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RESEARCH ARTICLE

ASSOCIATION OF *PLEKHA7 (rs11024074)* AND *ULK4 (rs1052501)* GENE POLYMORPHISM WITH ESSENTIAL HYPERTENSION RISK AMONG SOUTH INDIAN POPULATION

[1, 5, *]Vijayashree Priyadharsini Jayaseelan, [2]Karthikeyan Muthusamy, [3]Shridevi Venkatramani, [4]Paramasivam Arumugam, [5]Jayaraman Gopalswamy and [5]Santhiya Sathiyavedu Thiagarajan

[1]Central research laboratory, Meenakshi Ammal Dental College and Hospital, Meenakshi Academy of higher education and research, Maduravoyal, Chennai-95, India
[2]Department of Bioinformatics, Alagappa University, Karaikudi–630004, Tamil Nadu, India
[3]IIIT-Hyderabad, Gachibowli, Hyderabad, Telangana 500032, India
[4]Centre for Cellular and Molecular Biology, Hyderabad -500007, Telangana, India
[5]Department of Genetics, Dr. ALM PGIBMS, University of Madras, Taramani, Chennai-113, Tamil Nadu, India

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ABSTRACT

Essential or primary hypertension (EH) is one of the major risk factors leading to mortality due to non-communicable disease. A complex interplay of genes along with lifestyle habits defines the pathophysiology of EH. Genome wide association studies (GWAS) and candidate gene studies (CGS) have provided details on the vital genes associated directly or indirectly with this disease phenotype. The present case control study was designed to analyze the association of *PLEKHA* and *ULK4* gene polymorphisms in south Indian population. Subjects comprised of 568 hypertensive cases and 604 normotensive controls. A PCR-RFLP approach was applied to designate genotypes. Genotype and allele frequencies were compared between the normotensive and hypertensive groups. No significant association was recorded with each of the selected polymorphism in the population studied.

Key words: Essential hypertension, *PLEKHA7, ULK4*, Polymorphism, *RFLP.*

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INTRODUCTION

Essential hypertension is considered to the major risk factor associated with cardiovascular, renal and cerebrovascular diseases. Numerous reports have been published in the past two decades focusing on this field of hypertension genetics. GWAS, CGS, linkage analysis, twin studies have revealed association of novel genes with EH. Some of these genes may be directly involved in the pathophysiology of hypertension, whereas others may show indirect effect by acting synergistically or antagonistically with other candidate genes. Recently many reports have been recorded targeting genes with an ancillary role in etiology of EH such as cadherin 13 (Vijayashree *et al.,* 2016), solute carrier family 9 (Vijayashree *et al.,* 2017), mitofusin gene polymorphisms (Vijayashree *et al.,* 2016) etc.

Corresponding author: **Vijayashree Priyadharsini Jayaseelan,**
Central research laboratory, Meenakshi Ammal Dental College and Hospital, Meenakshi Academy of higher education and research, Maduravoyal, Chennai-95, India

The present study was also aimed to establish the association of two (*PLEKHA7* and *ULK4*) gene polymorphisms with EH. *PLEKHA7* was discovered as a novel protein component of epithelial adherens junctions (Meng *et al.,* 2008; Pulimeno *et al.,* 2010; Pulimeno *et al.,* 2011). Epithelial cells are characterized by an apical junctional complex, comprising tight junctions (TJ), adherens junctions (AJ) and desmosomes (Farquhar and Palade, 1963). TJ and AJ have critical role in the development and physiology of vertebrate epithelial tissues. TJ control the barrier function of epithelia and maintain cell polarity and AJ regulate cell-cell adhesion and morphogenesis (Gumbiner, 1996; Shin *et al.,* 2006).The molecular architecture of TJ and AJ by and large has been clarified in recent years. The junctions comprise transmembrane proteins that are linked to cytoskeletal filaments through a cytoplasmic plaque that contains scaffolding, adaptor and signaling proteins (Mitic and Anderson, 1998; Perez-Moreno and Fuchs, 2006; Guillemot *et al.,* 2008; Takai *et al.,* 2008; Meng and Takeichi, 2009). A recent study showed that E-cadherin, a major transmembrane

protein of AJ, associates with microtubules through a protein complex comprising p120 ctn and the newly identified proteins *PLEKHA7* and Nezha (Meng *et al.,* 2008). Protein kinases have been known to play crucial roles in cellular regulation, including cell growth, differentiation, metabolism and gene expression in the eukaryotic cells. An increasing number of protein kinase genes are being identified in the course of genome sequencing projects of various eukaryotes and to date protein kinases constitute one of the largest gene superfamily (Meharena *et al.,* 2013). *ULK4* codes for serine-threonine protein kinase which is found to have possible role in many signal transduction reactions. Autophagy is a dynamic and highly regulated process of self-digestion. In eukaryotic cells autophagy occurs constitutively at low levels to perform housekeeping functions such as destruction of dysfunctional organelles. Upregulation occurs in the presence of external stressors (e.g. starvation, hormonal imbalance, oxidative stress) and internal needs (e.g. removal of protein aggregates) suggesting that the process is an important survival mechanism. It plays an adaptive role to protect organisms against diverse pathologies (Mizushima *et al.,* 2008), including heart disease (Martinet *et al.,* 2007). Under rare circumstances the self-cannibalistic function of autophagy may be deleterious (Levine and Kroemer, 2008).

METHODOLOGY

Subjects were selected on the basis of 7th (2003) JNC report and WHOISH guidelines for management of hypertension (Chalmers *et al.,* 1999). The clinical investigations were carried out by qualified physicians and informed consent was obtained from all the patients and controls (Table 1). Five ml of venous blood was collected from hypertensive patients (n = 568) and controls (n = 604) between the age group of 20-82 years. Patients' samples were collected from hospitals and voluntary health care centers. Age and sex matched control samples were collected from healthy volunteers and hypertensive patients who visited outpatient clinics with minor ailments without hypertension in previous records. Patients with the history of diabetes mellitus, hyperlipidaemia, liver or renal disease, myocardial infarction and other causes of secondary hypertension were excluded from the study. All the subjects were recruited based on standard questionnaire and written informed consent was obtained. The study was approved by Institutional Human Ethical Committee.

Genotyping: Genomic DNA was extracted from the buffy coat of EDTA anti-coagulated blood by using Miller *et al.,* (1988) salting out method. Genotype analysis for the SNP marker was based on PCR-RFLP method. PCR was performed in 20 µl volumes using 100ng of genomic DNA, 200µM of dNTP, 5pmol/µl of forward and reverse primers (Eurofins MWG Operon, Bangalore, India) (Table 2a), 2mM $MgCl_2$ and 0.5U of Taq DNA polymerase (Prime Taq DNA polymerase, Korea) and was amplified following the PCR conditions (Table 2b) in master cycler gradient (Eppendorf, Hamburg, Germany). Genotyping was carried out by subjecting 15 µl of PCR product to appropriate restriction enzyme digestion (New England Biolabs, England) (Table 2c). The digested product was visualized on a 2% agarose gel and the results were documented. Random samples corresponding to each genotype was subjected to sequence analysis to confirm genotypes. Sequence chromatograms were analyzed using CHROMAS 2.31 software (Technelysium, Australia). The comparison of allele frequencies between different ethnic groups was performed from the data obtained from 1000 genome browser (http://browser.1000genomes.org/). *Statistical analysis:* All the continuous variables were expressed as mean ± standard deviation. Student's t-test was used for comparison of means of different variables. $\chi 2$ analysis was used to test for deviation of genotype distribution from Hardy-Weinberg equilibrium and to determine whether any significant differences in allele or genotype frequencies between cases and controls. The association between genotypes and hypertension risk was analyzed by calculating odds ratio (OR) at 95% 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

PLEKHA7 (pleckstrin homology domain, family A, member 7) (*rs11024074*) gene polymorphism: The genotypes and sequence chromatograms of the *PLEKHA7* gene polymorphism (*rs11024074*) are shown in figure 1. The allele frequency of the T and C allele in the study population was 83 and 17% respectively. This was found to be closely related to Asian population with the T and C allele frequency of 80 and 20% respectively (Fig. 2). There was no significant difference between case and control groups at χ^{2df}(p value = 0.402). The overall genotype distribution based on the models did not show any significant difference (Table 3). The observed and expected genotype frequencies of the control versus case groups were in good agreement with the predicted Hardy-Weinberg equilibrium values.

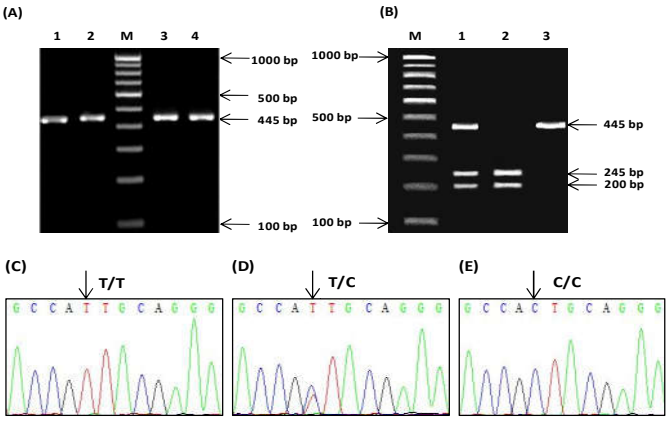

Figure 1. T/C polymorphism of *PLEKHA7* (*rs11024074*) gene: (A) PCR amplification showing 445 bp fragment (Lanes 1-4) [M = 100 bp DNA marker]. (B) *PstI*digestion of PCR amplified products for genotyping (Lanes 1 – TC, 2 – CC, 3 – TT). Sequence chromatograms of the genotypes: (C) Homozygous wild-type (TT); (D) Heterozygous (TC); (E) Homozygous variant (CC).

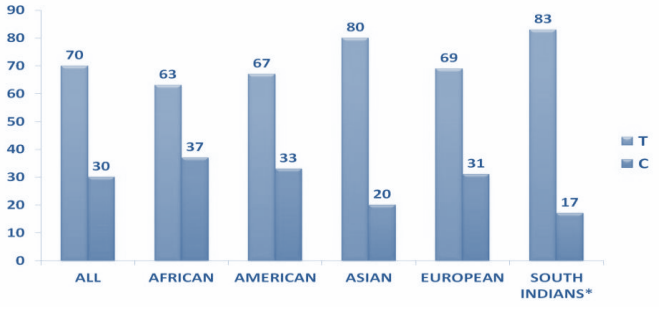

Figure 2. Ethnic distribution of *PLEKHA7* (*rs11024074*) allele frequencies among different populations with the present study group*

Table 1. Demographic 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/m^2)	N = 293	%	N = 295	%
Males (N)				
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)	N = 311	%	N = 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

Table 2a. List of primers used in the present study

Gene symbol	Reference SNP number	Primer sequences	Tm (°C)	PCR product size (bp)
PLEKHA7	*rs11024074*	VP5: F: 5'- CTGCACATCACTGACTCTGA - 3'	57.3	445
		VP6: R: 5'- TTCTTGGCTCAGCCAGTGTC - 3'	59.4	
ULK4	*rs1052501*	VP7: F: 5'- GGAGTGTGGGCCTGACCTGT - 3'	63.5	311
		VP8: R: 5'- AGGTCGGTGAAAGCTGCAGG - 3'	61.4	

Table 2b.PCR reaction conditions forpolymorphisms selected in the present study

Gene symbol/RefSNP No.	Initial Denaturation (Min)	Denaturation (S)	Annealing (S)	Extension (S)	Final Extension (M)	No. of Cycles
PLEKHA7 rs11024074	94°C - 4	94°C - 45	58°C - 45	72°C - 45	72°C - 7	30
ULK4 rs1052501	94°C - 4	94°C - 45	63°C - 45	72°C - 45	72°C - 7	30

Table 2c. RFLP reaction conditions for polymorphisms selected in the present study

Gene/ RefSNP No.	Variation	Restriction enzyme	Incubation		Product size (bp)		
			Temp (°C)	Time (h)	+/-	-/-	+/+
PLEKHA7 rs11024074	T ⟶ C	*PstI*	37	2	200, 245, 445	445	200, 245
ULK4 rs1052501	A ⟶ G	*PvuII*	37	2	120, 191, 311	311	120, 191

Table 3. Genotype frequencies of *PLEKHA7* (*rs11024074*) polymorphism among the cases and controls

Genotypes		T	C	HWE p value*
Cases N = 568 (%)				
TT	398 (70.1)	0.83	0.17	0.89
TC	149 (26.2)			
CC	21 (3.7)			
Controls N = 604 (%)				
TT	406 (67.2)	0.82	0.18	0.14
TC	179 (29.6)			
CC	19 (3.1)			

*For departure from Hardy-Weinberg equilibrium (HWE), chi square with one degree of freedom.
The genotype frequency of cases and controls do not differ significantly χ_{2df} (P = 0.402).

Table 4. Genotype frequencies of *ULK4 (rs1052501)* gene polymorphism among the cases and controls

Genotypes		A	G	HWE p value*
Cases N = 568 (%)				
AA	403 (71.0)	0.84	0.16	0.99
AG	149 (26.2)			
GG	16 (2.8)			
Controls N = 604 (%)				
AA	446 (73.8)	0.86	0.14	0.62
AG	146 (24.2)			
GG	12 (2.0)			

*For departure from Hardy-Weinberg equilibrium (HWE), chi square with one degree of freedom.
The genotype frequency of cases and controls do not differ significantly χ_{2df} (P = 0.431).

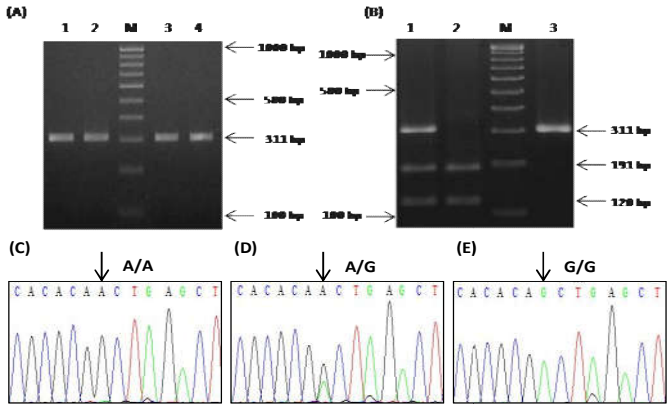

Figure 3. A/G polymorphism of *ULK4 (rs1052501)* gene: (A) PCR amplification showing 311bp fragment (Lanes 1-4) [M = 100 bp DNA marker]. (B) *PvuII*digestion of PCR amplified products for genotyping (Lanes 1 – AG, 2 – GG, 3 – AA). (C) Sequence chromatograms of the genotypes: Homozygous wild-type (AA); Heterozygous (AG); Homozygous variant (GG)

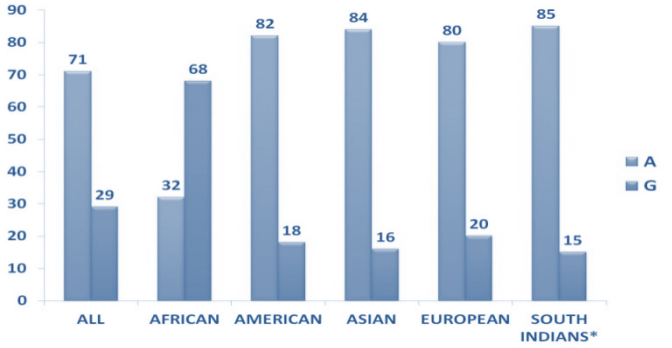

Figure 4. Ethnic distribution of *ULK4 (rs1052501)* allele frequencies among different populations with the present study group*

ULK4 (unc-51-like kinase 4)(*rs1052501*) gene polymorphism

The genotype and sequence chromatograms of the *ULK4* gene polymorphism *(rs1052501)* are shown in figure 3. The observed and expected genotype frequencies of the control and case group were in concordance with Hardy-Weinberg equilibrium (Table 4). There was no significant difference between case and control groups at χ^{2df}(p value = 0.431). The allele frequencies of the study population (A – 85% and G – 15%) were found to be similar to that Asian population (A – 86% and G – 14%) figure 4.

DISCUSSION

The advent of human genome sequencing has fastened the search for candidate genes associated with human essential hypertension. Several GWAS reports have pin-pointed crucial genes associated directly or indirectly with EH with both coherent as well as inconsistent results in various ethnic groups from around the world. The classical example being polymorphisms in angiotensiogen genes of the renin-angiotensin-aldosterone system (RAAS pathway), which exhibited both negative (Kato *et al.,* 2000) and positive (Chiang *et al.,* 1997) correlations in different populations/groups. Probably, this is because of the effects of variants under study might be effectively masked by effects of unknown variants that affect the phenotype (Moore and Williams, 2002). Therefore, cumulative effects of multiple candidate variations may provide more information in exploring hypertension susceptibility genes.

PLEKHA7 has been implicated in heart development and hypertension. Single nucleotide polymorphisms of *PLEKHA7* locus are known to be associated with diastolic high blood pressure in genome-wide studies on Caucasian and Asian ethnic groups (Levy *et al.,* 2009; Hong *et al.,* 2010; Lin *et al.,* 2011). The cellular mechanisms whereby mutations in the *PLEKHA7* locus lead to increased blood pressure are unknown. Knockdown studies show that the zebrafish homolog of *PLEKHA7* known as *Hadp1*, is required for cardiac contractility and morphogenesis, through a mechanism involving the regulation of intracellular Ca^{2+} handling (Wythe *et al.,* 2011). Two large-scale genome-wide association studies (GWAs) show that variants in *PLEKHA7* were significantly related to hypertension (Odds ratio and 95% confidence interval - 1.19 (1.01–1.41) in logistic regression analyses after adjusted by age, sex and BMI (Lin *et al.,* 2011).

PLEKHA7 gene encodes pleckstrin homology domain-containing protein, family A member 7. This protein was reported to be involved in maintaining integrity of zona adherens, an epithelial cadherin-based cell–cell junction (Meng *et al.,* 2008). Though it is difficult to determine the function of *PLEKHA7* on the regulation of blood pressure, it is found to promote the incorporation of cadherin clusters, including E-cadherin and p120- catenin, into the higher-order structure of the zonula adherens which plays a vital role in cell signaling (Pulimeno *et al.,* 2010). GWAS involving the CHARGE consortia and Global BPgen have together identified two markers of *PLEKHA7* gene, *rs381815* and *rs11024074* which showed positive association with SBP and DBP respectively. The marker *rs11024074* produced a significant association ($P = 2.8 \times 10^{-7}$) with diastolic BP (Levy *et al.,* 2009). The results of the present study for screening of *rs11024074* of *PLEKHA7* gene indicate that the genotype distribution did not differ significantly between hypertensive patients and normotensive subjects. *ULK4* encodes serine/theronine kinase of unknown function. The *rs1052501* marker of *ULK4* gene is a non-synonymous SNP marker leading to an amino acid change from alanine to threonine. The joint meta-analysis by CHARGE and Global BPgen showed suggestive evidence of association with diastolic blood pressure (Levy *et al.,* 2009). Very little information about the role of this gene in the etiology of blood pressure is available. Autophagy has emerged as a significant contributor of hypertension and cardiovascular diseases (Wang *et al.,* 2010). During hypertension or myocardial infarction, the heart undergoes a compensatory hypertrophic growth response. In such conditions, loss of autophagy in heart triggers cardiac dysfunction eventually leading to heart failure. Thus, the involvement of *ULK4* in the process of autophagy could vaguely explain its involvement in hypertension. Three linked nonsynonymous SNPs in this gene have been shown to be associated with diastolic blood pressure (Levy *et al.,* 2009).

Conclusion

Essential hypertension is a complex disorder which is influenced by multiple factors and genes. Population structure, ethnicity, demographics and other environmental factors may effectgene frequencies which may lead to association or lack of association of a particular genotype with the disease

phenotype. Hence, careful selection of genes and subjects is necessary to link putative genes with a disease phenotype. A meticulous study on candidate genes acquired through case control studies in a specific population and their functional analysis will bring about a dramatic change in the therapy of hypertension and related disorders.

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