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Research Article

STUDY OF ASSOCIATION OF *CYP11B2* C-344T GENE POLYMORPHISM WITH HYPERTENSION AND TYPE 2 DIABETES MELLITUS IN THE POPULACE OF J&K STATE

SHARMA MINAKASHEE[1], RAINA JYOTDEEP KOUR[1], BHAGAT MEENAKSHI[2], PANJALIYA RAKESH KUMAR[1], SETHI SURBHI[1] AND KUMAR PARVINDER*[1,2]

[1]Human Genetics Research cum Counselling Centre, University of Jammu, 180006, India
[2]Department of Zoology, University of Jammu, 180006, India
*Corresponding Author: Email- parvinderkb2003@rediffmail.com

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Abstract- Background: The polymorphisms of the Renin- angiotensin -aldosterone system have been extensively studied in various ethnic groups of different populations, but the results are not consistent in reference to its relationship with the risk of developing hypertension (HTN) and type 2 diabetes mellitus (T2DM). The present work was undertaken to assess the role of *CYP11B2C-344T* polymorphism in individual development of HTN and T2DM as well as their co-existence.
Material and Method: A case-control association study was conducted consisting of 200 cases (HTN: 89 hypertensives, HTN+T2DM: 67 with coexisting hypertension and type 2 diabetes mellitus, T2DM: 44 type 2 diabetics) and 100 randomly selected healthy controls. Genotyping was performed by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) technique. An ethically approved questionnaire was used to assess risk factors of HTN and T2DM followed by physical and biochemical examination with measurement of height weight, blood pressure, blood sugar and lipid profile.
Results: A significant difference was observed in non-genetic risk factors such as smoking, physical inactivity, alcohol intake, mental stress, anthropometry, fasting glucose levels and lipid parameters among all the patient groups in comparison to healthy controls. Overall, there was higher prevalence of variant T-allele in the studied population in comparison to wild C-allele. The frequency of T-allele was almost comparable in all the patient groups (HTN=0.61; HTN+T2DM=0.57; T2DM=0.51) and in controls (0.64). Logistic regression analysis of C vs T allele did not reveal any significant association of this polymorphism with the risk of HTN [OR=0.984, 95% CI (0.65-1.49); p=0.94], combined HTN & T2DM [OR=8.856, 95% CI (0.549-1.334); p=0.49] or T2DM alone [OR=0.683, 95% CI (0.412-1.132); p= 0.14].
Conclusion: Our findings did not support association of *CYP11B2C*-344T gene polymorphism with susceptibility of HTN and T2DM or their co-existence in populace of Jammu and Kashmir (J&K) state.

Keywords- *RAAS, Hypertension, Type 2 diabetes mellitus, CYP11B2, Hae III, RFLP.*

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Introduction

Hypertension (HTN) and type 2 diabetes (T2DM) are coexisting clinical conditions that are becoming increasingly common [1]. The existence of HTN causes a 7.2 times increase in mortality in diabetic patients [2]. Both HTN and T2DM are polygenic in nature and share common gene pool and non-genetic risk factors such as obesity, high caloric dietary intake, smoking, alcohol consumption, sedentary life style and stressful life [3].

Renin-angiotensin-aldosterone System (RAAS) is the utmost physiological pathway that helps in monitoring sodium homeostasis, blood pressure and insulin secretion & sensitivity. The genes operating RAAS pathway has been the foremost target for molecular analysis in evaluating the genetic predisposition for the development of HTN and T2DM [4]. There is a strong association of up regulation of RAAS with HTN and T2DM [5-6]. Of the known molecular variants of RAAS, the promoter region C-344 C>T (Rs 1799998) variation of the aldosterone Synthase gene (*CYP11B2*) is the most studied polymorphism involving the substitution of cytosine to thymidine and is believed to be a binding site for the steroidogenic transcription factor SF1 [7]. Aldosterone synthase gene positioned on the chromosome 8q22 [8] consists of 9 exons and 8 introns [9] This gene encodes an Aldosterone Synthase enzyme that participates in the biosynthesis of aldosterone, a hormone secreted by the adrenal cortex, which maintain saline and fluid balance in the body [10]. The functional implication of this polymorphism is linked to increased serum aldosterone levels [11] and glucose intolerance resulting in pathophysiological consequences like HTN [10] and DM [12].

A number of association studies regarding this polymorphism conducted on the different populations with different ethnic background revealed the contradictory results. Some studies reported the association of the C allele [13] while other studies claimed that the T allele is responsible for high levels of plasma aldosterone, HTN and DM [14-15]. However, no association of either allele with complex diseases has also been reported in some populations [16-17]. Not many studies have been carried out on Indian population to evaluate the association of this polymorphism with HTN and T2DM. Therefore, the present study was conducted with the aim to investigate the linkage of *CYP11B2C*-344T polymorphism with genetic predisposition to HTN and T2DM as well as their co-morbidity in the North Indian populace of Jammu region of the J&K State.

Materials and Methods

Ethics: The study design was approved by the Animal and Human.

Experimentation Ethical Committee (AHEEC), University of Jammu, Jammu and a proper written informed consent was taken from each subject prior to their recruitment for the study.

Study Area & Population

A total of 200 patients and 100 healthy controls were recruited for the present study. The patients were enrolled from the Outpatient Department of Super-specialty Hospital, Govt. Medical College, Jammu and Acharaya Shri Chander College of Medical Sciences and Hospital (ASCOMS), Sidhra, Jammu. The cases were divided into three groups: First group consisted of 89 individuals with essential hypertension (HTN) without T2DM or any other disease, the second group consisted of 67 individuals with both HTN and T2DM (HTN+T2DM) and a third group consisted of 44 individuals with T2DM without a history of HTN (T2DM). The unrelated healthy control individuals formed the fourth group, attending health camps and OPD with minor ailments without the previous history of HTN, DM, cardiovascular disease or dyslipidemia. A detailed information regarding the socio-demographic variables (age, gender), smoking status, onset and duration of disease, dietary pattern, salt consumption, anthropometric parameters and biochemical profiling was recorded from each studied participant in a pre-designed questionnaire.

Inclusion and Exclusion criteria

Hypertensive subjects were defined as per criterion given by JNC7: systolic blood pressure (SBP) of ≥140 mm Hg and/or diastolic blood pressure (DBP) of ≥90 mm Hg, or those currently receiving anti-hypertensive therapy for more than three months [18]. Individuals with T2DM were diagnosed on the basis of WHO recommendations, which classified DM individuals as those with fasting glucose (FG) greater than or equal to 126mg/dl or those receiving anti-diabetic agents [19]. Patients suffering from cardiovascular ailments, thyroid abnormalities, type 1diabetes and renal diseases were excluded.

Genotyping of *CYP11B2* C-344T polymorphism: 2-5ml of venous blood was collected in EDTA coated vials and stored at -20ºC. The DNA was isolated by phenol-chloroform (organic) method Sambrook and Russel [20] with minor modifications. The *CYP11B2*C-344T polymorphism was identified by PCR-RFLP method. The subjects were genotyped for (-344 C>T) promoter polymorphism using primer sequences:

Forward primer: *5'CAG GAG GAG ACC CCA TGT GAC3'*

Reverse primer: *5 'CCT CCA CCC TGT TCA GCC C 3'*

PCR was performed in thermal cycler of Applied Biosystems by life technology (Make Veriti) Singapore, using 25µl of reaction mixture containing 2 µl DNA, 5 µl flexi buffer, 0.5 µl dNTPs (10Mm), 0.3 µl Taq (1U/µl), 2.5ul $MgCl_2$ (25Mm), 0.5 µl each primers (100 uM/ ul), and 13.7 µl PCR water to make up the final volume of 25µl. The conditions used for the amplification were as follows: Initial denaturation at 94˚C for 5 min followed by 35 cycles of denaturation at 94˚C for 1 min, primer annealing at 67˚C for 1 min, extension at 72˚C for 1 min, at last final extension at 72˚C for 10 minutes. The amplified PCR products of 538bp were checked using 1.5% agarose gel electrophoresis [Fig-I] followed by restriction digestion with *HaeIII* restriction enzyme (New England Biolabs) by incubating the PCR product overnight at 37°C. The digested fragments were separated on 3.5% agarose gel and 274bp, 203bp, 138bp, 126bp, 71bp bands were obtained for heterozygous variant, 203bp, 138bp, 126bp, 71bp for wild type and 274bp, 138bp, 126bp for homozygous variant [Fig-II].

Statistical Analysis: Statistical analysis of crude datum was done using the statistical package for social sciences (SPSS version 21). Non-genetic variables were represented as mean and standard deviation. Statistical differences between the groups were calculated using student's unpaired t-test and one way annova. Genotype and allele frequencies were calculated by the gene counting method followed by Hardy-Weinberg calculations using chi-square analysis. The association of *CYP11B2*C-344T polymorphism with each of the three diseased groups was analyzed by calculating the odds ratio (OR) with 95% confidence interval (CI). A p-value <0.05 is considered as statistically significant.

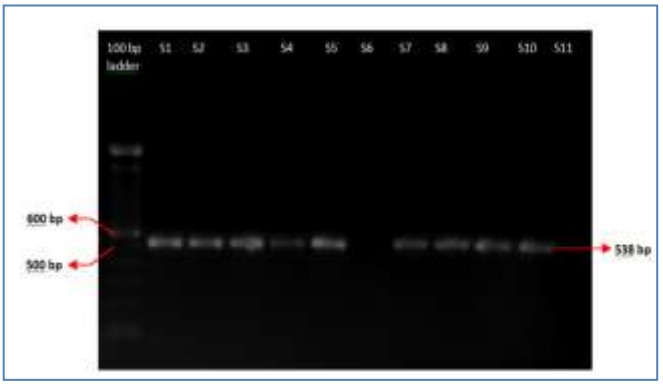

Fig-I Agarose gel image showing a 538bp *CYP11B2* PCR product in samples (S1-S11)

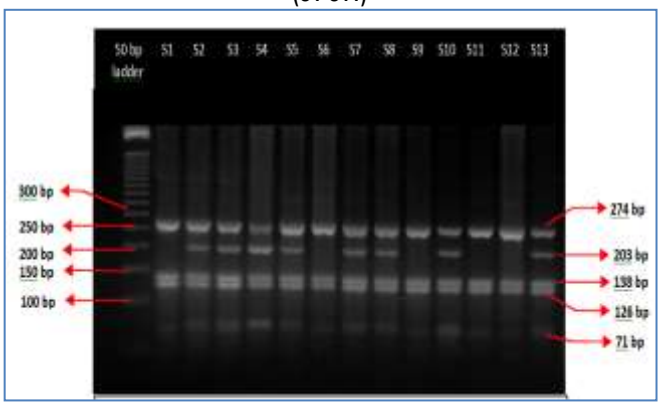

Fig-II Agarose gel image showing RFLP of *CYP11B2* C-3344T with *Hae III* enzyme
Sample 1, 6, 9, 11, 12 are mutant (TT)
Sample 2, 3, 4, 5, 7, 8, 10, 13 are heterozygous (CT)

Results

Non-genetic characteristics of the study subjects: The baseline characteristics, including personal, behavioral, dietary and clinical/metabolic risk factors of the study participants were described in the [Table-I and II]. The control subjects (n=100) consisted of 63 males and 37 females, whereas, HTN group (n=89) consisted of 48 males and 41 females, T2DM+HTN group (n=67) comprised of 43 males and 24 females and T2DM group (n=44) contained 30 males and 14 females. Total mean age of patients on the whole was 56.92±12.59 years and controls were 40.14±11.48 years. The mean duration of disease was higher in patients with hypertensive and diabetic co-morbidity (8.25± 15.12 and 8 ± 7.07) followed by patients with T2DM (7.62± 5.87) and essential hypertension (4.57 ± 4.62). Smoking, alcoholism, physical inactivity and stress appeared to have a significant risk associated with the diseased profile. The percentage of smokers was significantly higher in the diabetic group (p=0. 002) in comparison to other study groups. Alcohol intake was showing significantly higher risk of developing HTN+T2DM co-morbid (OR=3.2; p= 0.002) condition and isolated T2DM (OR=2.87; p= 0.01) than essential hypertension (OR=2.02, p=0.06). The prevalence of sedentary lifestyle was high in all the three disease categories with respect to controls (64.04%, 68.7%, 61.4% vs 33%). Stress had been implicated as a major predictor of HTN and T2DM in the present study (74.2% for HTN, 72.7% for T2DM and 76.1% for HTN+T2DM). The analysis of variance study (ANNOVA) [Table-II] comparing mean values of physiometric (SBP, DBP, PP and PR), metabolic (LDL, HDL, TC, TG and glucose) and anthropometric (BMI and WHR) among the four studied groups revealed highly significant (p = 0.0000) mean difference. This suggests that one or more groups (HTN, HN+T2DM, T2DM or controls) are different from other groups.

Genotype and Allele Frequency: The present study was designed to check the influence of *CYP11B2* (-344C>T) polymorphism in the HTN, HTN+ T2DM and T2DM patients. The PCR-RFLP image of *CYP11B2* was shown in [Fig-I and II].

Genotypic and allelic distribution of patients (HTN, T2DM and HTN+ T2DM) and controls was depicted in [Fig-III and IV]. However, the overall frequency of wild (C) and mutant (T) allele was 0.43 and 0.57 in patients and 0.36 and 0.64 in controls, respectively [Fig-IV]. The genotype frequency of cases and controls was: CC- 20%, CT- 46.5% and TT- 33.5% in all patients and CC- 17%, CT- 45% and TT- 38% in controls [Fig-III]. The genotypic frequencies were in agreement with Hardy Weinberg Equilibrium (cases: χ^2 = 0.5s6, p= 0.46 & controls: χ^2 = 0.34, p= 0.56). The observed frequency of TT genotype as well as T allele was almost alike in both patients and controls so, on the whole, C vs. T model suggested no association of *CYP11B2*C-344T polymorphism with the diseased phenotype [OR= 0.85, 95% CI (0.606-1.210); p= 0.380 (χ^2 = 0.77)]. The frequency of risk T-allele in observed separately in HTN, T2DM and HTN + T2DM group was also similar to the control group. The maximum strength of association was projected by HTN group (OR= 0.984; p= 0.94) followed by HTN+DM group (OR= 0.856; p= 0.49) and DM group (OR= 0.683; p= 0.14) but none of the group reached statistical significance level. Hence, it is clear that there was no significant association of *CYP11B2*C-344T polymorphism in all the three affected groups.

Table-I *Descriptive analysis on non-genetic risk factors for HTN and T2DM*

Parameters	GP1 (n= 89)	GP2 (n=67)	GP3 (n=44)	GP4 (n=100)
Gender *M/F*	48/41 (p=0.2)	43/24 (p= 0.8)	30/14 (p=0.5)	63/37
Age	55.03±14.42 (p<.0001)	58.73± 11.88 (p<.0001)	54.45± 11.32 (p<.0001)	40.14±11.48
Onset of HTN	49.28± 14.44	46.5± 15.72	-	-
Duration of HTN	4.57 ± 4.62	8.25± 15.12	-	-
Onset of DM	-	50.46± 10.81	48.43± 11.92	
Duration of DM	-	8 ± 7.07	7.62± 5.87	-
Smokers Non- smokers	30 (33.7%) 59 (66.3%) (OR: 2.03, p= 0.03)	17 (25.4%) 50 (74.6%) (OR: 1.36, p=0.41)	24 (54.5%) 20 (45.5%) (OR : 3.3, p=0.002)	20 (20%) 80 (80%)
Duration of Smoking (yrs.)	18.45±10.48 (p<0.0001)	16.59± 7.59 (p<0.0001)	16.5± 7.37 (p<0.0001)	6.4± 5.88
Alcoholics Non- Alcoholics	22 (24.7%) 67 (75.3%) (OR: 2.02, P=0.06)	23 (34.3%) 44 (65.7%) (OR: 3.2, p=0.002)	14 (31.8%) 30 (68.2%) (OR: 2.87, p=0.01)	14 (14%) 86 (86%)
Duration of alcohol intake (yrs.)	16.8± 7.07 (p=0.001)	16.73± 6.61 (p=0.004)	17.14± 6.19 (p=0.01)	11.43± 14.08
Stress *Yes* *No*	66 (74.2%) 23 (25.8%) (OR: 5.33, p<0.0001)	51 (76.1%) 16 (23.9%) (OR: 5.92, p<0.0001)	32 (72.7%) 12 (27.3%) (OR: 4.95, p=.0001)	35 (35%) 65 (65%)
Physical activity Sedentary	32 (35.96%) 57 (64.04%) (OR: 3.62, p<.0001)	21 (31.3%) 46 (68.7%) (OR:4.44, p<.0001)	17 (38.6%) 27 (61.4%) (OR: 3.2, p=0.001)	67 (67%) 33 (33%)
Dietary pattern *Vegetarian* *Non Vegetarian*	35 (39.3%) 54 (60.7%) (OR: 0.7, p=0.3)	31 (46.3%) 36 (53.7%) (OR: 0.5, p=0.08)	21 (47.7%) 23 (52.3%) (OR: 0.5, p=0.09)	33 (33%) 67 (67%)
Excessive Salt consumption	37 (41.3%) (OR:4.4, p= 0.0001)	25 (37.3%) (OR:3.9, p= 0.0008)	10 (22.7%) (OR:1.9, p=0.1)	13 (65%)

Abbreviations- GP: Group, M: Male, F: Female, OR: Odds Ratio, p: probability value

Table-II *Descriptive analysis of physiometric, biochemical and anthropometric variables among study participants*

Variables	HTN Mean± SD	HTN+DM Mean± SD	DM Mean± SD	Control Mean± SD	F-value	p-value
SBP	148.58± 19.10	145.09± 21.45	122.1± 8.14	120.92±9.91	66.206	0.0000
DBP	56.71± 16.46	91.78± 15.17	80.55± 4.85	79.24±8.95	34.545	0.0000
Pulse Pressure (PP)	56.71± 16.46	53.31± 22.53	50.66± 15.42	41.68± 7.81	15.659	0.0000
Pulse rate (PR)	82.51± 8.89	82.40±10.77	78.14± 5.70	73.23±3.21	30.214	0.0000
LDL	135.80± 68.80	111.27± 62.31	161.45±111.93	76.86± 21.18	21.977	0.0000
HDL	36.64± 11.09	35.56± 8.34	36.21± 10.16	51.22± 7.37	59.121	0.0000
TC	194.05± 69.46	173.52± 40.05	186.72± 70.43	127.89± 33.20	27.523	0.0000
TG	218.68± 86.76	190.0± 69.76	197.0± 83.22	121.12± 26.97	36.141	0.0000
Glucose (fasting)	103.3± 34.11	162.98± 37.62	162.98± 37.62	95.17± 14.17	111.330	0.0000
BMI	24.57±5.86	26.07±5.89	25.50±3.13	22.45±4.07	8.319	0.0000
WHR	0.98±0.08	0.99±0.09	1.01±0.08	0.95±0.06	7.582	0.0000

Abbreviations: SD: Standard deviations, SBP: Systolic blood pressure, DBP: Diastolic blood pressure, LDL: Low density lipoprotein, HDL: High density lipoprotein, TC: Total cholesterol, TG: Triglyceride, BMI: Body mass index, WHR: Waist to hip ratio

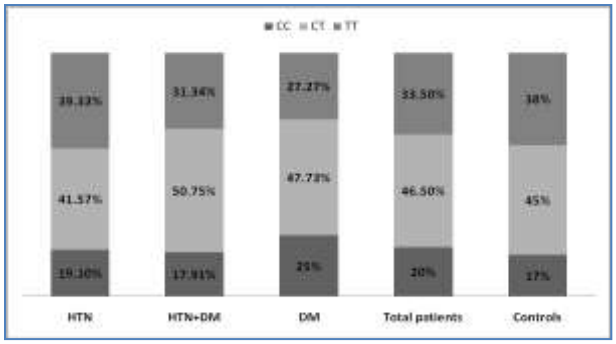

Fig-III Genotypic distribution of *CYP11B2* C-344T polymorphism in study groups.

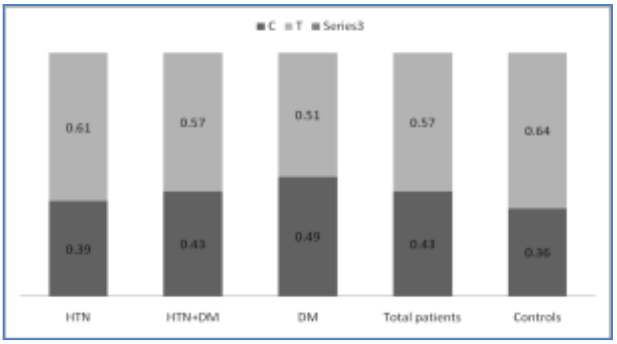

Fig-IV Allele distribution of *CYP11B2* C-344T polymorphism in study groups

Table-III *Genotypic and allelic distribution of patients (HTN, T2DM and HTN+ T2DM) and controls*

Grouping	Genotypes / alleles (*CYP11B2* C-344T)	Patient	Control	OR	95% CI	p-Value
GP4 (n=100)	*CYP11B2* CC	17	17	**1(Reference)**		
	CYP11B2 CT	37	45	0.82	0.37-1.83	0.63
Vs	*CYP11B2* TT	35	38	0.92	0.41-2.08	0.84
	CYP11B2 CT+TT (D)	72	83	0.87	0.41-1.82	0.70
GP1 (n=89)	*CYP11B2* CT+CC (R)	54	62	1.06	0.59-1.50	0.85
	CYP11B2 C	0.39	0.36	**1(Reference)**		
	CYP11B2 T	0.61	0.64	0.984	0.65-1.49	0.94
GP4 (n=100)	*CYP11B2* CC	12	17	**1(Reference)**		
	CYP11B2 CT	34	45	1.070	0.45-2.54	0.88
Vs	*CYP11B2* TT	21	38	0.78	0.315-1.95	0.59
	CYP11B2 CT+TT(D)	55	83	0.94	0.42-2.1	0.88
GP2 (n=67)	*CYP11B2* CT+CC (R)	46	62	0.74	0.39- 1.43	0.37
	CYP11B2 C	0.43	0.36	**1(Reference)**		
	CYP11B2 T	0.57	0.64	0.856	0.549-1.334	0.49
GP4 (n=100)	*CYP11B2* CC	11	17	**1(Reference)**		
	CYP11B2 CT	21	45	0.72	0.29-1.81	0.48
Vs	*CYP11B2* TT	12	38	0.49	0.18-1.32	0.155
	CYP11B2 CT+TT(D)	33	83	0.61	0.26-1.45	0.263
GP3 (n=44)	*CYP11B2* CT+CC (R)	32	62	0.61	0.28-1.33	0.21
	CYP11B2 C	0.49	0.36	**1(Reference)**		
	CYP11B2 T	0.51	0.64	0.683	0.412-1.132	0.14

Abbreviations- D: Dominant model; R: Recessive model.

Discussion:

The present study examined the potential interaction between genetic and environmental factors in the etiology of HTN, T2DM and coexisting HTN-T2DM in the North Indian population of Jammu region. Insignificant association was observed in gender and dietary pattern in all the three patient groups. Similar findings were reported by multiple studies from different regions of India [21-22]. The difference of SBP and DBP in diabetics and control are in agreement with those reported by Badr *et al* [23] in Egyptian population. A highly significant difference was observed in systolic blood pressure (SBP), diastolic blood pressure (DBP), pulse rate (PR), body mass index (BMI) and waist hip ratio (WHR) in hypertensive patients with/without diabetes which are in agreement with the findings of Vasudevan *et al* [24] and Ramachandran *et al* [25]. The majority of the patients in our study belonged to advanced age (above 50 years), as the person's predisposition to HTN or T2DM increases with the age [25-26]. A significant difference was observed in biochemical parameters (TC, TG, LDL and HDL) in patient groups when compared to control group in the present study which was consistent with the previous studies [4, 27]. Stress appeared to be a potent risk factor for HTN and T2DM in the present study. These findings were in line with several other studies done by Mooy *et al*, Rasool *et al*, Nehra *et al* and Falco *et al* [28-31]. Among patients, alcoholism was observed as a prominent risk factor for diabetic individual with/without hypertension, which is supported by the previous findings [32-33]. The percentage of smokers was significantly higher in diabetic group, whereas, previous studies on the association between smoking and diabetes reported contradictory results [34-36].

Sedentary lifestyle or lack of physical activity also enhances the risk of HTN and T2DM [23]. The same was observed in the present study. The role of excessive intake of dietary sodium in the development of hypertension was observed in our findings, which were also confirmed by Radhika *et al* [37] and Takase *et al* [38].

The *CYP11B2* C-344T gene polymorphism was found to be associated with elevated plasma aldosterone levels [39], glucose levels and glucose intolerance [40], myocardial infarction [41], hypertension [42] and type 2 diabetes [43]. Several researchers investigated the association of this polymorphism independently with HTN and T2DM [44-45] but, the results are not always universal among the different ethnic groups due to interethnic variations. Though few studies on the linkage of C-334T polymorphism with HTN [14], T2D [46] and ESRD [47] are available from Indian populations, data regarding its impact on combination of HTN and T2D is still missing. In accordance to the present study, *CYP11B2* C-344T gene polymorphism was not observed to be a contributing risk factor for HTN, T2DM or combined HTN+T2DM in population of Jammu region (J&K). The same observations were obtained in Malaysian population [25], Northern Han Chinese population [42] and Japanese population [17]. The present work revealed that the T allele variant might not be the potential risk factor in the causation of HTN and T2DM in our population. Likewise, a study on North Indian population was failed to locate a potential association of the said polymorphism in diabetes [4]. Contradictory to our findings, T-allele of *CYP11B2* C-344T gene polymorphism was found to be positively associated with development of HTN [14] and T2DM [46] in South Indian and Asian Indian populations respectively. Further, possible link between C-allele and essential hypertension was confirmed from various other studies reported from different populations [22, 44, 48].

Conclusion

In conclusion, the present study was in agreement of lack of association of *CYP11B2C*-344T gene polymorphism with HTN and T2DM or their co-existence in the North Indian population of Jammu region (J&K).

Limitations of the study:

(i) The cross-sectional sampling design do not allow inferences to be drawn with respect to casual relationships among the variables

(ii) The sample size is small. Further investigation is needed on a larger sample size to reach to an accurate conclusion.

(iii) All studied variables are age dependent; however, age grouping analysis has not been done.

(iv) Only single polymorphism has been investigated in the present study. More number of Single nucleotide polymorphisms involved in RAAS pathway are needed to better comprehend the genetics of coexisting HTN+T2DM.

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