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Gender-specific Association of *ATP2B1* (*rs2681472*) Gene Polymorphism with Essential Hypertension in South Indian Population

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ABSTRACT Essential hypertension (EH) makes up ~ 95 percent of all hypertensive cases and has no clear identifiable cause. Although EH has a strong genetic basis, identification of genes associated with it has been difficult because of the complexity of regulation of blood pressure, its multifactorial nature, and the presence of multiple susceptibility genes. The present case–control association study investigates the possible involvement of the *ATP2B1 (rs2681472)* polymorphism in EH patients. The study group included 568 hypertensive cases and 604 normotensive controls, whose DNA were genotyped by the PCR-RFLP method. A significant association was observed between the T allele ($p = 0.038$) and the TT genotype ($p = 0.051$) with EH. Gender-wise segregation evidenced a significant association of T allele with EH in female patients ($p = 0.053$) rather than male patients. Hence, it is concluded that females with TT genotype are more susceptible to EH, and T allele could be the risk allele for EH in south India.

INTRODUCTION

Essential hypertension (EH) represents one of the potential risk factors associated with cardiovascular diseases (CVD), posing a major public health problem for socio-economic and epidemiological transition. Hypertension (HT) is the major risk factor contributing to the overall mortality, which accounts for 20–50 percent of all deaths. The global burden of CVD is continuing to rise with simulations by the World Health Organization (WHO) suggesting that the number of individuals with HT will cross 1.5 billion by 2020 (Murray et al. 2002). In India, death due to cardiovascular diseases (CVD) in 1990 reached the figure of 2.3 million deaths, which is projected to double by the year 2020 (Gupta, 2004). In spite of the development of effective antihypertensive therapies, HT remains poorly controlled (Lewington et al. 2002). Treatment of HT at an early stage is associated with a 40 percent reduction in the risk of stroke and a 15 percent reduction in the risk of myocardial infarction (Whitworth 2003). Although 10–25 percent of people achieve the current blood pressure (BP) target, 10 percent of them show resistance to the current treatment modalities (Petersen et al. 2005). This discrepancy in the response is attributed to the fact that the physiological systems involved in BP regulation that have been tested as therapeutic targets have failed. Therefore, new approaches to identify broad-range targets based on an individual's genetic constitution will benefit millions suffering from CVD worldwide (Munroe et al. 2009).

Altered calcium homeostasis plays a vital role in the pathogenesis of EH (Mateus-Hamden et al. 2014). Different cell types of hypertensive subjects have shown increased intracellular Ca^{2+} levels. Plasma membrane calcium ATPase

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(PMCA) represents the most vital Ca^{2+}-ejection system. Any change in the PMCA pump is assumed to increase the contractile tone of small vessels and promote a rise in BP (Benkwitz et al. 1999). In mammals, the PMCAs are encoded by four distinct genes (*ATP2B1–4*) located on the human chromosomal loci 12q21-q23, 3p25-p26, Xq28, and 1q25-q32[7] respectively[7] (Leva et al. 2008). The basic function of PMCA is to maintain a ~10,000-fold Ca^{2+} gradient across the plasma membrane by active extrusion of Ca^{2+} from the cell. The PMCA pump transports one Ca^{2+} ion per molecule of ATP hydrolyzed, and this energy expenditure is necessary to maintain a relatively low intracellular concentration of Ca^{2+}. Unstimulated PMCA pumps have poor affinity for Ca^{2+} and would be inactive at physiological sub-μM cytoplasmic Ca^{2+} concentrations. Calmodulin serves as an activator and regulator of PMCA pump (Jarrett and Penniston 1977). Ca^{2+} influx across the plasma membrane or release from intracellular organelles is required for excitation–contraction coupling and receptor-mediated Ca^{2+} signaling (Hussain and Inesi 1999). A high concentration of intracellular calcium causes endothelial cells to contract, constricting the blood vessel and reducing blood flow. Incidentally, calcium channel blockers are frequently prescribed to lower BP.

In vascular smooth muscle cells (VSMCs), contractility increases due to altered function of Ca^{2+} channels, which is known to be a vital process in the development of EH. Studies of animal models have revealed that Ca^{2+} influx is greater in VSMCs from spontaneously hypertensive rats compared to normotensive Wistar-Kyoto control rats (van Breemen et al. 1987). Large-scale genome-wide association studies by the CHARGE, Global BPgen consortium and other candidate gene studies in the European (Levy et al. 2009), Korean (Hong et al. 2010a; Hong et al. 2010b), East Asian (Xi et al. 2012), and Japanese (Takeuchi et al. 2010) populations have identified *ATP2B1* to be an HT susceptibility gene. The variants of this gene have been linked to all the three BP traits, including systolic BP, diastolic BP, and HT.

A recent study using chronic angiotensin II administration mice models revealed elevated levels of albuminuria and BP along with increasing levels of renal PMCA1 mRNA and protein expression (Wakui et al. 2017). Subsequent studies have established the pivotal role of calcium PMCA1 pump in the development of EH. BP was consciously measured using pressure myography in PMCA1 heterozygous null mice ($PMCA1^{Ht}$) at 6–18 months of age. Initially, between 6 and 9 months the null mice exhibited normal BP but over time developed significantly higher BP levels compared to age-matched wild-type controls at >12 months of age (Little et al. 2017).

METHODOLOGY

Sample Collection

All the samples were selected based on the 7th (2003) JNC report and the WHO/ISH guidelines for management of HT (Chalmers et al. 1999). Clinical investigations were carried out by qualified physicians and informed consent was obtained from all the patients and controls. 5 ml of venous blood was collected from hypertensive patients ($n = 568$) and controls ($n = 604$) between the age group of 20 and 82 years. Patients' samples were collected from four different areas: 1. Govt. Hospital, Headquarters Dindigul, Tamil Nadu, 2. K.S. Hospital, Kilpauk, Chennai, Tamil Nadu, 3. Government Hospital, Headquarters Chennai, Tamil Nadu, India, and 4. Voluntary Health Services, Adyar, Chennai, Tamil Nadu, India. Age- and sex-matched control samples were collected from healthy volunteers and patients who visited outpatient clinics with minor ailments without HT in previous records. Patients with a history of diabetes mellitus, hyperlipidemia, liver or renal disease, myocardial infarction, and other causes of secondary HT were excluded from the study. All the subjects were recruited based on a standard questionnaire and written informed consent was obtained. The study was approved by the Institutional Human Ethical Committee.

Genotyping

Genomic DNA was extracted from the buffy coat of EDTA anti-coagulated blood using the Miller et al. (1988) salting out method. Genotype analysis for the SNP marker was based on PCR-RFLP method. PCR was performed in master cycler gradient (Eppendorf, Hamburg, Germany). PCR was performed in 20 μl volumes using 100 ng of genomic DNA, 200 μM of dNTP, 5 pmol/μl of forward: VP3: 5'-TGGCAGGGTGGCATAT-

CAGG-3' and reverse: VP4: 5'-CACAGCCCAG-TAAGGCCA- 3' primers (Eurofins MWG Operon, Bangalore, India), 2 mM $MgCl_2$ and 0.5 U of Taq DNA polymerase (Prime Taq DNA polymerase, Korea) and was amplified following PCR conditions, which involved an initial denaturation at 94 °C for 4 min, annealing at 58^0C for 45 sec, extension at 72^0C for 45 sec, and a final extension at 72°C for 4 min. 5 µl of PCR product was checked on a 1% agarose gel (Fig. 1A). 15 µl of PCR product was digested using *PvuII* restriction enzyme procured from New England Biolabs, England. Digestion was carried out at 37°C for 2 h. The digested product was visualized on a 2% agarose gel and the results were documented (Fig. 1B). Sequencing analysis was performed to confirm the genotypes and the sequence chromatograms (Fig. 1C, D, E) were analyzed using CHROMAS 2.31 software (Technelysium, Australia). Comparison of allele frequencies between different ethnic groups was performed from the data obtained from Ensembl genome browser.

Statistical Analysis

The continuous variables were expressed as mean ± standard deviation. Student's t-test was used for comparison of means of different variables. Chi-square analysis was used to test for deviation of genotype distribution from Hardy–Weinberg equilibrium and to determine any significant differences in allele or genotype frequencies between cases and controls. The association between genotypes and HT risk was analyzed by calculating the odds ratio (OR) at 95 percent confidence interval (95% CI). Statistical tests, including logistic regression analysis, were performed using the statistical package SPSS 14.0 version (SPSS Inc., Chicago, Illinois, USA). p value < 0.05 was considered to be statistically significant.

RESULTS

Table 1 shows the baseline features found in the controls and patients. The mean values of

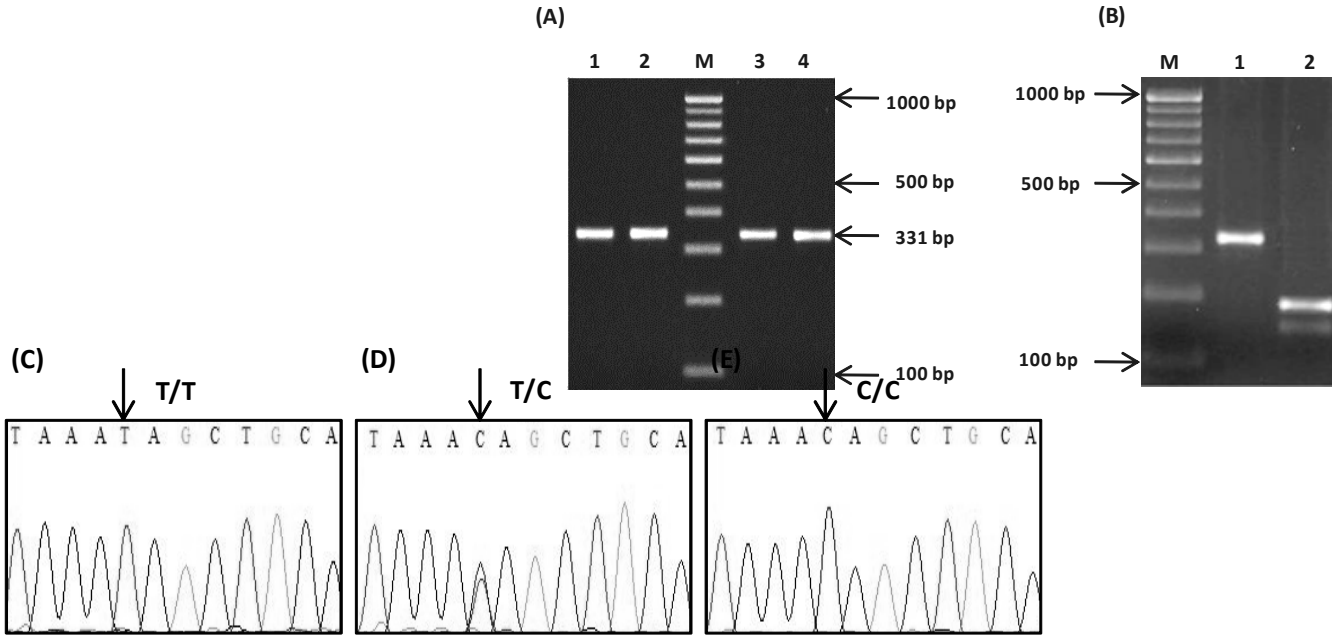

Fig. 1. T/C polymorphism of *ATP2B1* (*rs2681472*) gene: (A) PCR amplification showing 331bp fragment (Lanes 1-4) [M = 100 bp DNA marker]. (B) *PvuII* digestion of PCR amplified products for genotyping (Lanes 1 – TT, 2 – CC, 3 – TC). Sequence chromatograms of the genotypes: (C) Homozygous wild-type (TT); (D) Heterozygous (TC); (E) Homozygous variant (CC)

Table 1: Base-line data of normotensive controls and hypertensive patients

		Controls (N=604)		*Patients (N=568)*	
Sex (M:F)		*1: 1.06*		*1.08: 1*	
Age (Years)					
Males	Mean±SD	54.4± 12.10		54.5± 11.27	
Females	Mean±SD	54.4± 12.87		54.5± 11.55	
Systolic Blood Pressure (SBP) mmHg	Mean±SD	116.8± 7.54		154.0± 19.93*	
Diastolic Blood Pressure (DBP) mmHg	Mean±SD	77.9± 4.69		94.7± 12.36*	
Body Mass Index (BMI) (kg/m²)		*N*	*%*	*N*	*%*
Males (N)		*293*		*295*	
Underweight		16	5.46	24	8.14
Normal		177	60.41	143	48.47*
Overweight		87	29.69	103	34.92
Obese		13	4.44	25	8.47*
Females (N)		*311*		*273*	
Underweight		31	9.97	20	7.32
Normal		180	57.88	129	47.25*
Overweight		87	27.97	100	36.64*
Obese		13	4.18	*24*	8.79*

* p value less than 0.01

SBP and DBP were significantly higher in cases compared to the control group ($P < 0.0001$). Allele frequencies in hypertensive cases and normotensive controls were in good agreement with the Hardy-Weinberg equilibrium with a *p* value greater than 0.05 (Table 2). The genotype frequency when segregated based on dominant and recessive models showed HT risk to be associated with TT homozygotes with a significant *p* value of 0.051 [OR = 1.257, 95% CI = 0.9983 – 1.5817]. The T allele also showed a significant difference in frequency between the case and the control groups with a *p* value of 0.038 [OR = 1.213, 95% CI = 1.0103 – 1.4567] (Table 2).

Gender-wise distribution showed that in female subjects, the T allele exhibited a significant *p* value of 0.053 [OR = 1.291, 95% CI = 0.9958 – 1.6753] (Table 4). Thus, from the above results, it is evident that the TT genotype and T allele make individuals susceptible to HT. Male subjects did not show any significant difference for the genotypes analyzed (Table 3). To the best of the researchers' knowledge, the present study is the first to report a significant association of *rs2681472* polymorphism of *ATP2B1* gene in female hypertensive patients in India.

A comparison on allele frequency of *rs2681472* polymorphism in south Indian population (73%) as addressed from the present study with other populations from around the globe, indicates a high T allele frequency in African population (91%), followed by American (89%), European (85%), Asian (68%), and south Asian (66%) (www.ensembl.org).

DISCUSSION

EH arises from a complex combination of genetics and environmental risk factors, which accounts for 95 percent of all cases of HT (Carretero and Oparil 2000). Family studies suggest that the heritability of EH is in the range of 30–50 percent (Marteau et al. 2005). Such a complex phenotype demands analysis of various genes involved in the pathophysiological mechanism of EH. In the past few decades, various genes of renin-angiotensin-aldosterone system, adducin, β-adrenoceptors, G protein subunits, regulators of G protein signaling (RGS) proteins, rho kinases, and G protein receptor kinases were examined in different populations (Aguayo and Fardella 2009). In recent times, genes that are indirectly involved in the pathophysiology of HT have gained focus due to their interactions with known biological markers reported to be involved in the establishment of the disease phenotype.

PMCA isoform 1 is encoded by *ATP2B1,* which plays a critical role in intracellular calcium homeostasis. In addition, it is also suggested

Table 2: Overall genotype distribution between normotensive controls and hypertensive patients

Genotype	*Hypertensive N=568 (%)*	*Normotensive N=604 (%)*
T T	320 (56.3)	306 (50.7)
TC	211 (37.1)	247 (40.9)
CC	37 (6.5)	51 (8.4)
p value	0.118	
	Dominant	
T T	320 (56.3)	306 (50.7)
TC+CC	248 (43.7)	298 (49.3)
Unadjusted OR	1.257	
95% CI	0.9983 -1.5817	
p value	0.051	
Adjusted OR*	1.237	
95% CI	0.982 – 1.559	
p value	0.071	
	Recessive	
CC	37 (6.5)	51 (8.4)
TT + TC	531 (93.5)	553 (91.6)
Unadjusted OR	0.756	
95% CI	0.4867-1.1728	
p value	0.212	
Adjusted OR*	0.768	
95% CI	[0.494-1.194]	
p value	0.240	
	Allele	
T	851 (74.9)	59 (71.1)
C	285 (25.1)	349 (28.9)
OR	1.213	
95% CI	1.0103-1.4567	
p value	0.038	

* Values obtained after adjusting for BMI

creased Ca^{2+} influx in VSMC may contribute to increased contractility and promote rise in BP (van Breemen et al. 1986; van Breemen et al. 1987). Genetic alterations may affect the activity or affinity of the isoform contributing to a hypertensive state (Benkwitz et al. 1999). Mice with VSMC-specific knockout (KO) of *ATP2B1* were generated to test the relationship between *ATP2B1* and HT. The KO mice expressed significantly lower levels of *ATP2B1* mRNA and protein in the aorta compared to the controls. Subsequently, a higher systolic BP was also recorded in the KO mice. The cultured VSMC of KO mice showed increased intracellular calcium concentrations (Kobayashi et al. 2012).

Genetic variants of the *ATP2B1* gene have been established as one of the strong markers associated with HT. Recent GWAS have revealed their positive association in the European, Japanese, and Korean populations and a negative association in the Chinese population (Kato et al. 2011; Ji et al. 2013; Xu et al. 2016). The genome-wide association study of six individual cohorts of the CHARGE consortium (AGES, n = 3,219; ARIC, n = 8,047; CHS, n = 3,277; FHS, n = 8,096; RS, n = 4,737; RES, n = 1760) investigated the association of SNP markers with EH. Among the top ten loci analyzed in the European population by CHARGE consortia, eight loci attained a genome-wide significance ($p < 5 \times 10^{-8}$). The *ATP2B1* markers demonstrated a significant association with all the three traits involving SBP, DBP, and HT. The *rs2681472* marker showed a strong association with diastolic BP and HT (Levy et al. 2009).

A meta-analysis involving 15,909 cases and 18,529 controls performed to assess the association of *rs2681472* marker of *ATP2B1* gene with HT risk in East Asians also showed a significant association (OR = 1.18; 95% CI: 1.10-1.27; p≤ 0.001) with HT susceptibility (Xi et al.2012). Another meta-analysis by the Amagasaki cohort, Fukuoka cohort, and the Kita-Nagoya Genomic

Table 3: Genotype distribution between male normotensive controls and hypertensive patients

Genotypes	*Hypertesive N=295 (%)*	*Normotensive N=293 (%)*	*Unadjusted OR [95% CI]*	*p-Value*	*Adjusted OR*[95% CI]*	*p-value*
Dominant						
TT	164 (55.6)	151 (51.5)	1.177	0.324	1.185	0.308
TC+CC	131 (44.4)	142 (48.5)	[0.8511 – 1.6285]		[0.855 – 1.642]	
Recessive						
CC	20 (6.8)	23 (7.8)	0.854	0.619	0.888	0.709
TC+TT	275 (93.2)	270 (92.2)	[0.4582 – 1.5908]		[0.475 – 1.1659]	
Alleles						
T	439 (74.4)	421 (71.8)	1.139	0.322	-	-
C	151 (25.6)	165 (28.2)	[0.8803 – 1.4749]			

* Values obtained after adjusting for BMI

Table 4: Genotype distribution between female normotensive controls and hypertensive patients

Genotypes	*Hypertesive N=273 (%)*	*Normotensive N=311 (%)*	*Unadjusted OR [95% CI]*	*p-Value*	*Adjusted OR*[95% CI]*	*p-value*
Dominant						
TT	156 (57.1)	155 (49.8)	1.342	0.078	1.287	0.134
TC+CC	117 (42.9)	156 (50.2)	[0.9677 – 1.8608]		[0.925 – 1.791]	
Recessive						
CC	17 (6.2)	28 (9.0)	0.605	0.116	0.670	0.212
TC+TT	256 (93.8)	283 (91.0)	[0.3230 – 1.1322]		[0.357 – 1.258]	
Alleles						
T	412 (75.5)	438 (70.4)	1.291	**0.053**	-	-
C	134 (24.5)	184 (29.6)	[0.9958 – 1.6753]			

* Values obtained after adjusting for BMI

Epidemiology [KING] study cohort in a randomly selected Japanese general population revealed a significant association of the *rs2681472* marker with HT (OR = 1.10; 95% CI: 1.04 – 1.17; $p \leq 0.01$) (Takeuchi et al. 2010).

A cross-sectional study on the She ethnic minority of China, ranging from 20 to 80 years of age, indicated a significantly higher minor allele frequency for the *rs2681472* marker in prehypertensive and hypertensive groups compared to normotensive subjects. Despite this fact, the marker had no association with both BP traits and HT in the She population after adjusting for age, sex, and BMI (Lin et al. 2011). A recent study on women of the Northern Han Chinese population has reported that the SNP marker *rs2681472* has been significantly associated with early onset preeclampsia, a condition characterized by HT and proteinuria (Wan et al. 2014).

The present study indicates that the TT homozygous genotype of *rs2681472* polymorphism (*ATP2B1* gene) acts as an independent risk factor for EH. The results of the study show that individuals with TT homozygous genotype are 1.2 times more susceptible to EH compared to individuals with CC or CT genotypes. Since the distribution of *ATP2B1* gene polymorphisms were in concordance with the Hardy–Weinberg equilibrium, the results of this study are unlikely to be biased by population stratification or admixture for EH.

CONCLUSION

PMCA is a potential genetic marker showing a strong association with EH in several ethnic groups of the world population. Although the several polymorphisms analyzed in this gene showed contradictory results in different ethnic groups, the majority of the data support the fact that *ATP2B1* plays a pivotal role in BP regulation and HT etiology, thereby emphasizing the significance of carrying out further investigations to clarify the functional basis of the associations. The physiological functions of the protein in KO mouse models have provided insight into pathways leading to elevated BP driven by improper functioning of PMCA1 protein. Although there are only a limited number of studies based on gender for this polymorphism, reports have substantiated their association with EH in pre-eclampsia cases. Moreover, the polymorphism selected for this study is an intronic variant that may affect the splicing regulatory elements leading to aberrant splicing. These may either introduce or erase splice sites. Hence, intronic variants are equally important in establishing a disease phenotype.

Global reports on the candidate genes related to EH account for only a minimal increase in the BP values. Hence, understanding the role of genes indirectly involved in the development of EH would provide a clue about the potential drug targets. To the best of the researchers' knowledge, the present study represents the first report to analyze the association of the genetic marker of *ATP2B1* gene with EH in the south Indian population. Further studies are required to consider the joint effects of several candidate genes to dissect the genetic framework and the gender-specific association of EH.

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