Therefore, the aim of this study is to evaluate the relation between

*CYP11B2* polymorphism and Type 2 Diabetic Nephropathy on Hemodialysis patients in Indian population and the genotype and allele frequency of *CYP11B2* gene (-344 C>T) promoter polymorphism.

### Materials and Methods

This is a cross-sectional case control study. Ethical committee clearance was obtained from the respective medical institutions prior to the recruitment of subjects in this study. An informed consent was obtained from all the participants prior to their recruitment for the study.

### Subjects

The study participant were 84 type 2 diabetic nephropathy on hemodialysis (T2DNH), 122 type 2 diabetes patients without nephropathy (T2DM) and 118 healthy controls (H.CON). The detection of Type 2 diabetic and nephropathy patients was based on physician's recommendation, registered patient for dialysis and a detailed medical history of each patient was recorded accordingly. The healthy unrelated controls were randomly selected and recruited from local community centers. Subjects for the study were recruited at the participating medical institutions namely Calcutta Medical College (Kolkata), B.P. Poddar Hospital and research Centre (Kolkata).

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## Biochemical analysis

10 ml venous blood was collected from each individual included in the study for biochemical (5 ml) and genetic analysis (5 ml). Biochemical analyses to determine Total Cholesterol (mg/dl), Triglyceride (mg/dl), HDL Cholesterol (mg/dl), LDL Cholesterol (mg/dl), Creatinine (mg/dl), BUN (mg/dl), Uric Acid (mg/dl), Total Protein (g/dl), Albumin (g/dl), Chloride by using automated analyzer (EM 360, TRANSASIA).

## Molecular analysis of the aldosterone synthase (CYP11B2) gene

Approximately 5 ml of venous blood was drawn from each of the subjects in EDTA vials and genomic DNA was extracted from whole fresh blood using standard salting out method using phenol-chloroform [36]. The CYP11B2 (-344 C>T) polymorphism was identified by PCR-RFLP method. Subjects were genotyped for the (-344 C>T) promoter polymorphism using primers CAGGAGGAGACCCCATGTGA (sense) and CCTCCACCCTGTTTCAGCCC (antisense). Standard PCR amplification was performed in a final volume of 10  $\mu$ l reaction mixture containing 50 ng of genomic DNA, 20 pmol of each primer, 10X Taq PCR buffer, 25 mM MgCl<sub>2</sub>, 100 mM of each dNTPs and 0.5 U/ $\mu$ l of Red Taq polymerase. PCR amplification was performed in a DNA thermo cycler (Gene Amp PCR 9700 - Applied Biosystems, USA). PCR was carried out with a Gradient standardize PCR condition with an initial denaturing time at 95°C for 5 min. Then the DNA was amplified for 35 cycles with denaturation at 94°C for 1 min, annealing at 69°C for 1:30 min and extension at 72°C for 1:30 min and final extension 72°C for 10 min. The PCR products were checked by 1% agarose gel electrophoresis with ethidium bromide staining and directly visualized in UV light.

Restriction fragment length polymorphism analysis (RFLP) was performed by adding 5 U of restriction endonuclease *Hae*III (Fermentas) in the appropriate buffer to 4.5  $\mu$ l of amplified 541 bp PCR product and by incubating at 37°C for 3 h and 30 min. Electrophoresis of the digested samples were done in 2.5% agarose gel with ethidium bromide stained, and analyzed under UV light. The C alleles are detected as fragments of 202 bp and the T alleles as fragments of 273 bp plus smaller fragments (138 bp, 125 bp, and 71 bp) in each case (Figure 1).

## Statistical analysis

Data were analyzed using statistical package for Social Sciences statistical software (SPSS Version 16, Chicago, Illinois, USA). Data were expressed as mean and SD (continuous variables) or percentage. Statistical differences between groups were assessed by analysis of variance (ANOVA) or t test. Genotypes and allele frequencies of CYP11B2 gene polymorphism were compared between type 2 diabetic

patients (T2DM), type 2 diabetic nephropathy on hemodialysis patients (T2DNH) and healthy match controls using  $\chi^2$ -test (MINITAB 11). Allele frequencies were calculated for the SNP and tested for Hardy-Weinberg equilibrium and allelic association with disease (exact tests, model tests) using PLINK (<http://pnuu.mgh.harvard.edu/~purcell/plink/>). For comparing the allelic distributions between study groups the odds ratio (OR) with 95% confidence interval (CI) were also calculated. A level of  $p < 0.05$  was assumed statistical significance.

## Results and Discussion

In this cross sectional case control study, 84 T2DNH subjects were compared with 122 T2DM subjects and 118 healthy control subjects. For the control subjects consisted of 61.9 % male and 38.1% female and the T2DNH and T2DM cases 53.6%, 52.5% male subject and 46.4%, 47.5% female respectively. Mean age for the case samples was higher than control samples.

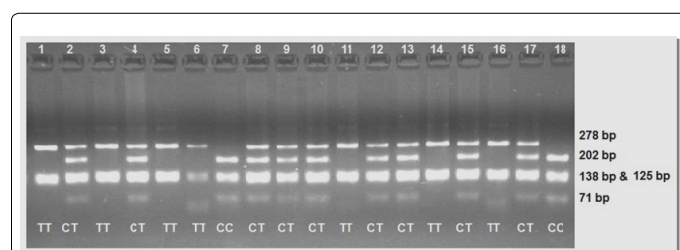
## Clinical characteristics of all subjects

The descriptive, ANOVA and Post Hoc tests had been done in order to find the means, SD, mean differences and significant difference of the base line clinical characteristics. Distribution of base line Clinical characteristics of the CONTROL, T2DM, and T2DNH are shown in Table 1 and mean differences of multiple Comparisons are shown in Table 2. There was a significant difference in all (age, cholesterol, triglycerides, HDL, creatinine, BUN, Uric acid, total protein, Albumin, SBP, DBP) variables except LDL Cholesterol among the study groups. In the analysis of multiple comparisons for mean difference of different groups shows a different picture. Further, we compare between Control and T2DM, T2DNH and T2DM and T2DNH. Age, cholesterol, triglycerides, HDL, BUN, Uric acid, total protein, Albumin, SBP were significant while LDL, creatinine and DBP were not significant when compared between control and T2DM. Age, HDL, creatinine, BUN, Uric acid, total protein, Albumin, SBP, DBP were significant while cholesterol, triglycerides, LDL were not significant when compared between control and T2DNH. Creatinine, BUN, total protein, albumin, SBP was significant while age, cholesterol, triglycerides, HDL, LDL, Uric acid and DBP were not significant when compared between T2DM and T2DNH. Among the T2DNH case, the significant difference of SBP showed that increase in SBP was likely to develop T2DNH and supports the findings of [8,31,37] as they were all reported that the association of (-344 C>T) polymorphism and hypertensive renal failure population. Table 3 shows the comparison of baseline clinical and biochemical characteristics of T2DNH opatients according to genotype of CYP11B2 gene promoter polymorphism. We could not observed significant different between three genotypes.

In this study, older age can be said to be more susceptible to get T2DM and T2DNH as the means and standard deviation of T2DM and T2DNH case samples showed higher value ( $54.90 \pm 11.28$  and  $53.97 \pm 8.91$ ) than control ( $48.16 \pm 7.28$ ).

## Genotypic and allele frequency

Our study was designed to test the hypothesis that the prevalence of type 2 diabetic nephropathy may be influenced by the aldosterone synthase (CYP11B2) gene polymorphism of the RAAS. Figure 1 shows the PCR-RFLP digestion product size in agarose gel electrophoresis stained with ethidium bromide of CYP11B2 gene (-344 T>C) polymorphism. Genotype and allele frequencies for the polymorphism of CYP11B2 in T2DNH, T2DM and Healthy controls are presented in Table 4. The frequencies of CYP11B2 gene CC, CT, TT genotypes among the T2DNH are 16.67 %, 51.19 %, 32.14 %; in T2DM patient



[Lane 1, 3, 5, 6, 11, 14, 16 represent TT genotype; lane 7 & 18: CC genotype and lane 2, 4, 8, 9, 10, 12, 13, 15, 17: CT genotype]

**Figure 1:** Agarose gel electrophoresis stained with ethidium bromide, showing the PCR- RFLP digestion for CYP11B2 (-344 T > C) polymorphism.

10.66 %, 52.46 %, 36.88 % and 16.95%, 38.14%, 44.91% in controls respectively. In our study, we could not observe significant differences in both genotype ( $\chi^2=7.289$ ,  $p=0.121$ ) and allele frequency ( $X^2=1.82$ ,

$p=0.403$ ) of the *CYP11B2* gene (-344 T>C) polymorphism between T2DNH, T2DM patients and Controls subjects. The CT genotype was the highest percentage among T2DNH and T2DM subject (51.19%,

Variables	Control (n=118)	T2DM (n=122)	T2DMH (n=84)	F	p
	Mean ± SD	Mean ± SD	Mean ± SD		
Age (years)	48.16 ± 7.29	54.90 ± 11.29	53.98 ± 8.91	17.516	0.000*
Cholesterol (mg/dl)	159.71 ± 35.87	179.90 ± 42.10	164.09 ± 50.58	7.396	0.001*
Triglycerides (mg/dl)	151.38 ± 74.09	183.09 ± 88.64	155.81 ± 68.27	5.579	0.004*
HDL Cholesterol (mg/dl)	41.45 ± 15.00	50.75 ± 17.39	50.45 ± 16.20	11.99	0.000*
LDL Cholesterol (mg/dl)	96.10 ± 23.56	97.41 ± 30.28	94.80 ± 31.15	0.216	0.806
Creatinine (mg/dl)	0.97 ± 0.14	1.25 ± 0.51	1.72 ± 1.81	14.623	0.000*
Bun (mg/dl)	7.74 ± 2.68	13.30 ± 6.65	27.45 ± 18.69	89.365	0.000*
Uric acid (mg/dl)	5.72 ± 1.39	5.80 ± 1.20	6.32 ± 1.62	5.151	0.006*
Total protein (g/dl)	6.99 ± 0.51	8.08 ± 1.07	7.61 ± 1.16	41.487	0.000*
Albumin (g/dl)	3.87 ± 0.23	4.40 ± 0.55	4.12 ± 0.95	22.765	0.000*
Systolic blood pressure (mmHg)	123.57 ± 21.00	137.19 ± 20.11	151.49 ± 25.95	39.557	0.000*
Diastolic blood pressure (mmHg)	82.89 ± 10.68	85.02 ± 10.11	88.08 ± 13.76	5.128	0.006*

\*Significant at the  $P<0.05$  level

Table 1: Distribution of Clinical and Biochemical characteristics of Healthy Control, T2DM and T2DNH patients.

Variables	Group (I)	Group (J)	Mean Difference (I-J)	p
Age (years)	Control	T2DM	6.7406	0.000*
		T2DNH	5.8152	0.000*
	T2DM	T2DNH	0.9254	0.785
Cholesterol (mg/dl)	Control	T2DM	20.1903	0.001*
		T2DNH	4.3791	0.770
	T2DM	T2DNH	15.8112	0.033*
Triglycerides (mg/dl)	Control	T2DM	31.7099	0.008*
		T2DNH	4.4299	0.925
	T2DM	T2DNH	27.2800	0.051
HDL Cholesterol (mg/dl)	Control	T2DM	9.3038	0.000*
		T2DNH	9.0020	0.001*
	T2DM	T2DNH	0.3018	0.991
LDL Cholesterol (mg/dl)	Control	T2DM	1.3159	0.937
		T2DNH	1.2974	0.950
	T2DM	T2DNH	2.6133	0.809
Creatinine (mg/dl)	Control	T2DM	0.2742	0.095
		T2DNH	0.7507	0.000*
	T2DM	T2DNH	0.4765	0.003*
Bun (mg/dl)	Control	T2DM	5.5620	0.000*
		T2DNH	19.7090	0.000*
	T2DM	T2DNH	14.1470	0.000*
Uric acid (mg/dl)	Control	T2DM	0.0883	0.886
		T2DNH	0.6033	0.010*
	T2DM	T2DNH	0.5150	0.034
Total protein (g/dl)	Control	T2DM	1.0970	0.000*
		T2DNH	0.6177	0.000*
	T2DM	T2DNH	0.4792	0.002*
Albumin (g/dl)	Control	T2DM	0.5270	0.000*
		T2DNH	0.2471	0.018*
	T2DM	T2DNH	0.2799	0.005*
Systolic blood pressure (mmHg)	Control	T2DM	13.6207	0.000*
		T2DNH	27.9203	0.000*
	T2DM	T2DNH	14.2996	0.000*
Diastolic blood pressure (mmHg)	Control	T2DM	2.1348	0.348
		T2DNH	5.1935	0.006*
	T2DM	T2DNH	3.0587	0.166

\*The mean difference is significant at the 0.05 level

Table 2: Multiple Comparisons for Mean difference of different groups.

Variables	CC (N=14)	CT (N=43)	TT (N=27)	F	p
	Mean ± SD	Mean ± SD	Mean ± SD		
Age (years)	57.14 ± 9.6	54.67 ± 10.11	51.22 ± 5.34	2.38	0.099
Cholesterol (mg/dl)	166.85 ± 46.29	162.42 ± 50.04	165.32 ± 55.15	0.051	0.950
Triglycerides (mg/dl)	164.05 ± 66.91	143.95 ± 59.04	170.43 ± 80.74	1.382	0.257
HDL Cholesterol (mg/dl)	45.94 ± 16.59	52.35 ± 15.79	49.76 ± 16.73	0.861	0.423
LDL Cholesterol (mg/dl)	93.11 ± 27.12	94.18 ± 29.24	96.67 ± 36.66	0.076	0.927
Creatinine (mg/dl)	1.44 ± 1.37	1.47 ± 1.38	2.27 ± 2.44	1.862	0.162
Bun (mg/dl)	25.9 ± 17	25.79 ± 18.09	30.87 ± 20.59	0.662	0.519
Uric acid (mg/dl)	7.13 ± 1.26	6.13 ± 1.49	6.2 ± 1.9	2.174	0.120
Total protein (g/dl)	7.94 ± 1.27	7.7 ± 1.15	7.285 ± 1.1	1.774	0.176
Albumin (g/dl)	4.19 ± 0.99	4.19 ± 0.88	3.97 ± 1.04	0.474	0.624
Systolic blood pressure (mmHg)	153.14 ± 20.87	148.7 ± 28.52	155.07 ± 24.29	0.529	0.591
Diastolic blood pressure (mmHg)	92 ± 15.18	87.86 ± 14.37	86.41 ± 12.02	0.769	0.467

**Table 3:** Comparison of clinical and Biochemical characteristics of type 2 diabetic nephropathy on dialysis Patients according to CYP11B2 gene polymorphism.

Group Studied	Genotypes			Allele frequencies	
	CC	CT	TT	C	T
Control(N=118)	20 (16.95)	45 (38.14)	53 (44.91)	85 (36.02)	151 (63.98)
T2DM(N=122)	13 (10.66)	64 (52.46)	45 (36.88)	90 (36.88)	154 (63.12)
T2DNH (N=84)	14 (16.67)	43 (51.19)	27 (32.14)	71 (42.26)	97 (57.74)
X <sup>2</sup> = 7.289 , P= 0.121			X <sup>2</sup> = 1.82 , P= 0.403		

**Table 4:** Genotype and Allele Frequencies of the CYP11B2 (-344 C >T) Polymorphism in Controls, T2DM and T2DNH Patients

Study Group	MAF	General Association					HWE P	Binary logistic regression					
		Group	MAF	X <sup>2</sup>	p	OR (95% CI)		Additive		Dominant		Recessive	
								P	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)
Total study Samples	0.379	Aff	0.39	0.597	0.439	1.139 (0.818 - 1.587)	0.241	0.44	1.14	0.08	1.51	0.35	0.73
		U Aff	0.36						0.82 - 1.59		0.96 - 2.41		0.39 - 1.39
T2DM & CON	0.379	Aff	0.37	0.039	0.843	1.038 (0.716 - 1.506)	0.823	0.85	1.04	0.21	1.39	0.16	0.58
		U Aff	0.36						0.72 - 1.49		0.83 - 2.34		0.27 - 1.24
T2DNH & CON	0.386	Aff	0.42	1.615	0.204	1.3 (0.87 - 1.95)	0.823	0.22	1.28	0.07	1.72	0.96	0.98
		U Aff	0.36						0.86 - 1.88		0.96 - 3.09		0.46 - 2.07

MAF: Minor Allele Frequency; Aff = Affected; U Aff: Un-affected

**Table 5:** Summary statistical analysis of SNP (-344 C >T; rs1799998) of CYP11B2 gene by General association, HWE and binary logistic regression.

52.46 % respectively) than the control subjects (38.14%). The CC genotype was almost same percentage among the T2DNH and Control subject (16.67%, 16.95% respectively) and the TT genotype was the highest among the control (44.91%). From the Table 5 it is evident that no significant association was observed for all the study groups with affected and non-affected MAF through Plink association test. From the analysis of HWE, it was found that the SNP (C -344 T) was in Hardy - Weinberg equilibrium. The association was further verified through 3 model - additive, dominant and recessive models to confirm which of these 3 models best predict the association at micro level. On the whole it was observed that dominant model more predictable than additive and recessive model, but there are no significant association in these 3 models.

Several studies of the association between this polymorphism and hypertension [10,27,38,39] left ventricle size and mass [18,40] and myocardial infarction [28,37] in the general population and hypertensive individuals with normal renal function have been performed. Various studies had reported the CYP11B2 gene polymorphism associated with CRI [5]; renal insufficiency in the hypertensive population [10,31] and [30] reported that there was no association between the CYP11B2

genotype and progression of renal failure among the diabetic ESRD patients.

In ESRD patients, however, studies of association of CYP11B2 -344C>T polymorphism and left ventricular hypertrophy and cardiovascular morbidity are few. It is also possible that the type 2 diabetic patients with high risk genotype may be excluded from the present study because of premature mortality due to cardiovascular influences by CYP11B2 polymorphism. Thus, further prospective investigation is needed to explore the role of CYP11B2 polymorphism in the susceptibility of diabetes and cardiovascular effect in type 2 diabetic patients.

## Conclusion

Our findings do not support the hypothesis that CYP11B2 polymorphism is associated with prevalence of Type 2 Diabetes and diabetic nephropathy patients in Indian populations. The minor T allele of CYP11B2 gene polymorphism is not associated with T2DNH in Indian subjects. Therefore, CYP11B2 gene (-344 C>T) polymorphism may not be a genetic marker and might not be considered as a genetic risk factor for Indian Type 2 Diabetic nephropathy patients.

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