International Journal of Current Advanced Research
ISSN: O: 2319-6475, ISSN: P: 2319 – 6505, Impact Factor: SJIF: 5.995
Available Online at www.journalijcar.org
Volume 6; Issue 3; March 2017; Page No. 2608-2612
DOI: http://dx.doi.org/10.24327/ijcar.2017.2612.0056

Research Article

ASSOCIATION OF SLC6A9 (rs2286245) POLYMORPHISM WITH ESSENTIAL HYPERTENSION IN SOUTH INDIAN POPULATION

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A R T I C L E I N F O

Article History:

Received 18th December, 2016
Received in revised form 16thJanuary, 2017
Accepted 26th February, 2017
Published online 28th March, 2017

Key words:

Essential hypertension, *SLC6A9*, SNS, SNP, PCR-RFLP

A B S T R A C T

Essential hypertension (EH), is the most common form of hypertension resulting in the elevation of blood pressure due to unknown cause. EH is also known to elevate the risk of cerebral, cardiac and renal disorders. In a genetic perspective, several polymorphisms have been found to be associated with EH along with epigenetic factors. Recent findings in this field provide concrete evidence about the involvement of hereditary factors which might precipitate as susceptibility to EH. In view of the above facts, a case-control association study was conducted to investigate the possible involvement of *SLC6A9* (*rs2286245)* polymorphism in essential hypertensive patients of south India. A total of 568 cases and 604 controls were recruited for this study. Genotyping was performed using PCR-RFLP method. The overall geno type distribution revealed a p value of 0.861.Hence, the present study shows that *SLC6A9* gene polymorphism (*rs2286245)*is not associated with essential hypertension in south Indian population.

INTRODUCTION

Human essential hypertension is a complex, multifactorial, quantitative trait under a polygenic control. EH is defined as a systolic blood pressure ≥140 mm Hg, a diastolic blood pressure ≥90 mm Hg, or taking antihypertensive medications.Hypertension (HT) is directly responsible for 57% of all death due to stroke and 24% of all deaths due to coronary heart disease (Gupta and Gupta, 2009). Treatment of HT at an early stage has been associated with 40% reduction in the risk of stroke and 15% reduction in the risk of myocardial infarction (Whitworth, 2003). Monogenic forms of hypertension, familial studies, twin studies and cross transplantational studies in experimental models have revealed the association of genetic components with essential hypertension. Existing evidences also suggest that the genetic contribution to blood pressure variation is about 30-50% (Marteau *et al.,* 2005). Abdominal obesity, dyslipideamia, glucose intolerance, hyperinsulinemia and hyperuremia aggregates with EH possibly because of the common physiological mechanisms involved (Staessen*et al.,* 2003).

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Although several strategies have been developed over the last. The decade to dissect genetic determinants of hypertension, the idiopathic origin of EH and environmental factors hampers the process of identifying the crucial genes responsible for the phenotype in a population. Variants in regulatory regions are predicted to play a vital role in disease susceptibility of common complex disorders. Recent evidences demonstrate the effect of 3'-UTR variants on gene expression patterns. They exert this effect by influencing mRNA stability and translation (Akhter *et al.,* 2012). The cis-acting determinants in the 3′UTR to which proteins bind either stabilize or destabilize the mRNA. These determinants in the 3′UTR may also interact with other sequences within the same mRNA. Therefore, any variation in these cis-acting determinants may affect the expression of a particular gene (Misquitta*et al.,* 2001). Mammalian miRNA (miRNA) have been shown to target endogenous mRNA through 3'-UTR and interfere with translational output (Lytle *et al.,* 2007). Polymorphisms in microRNA binding sites have been shown to disrupt the ability of miRNAs to target genes resulting in differential mRNA and protein expression. Many of the polymorphisms studied in recent years encompass these types of variants which are considered to be crucial in gene regulation and expression.The present study was designed to investigate the possible role of a 3'-UTR polymorphism *(rs2286245)* in *SLC6A9* gene.

Solute carriers are membrane proteins that control the uptake and efflux of various solutes, including amino acids, sugars and drugs (Hediger*et al.,* 2004). The Human Gene Nomenclature Committee (HGNC) of the Human Genome Organization (HUGO) has classified ~400 human solute carriers into 47 families (Povey*et al.,* 2001). The SLC6 family of proteins has 20 members in the human genome. It acts as specific transporters for neurotransmitters, aminoacids and osmolytes (Chen *et al.,* 2004).

SLC6 transporters can be divided in 4 subgroups based on the substrate they translocate. The neurotransmitter transporters (NTT) which include 3 γ-aminobutyric acid (GABA) transporters (GAT), 2 glycine transporters (GLY) and the monoamine (dopamine (DAT), serotonin (SERT) and norepinephrine (NET) transporters; the amino acid transporters which include proline (PROT, IMINO), cationic and neutral amino acid transporters (AA^0, $AA^{0,+}$); the osmolyte transporters which include the betaine (BGT1), taurine (TauT) transporters and creatine transporters (CT) and 1 orphan transporter (Amara and Kuhar, 1993).

In humans, the SLC6 family of transporters is one of the most clinically relevant protein groups with links to orthostatic intolerance, attention deficit hyperactivity disorder (ADHD) (Mazei-Robison *et al.,* 2008), addiction, osmotic imbalance, X-linked mental retardation (Martinez-Munoz *et al.,* 2008), Hartnup disorder, hyperekplexia, Tourette syndrome, schizophrenia, Parkinson disease (PD), autism and mood disorders such as depression, anxiety, obsessive compulsive disorder (OCD) and post-traumatic stress disorder (PTSD) (Hahn and Blakely, 2007). *SLC6A9,* a member of this family encodes Na^+ and Cl^- dependent glycine transporter which is involved in the inhibitory glycinergic transmission.

Human *SLC6A9* gene has been mapped to chromosome band 1p33 by *in situ* hybridization (Jones *et al.,* 1995). Two different high affinity plasma membrane transporters GlyT1 (*SLC6A9*) and GlyT2 (*SLC6A5*) have been discovered so far (Guastella*et al.,* 1992; Liu *et al.,* 1992). Both the transporters are encoded by different genes and multiple splice variants have also been described (Adams *et al.,* 1995; Ebihara*et al.,* 2004). GlyT1 shows a broader expression pattern in the glial cells of the brain stem and spinal cord, the regions which are rich in glycinergic neurotransmission (Zafra*et al.,* 1995). About half of the transporters in SLC6 family co-transport their substrates together with 2 Na^{2+} ions and one Cl^- ion. The number of Na^{2+} ions could vary from one to three, for eg., GlyT1 requires $2Na^{2+}$ and $1Cl^-$ per transport cycle whereas GlyT2 requires $3Na^{2+}$ and $1Cl^-$ for transport (Roux and Supplisson, 2000). Therefore, the accumulative power of GlyT1 is less than GlyT2.

Solute carrier family 6, member 9 and hypertension:

The sympathetic nervous system (SNS) plays a vital role in the regulation of arterial pressure. SNS hyperactivity has been implicated as a primary precursor of essential hypertension in both humans and animal models of the disease (Wyss, 1993). The principal activity of *SLC6A9* protein is the termination of synaptic activity through the removal of neurotransmitters. GlyT1 maintains significant extracellular glycine concentration which is sufficient to co-activate NMDA (N-methyl D-aspartate) receptors through the glycine site. Any change in the concentration or membrane potential results in the removal of glycine thereby modulating glutamatergic neurotransmission. Thus, GlyT1 is essential for regulating glycine levels at synapses. In this context, the gene polymorphism *rs2286245* which spans the 3'-UTR of *SLC6A9* gene seems to be a promising target for investigating the genetic component underlying essential hypertension since they play an important role in maintaining sympathetic activity in the central nervous system (Ueno *et al.,* 2009).

MATERIALS AND METHODS

All the samples were selected based on the 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. 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 four different areas: 1. Govt. Hospital, Headquarters Dindigul, Tamilnadu, 2. K.S. Hospital, Kilpauk, Chennai, Tamilnadu, 3. Government Hospital, Headquarters Chennai, Tamilnadu, India and 4. Voluntary Health Services, Adyar, Chennai, Tamilnadu, India. Age and sex matched control samples were collected from healthy volunteers and 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 (Table 1). The study was approved by Institutional Human Ethical Committee.

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)				
Male	54.4 ± 12.10		54.5 ± 11.27	
Female	54.4 ± 12.87		54.5 ± 11.55	
Systolic blood pressure (SBP) mmHg	116.8 ± 7.54		154.0 ± 19.93*	
Diastolic blood pressure (DBP) mmHg	77.9 ± 4.69		94.7 ± 12.36*	
Body Mass Index (BMI) (kg/m²)	**N**	**%**	**N**	**%**
	293		**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*
	N	**%**	**N**	**%**
	311		**273**	
Females (N)				
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

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 both the SNP marker was based on PCR-RFLP method. PCR was performed in mastercycler gradient (Eppendorf, Hamburg,

Germany). PCR was performed in 20 µl volumes using 100ng of genomic DNA, 200µM of dNTP, 5pmol/µl of forward: VP26: 5'- AAGGTGACAGCAGCCGTC – 3', reverse: VP27: 5'- ATGCCTACCTCATGCAGC – 3' (Eurofins MWG Operon, Bangalore, India), 2mM $MgCl_2$ and 0.5U of Taq DNA polymerse (Prime Taq DNA polymerase, Korea) and was amplified following the PCR conditions which involves an initial denaturation at 94°C for 4 min, annealing at 58°C for 45 secs, extension at 72°C for 45 secs and a final extension at 72°C for 4 min. 3 µl of PCR product was checked on a 1% agarose gel (Figure 1A). 15 µl of PCR product was digested using restriction enzyme *StyI*procured from New England Biolabs, England. Digestion was carried out at 37°C for 2 hours. The digested product was visualized on a 2.5% agarose gel and the results were documented (Figure 1B). Sequencing analysis was performed to confirm genotypes and the sequence chromatograms (Figures C,D,E) 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/) (Figure 2).

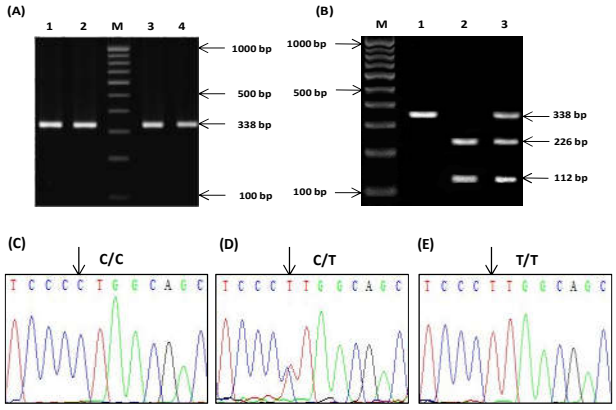

Figure 1 C/T polymorphism of *SLC6A9* (*rs2286245*) gene: (A) PCR amplification showing 338 bp fragment (Lanes 1-4) [M = 100 bp DNA marker]. (B) *StyI*digestion of PCR amplified products for genotyping (Lanes 1 – CC, 2 – TT, 3 – CT) (C) Sequence chromatograms of the genotypes: Homozygous wild-type (CC); Heterozygous (CT); Homozygous variant (TT).

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

The genotype and sequence chromatograms of the *SLC6A9* gene polymorphism *(rs2286245)* are shown in figure 1. The allele frequency of the study population (C-90% and T-10%) were in concordance with the Asian population (C-89% and T-11%) (Figure 2). The observed and expected genotype frequencies of the control and case group were in agreement with Hardy-Weinberg equilibrium (Table 2). There was no significant difference between case and control groups at χ^{2df} (p value = 0.861).

Table 2 Genotype frequencies of *SLC6A9* (*rs2286245*) gene polymorphism among the cases and controls

Genotypes	CC	CT	TT	HWE p value*
Cases N = 568 (%)	460 (81.0)	102 (18.0)	6 (1.1)	0.89
Controls N = 604 (%)	496 (82.1)	103 (17.1)	5 (0.8)	0.89

*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 = 0.861).

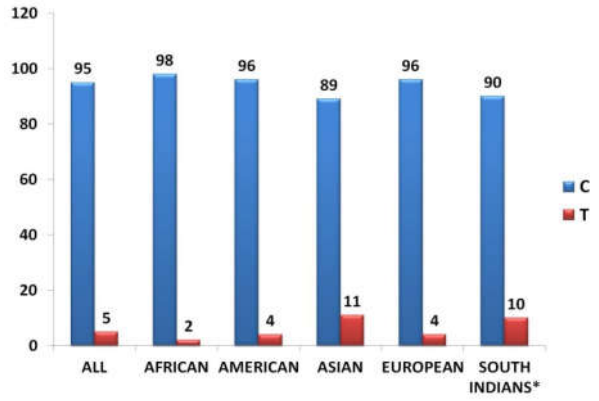

Figure 2 Ethnic distribution of allele frequencies among different populations with the present study group*

Overall genotype distribution (Table 3a) and gender specific distribution did not show any significant difference in the genotype or allele frequencies between case and control groups of both male (Table 3b) and female subjects (Table 3c).

Table 3a Overall genotype distribution of the *SLC6A9* gene polymorphism *(rs2286245)* in cases and controls

	Cases N=568 (%)	Controls N=604 (%)	Unadjusted OR [95% CI]	P-Value	Adjusted OR* [95% CI]	P-value
Dominant						
CC	460 (81.0)	496 (82.1)	0.927 [0.6902 - 1.2461]	0.617	0.919 [0.683 – 1.237]	0.578
CT + TT	108 (19.0)	108 (17.9)				
Recessive						
TT	6 (1.0)	5 (0.8)	1.279 [0.3882 - 4.2143]	0.686	1.257 [0.379 – 4.167]	0.708
CT + CC	562 (99.0)	599 (99.2)				
Additive						
C	1022 (90.0)	1095 (90.6)	0.925 [0.7036 - 1.2165]	0.578	-	-
T	114 (10.0)	113 (9.4)				

*Odds ratio according to genotypes were estimated after adjusting the confounding variables for BMI.

DISCUSSION

SLC6A9 gene encodes glycine transporter,whoseprincipal activity is the termination of synaptic activity through the removal of neurotransmitters. Recent reports have revealed the fact that an early stage of essential hypertension is accompanied by sympathetic hyperactivation. A genetic case control study in a Japanese population also found marginal

association between hypertensives and normotensives.The OR was estimated to be 1.26 (95% CI: 0.99 – 1.62; P = 0.06) after BMI and age adjustment (Ueno *et al.,* 2009).

Table 3b Gender specific distribution of *SLC6A9* (*rs2286245*) gene polymorphism in male subjects

	Cases N=295 (%)	Controls N=293 (%)	Unadjusted OR [95% CI]	P-Value	Adjusted OR* [95% CI]	P-value
Dominant						
CC	232 (78.6)	238 (81.2)	0.851 [0.5680 - 1.2751]	0.434	0.823 [0.548 – 1.236]	0.348
CT + TT	63 (21.4)	55 (18.8)				
Recessive						
TT	5 (1.7)	3 (1.0)	1.667 [0.3946 - 7.0388]	0.487	1.797 [0.425 – 7.601]	0.426
CT + CC	290 (98.3)	290 (99.0)				
Additive						
C	522 (88.5)	528 (90.1)	0.843 [0.5821 - 1.2216]	0.367	-	-
T	68 (11.5)	58 (9.9)				

*Odds ratio according to genotypes were estimated after adjusting the confounding variables for BMI.

Table 3c Gender specific distribution of *SLC6A9* (*rs2286245*) gene polymorphism in female subjects

	Cases N=273 (%)	Controls N=311 (%)	Unadjusted OR [95% CI]	P-Value	Adjusted OR* [95% CI]	P-value
Dominant						
CC	228 (83.5)	258 (83.0)	1.041 [0.6734 - 1.6087]	0.857	1.065 [0.686 – 1.653]	0.778
CT + TT	45 (16.5)	53 (17.0)				
Recessive						
TT	1 (0.4)	2 (0.6)	0.568 [0.0512 - 6.2992]	0.645	0.435 [0.037 – 5.051]	0.506
CT + CC	272 (99.6)	309 (99.4)				
Additive						
C	500 (91.6)	567 (91.2)	1.054 [0.7000 - 1.5882]	0.800	-	-
T	46 (8.4)	55 (8.8)				

*Odds ratio according to genotypes were estimated after adjusting the confounding variables for BMI.

The polymorphic marker of *SLC6A9* gene *(rs2286245)* did not produce significant association with essential hypertension in the south Indian population studied. The genotype frequency was found to be roughly the same in both normotensives and hypertensive groups. Gender-wise analysis also did not show any significant difference between the case and control groups of the population studied. Though the *SLC6A9* gene has a strong functional role in the etiology of essential hypertension, the outcome of the present study could not be compared to other global populations due to lack of reports.

CONCLUSION

Management of blood pressure is essential for prevention of complications due to essential hypertension. Anti-hypertensive drugs currently in use are diuretics, beta-blockers, angiotensin converting enzyme inhibitors, calcium channel blockers and angiotensin receptor blockers (Minushkina*et al.,* 2005). Despite the plethora of treatment options, the blood pressure control rates are less than 50%. This fact clearly establishes the inability to choose the antihypertensive drug likely to be most effective for an individual patient (Johnson, 2012). Inter individual variation in terms of genetic polymorphisms has been found to underlie pathophysiology of diseases which can also affect the efficacy of therapy.

The goal of hypertension pharmacogenomics relies on the genetic information along with clinical and demographic data to select antihypertensive regimen which is likely to provide greatest efficacy with minimal risk of adverse effects. Many of the pharmacogenetics studies have focused on single nucleotide polymorphisms (SNPs) within genes involved in the pathophysiology of EH. Polymorphic genes that encode elements of metabolism, absorption, transport, elimination of drugs and receptor systems are considered to be main candidates for pharmacogenetic studies. The single nucleotide variants spanning the genome may produce pathogenic effect or remain silent without causing any damaging result in an individual harboring it.

Personalized therapy targeted towards the specific pathway based on the genetic profile and ethnicity may improve drug response thereby decreasing the global burden of EH. Additionally, whole genome mapping of hypertension and blood pressure traits coupled with the understanding of pharmacogenetics and pharmacokinetics of current drugs will enable the discovery of new drug targets in future.In conclusion, the lack of association of *SLC6A9* polymorphism may be attributed to genetic heterogeneity among different populations.Additional studies in other ethnic groups and functional analysis of the candidate gene are required to further elucidate the intricate mechanisms underlying the pathophysiology of hypertension.

Acknowledgement

We wish to thank all the staff members of Govt. Hospital, Dindigul, KS Hospital, Chennai, Govt. Hospital, Chennai and VHS, Chennai for extending their support towards this research. This work was assisted by DST-PURSE programme in the form of junior and senior research scholarship. Financial support by UGC-SAP (DRS- II) and DST-FIST to the Dept. of Genetics is acknowledged. We thank all the patients and volunteers for participating in this study. We thank Mr.K.Boopathi, technical assistant, National Institute of Epidemiology for helping us in the statistical analysis.

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Please cite this article in press as:

SanthiyaSathiyavedu Thiagarajan *et al*(2017), 'Association of slc6a9 (rs2286245) polymorphism with essential Hypertension in south indian population', *International Journal of Current Advanced Research,* 6(3), pp. 2608-2612.
http://dx.doi.org/10.24327/ijcar.2017. 2612.0056

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