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Evaluation of I/D polymorphism of ACE in association with development of ischemic stroke in a North Indian population

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Abstract

Previous studies have demonstrated that stroke has a strong genetic component. In the current study, we carried out an investigation to evaluate the angiotensin converting enzyme insertion/deletion (ACE I/D) variant of the ACE gene with the development of ischemic stroke and its subtypes. Three hundred ischemic stroke patients and an equal number of healthy controls were included in this case-control study. Blood samples were collected and the presence of ACE gene polymorphism (I/D) was detected by the polymerase chain reaction (PCR) technique. Ischemic stroke subtypes were classified according to TOAST (Trial of Org 10172 in Acute Stroke Treatment) classification. The genotypic distribution and allelic frequencies between patients and controls were found to be statistically significant ($p<0.001$). ACE DD genotype and D allele associated significantly with increased susceptibility to ischemic stroke in patients from the Malwa region of Punjab. In addition, D allele associated significantly with large artery atherosclerosis including both intracranial large artery as well as large extracranial large artery ($p<0.001$). Multiple logistic regression analysis with the potential confounding risk factors and ACE I/D gene variant revealed that the I/D polymorphism in ACE gene is significantly [$p = .015$] associated with ischemic stroke. We find significant association of this polymorphism with intracranial large artery atherosclerosis and extracranial large artery atherosclerosis. Our results indicate that the individuals bearing ID+DD genotype of ACE gene are at greater risk of developing stroke than II genotype.

1. Introduction

Stroke is defined as the focal and /or global dysfunction of the brain, spinal cord, and retina, lasting more than 24 hours (Sacco et al., 2013). It has been ranked as 2nd most common cause of death and a leading cause of adult disability worldwide (Krishnamurthi, Ikeda, & Feigin, 2020). Hypertension is a major risk factor in the development of vascular disorders including stroke. The blood pressure in the circulation is maintained by the orchestration between the various components of theRAAS (renin-angiotensin-aldosterone system) pathway (te Riet, van Esch, Roks, van den Meiracker, & Danser, 2015). Hypotension triggers the juxtaglomerular cells to release protease enzyme, renin which hydrolyzes angiotensinogen to angiotensin I in the circulation. Angiotensin I is a substrate for dipeptidyl carboxypeptidase, angiotensin converting enzyme (ACE) and gets converted to angiotensin II (Ang II). Ang II**,** the effector octapeptide of the RAAS pathway is a potent vasoconstrictor leading to increased blood volume within the vessels (Li et al., 2016). Ang II acts on transmembrane angiotensin II type1 receptor (AT1R) receptor which through certain signal transduction pathways including kinases and Gq/PLCpromote events like the release of aldosterone from adrenal glands, sodium and water reabsorption from kidneys leading to increased blood flow and hypertension (Forrester et al., 2018).

The use of FDA-approved ACE inhibitors in the mitigation and management of hypertension underscores the crucial role of the ACE enzyme in engendering pathological vascular changes leading to ischemic stroke. The genomic changes in the ACE gene modulate the level of circulating ACE enzyme leading to an increased level of AngII. This triggers hypertension, and inflammation in brain cells and thereby increases the risk of development of

ischemic stroke. The association between ACE gene variants and the development of ischemic stroke has been studied in different ethnic groups (Vasudeva, Balyan, & Munshi, 2020). These studies have provided compelling evidence of the involvement of ACE gene polymorphisms especially I/D polymorphism in intron16 with the activity of ACE enzyme as well as the development of stroke (Kumar et al., 2014; Malueka et al., 2018; Martínez-Rodríguez et al., 2013). We have already established the association of ACE I/D polymorphism with susceptibility to ischemic stroke and also found that higher levels of circulating ACE result in an increased risk of hemorrhagic stroke in patients from Andhra Pradesh (South India) (Satrupa Das et al., 2015; Pera, Slowik, Dziedzic, Wloch, & Szczudlik, 2006). In this current study, we investigated the association of I/D polymorphism of the ACE gene with ischemic stroke and its subtypes in patients from the Malwa region of Punjab, North India.

2. Materials and methods

2.1 Subjects

Since the study involved human blood samples, approval from the Institutional Ethics Committee was taken for the current study. In this case-control study, three hundred patients with ischemic stroke were enrolled from the department of neurology, Guru Gobind Singh Singh Medical College and Hospital, Faridkot (a tertiary level hospital) in the Malwa region of Punjab in the study from May 2019 to July 2020. Inclusion criteria: All the ischemic stroke patients included in the study were screened by a qualified stroke neurologist. Ischemic stroke and its subtypes were confirmed by evaluating the computed tomography (CT) and magnetic resonance imaging (MRI) reports of the patients. Exclusion criteria -Patients who at the time of sample collection were suffering from or reported a history of cancer, hepatic, renal, cardiac, and skeletal disorder were considered disqualified for the study. The equal number of sex and age-matched healthy individuals (male: female=200:100) with no history of any cerebrovascular disorder were recruited from the same geographic area. The blood samples were withdrawn from individuals only after getting the written informed consent. Information on demographic features, risk factors, clinical details, and stroke subtype was collected using a structured proforma or questionnaire. The study was approved by ethical committee of the hospital with letter vide no. UCER/BFUHS/2019/No. 2552-67 as well as Central University of Punjab with letter vide no. CUPB/CC/16/929.The power calculation for sample size was calculated by Open epi Software (Open Epi Version 2.3.1 from Department of Epidemiology, Rollins School of Public Health, Emory University, Atlanta, GA 30322, USA), the power of the study was found to be above 80 percent.

2.2 DNA isolation and genotypic of ACE I/D polymorphism

Three ml of blood was obtained by venipuncture from the subjects and collected in ethylenediaminetetraacetic acid (EDTA) coated vial. Genomic DNA was isolated using the standard phenol-chloroform method. Qualitative and quantitative analysis of DNA was carried out on 0.8 % agarose gel and using Thermo Scientific NanoDrop™ 1000 spectrophotometer respectively. DNA samples were diluted with Tris-EDTA (TE) buffer to get a concentration of 10ng/µl. The polymorphism in ACE gene bearing I/D polymorphism was detected using polymerase chain reaction (PCR). The following primers were used for the amplification of the ACE gene region bearing intron 16 polymorphism forward 5'-CTGGAGACCACTCCCATCCTTTCT-3' and reverse 5'-GATGTGGCCATCACATTCGTCAGAT 3'. The amplified PCR product was separated on 2 % agarose gel. The DD genotype was observed as 190 bp fragment whereas the II genotype was observed as 490 bp fragment.

2.3 Statistical analysis

The association of alleles and all the genotypes with increased risk of ischemic stroke was evaluated by the Open EPI6 software (Open Epi Version 2.3.1 from Department of Epidemiology, Rollins School of Public Health, Emory University, Atlanta, GA 30322, USA). The p-value, chi-square, and odds ratio with 95 % confidence interval

(CI) was observed for different genotypic models. The relationship between ACE I/D genotype and disease and confounding risk factors was also evaluated by multiple logistic regression (MLR method) using SPSS software. The independent variables were decoded as the following dummy variables: genotype (0 = normal homozygous, 1 = heterozygous and mutant homozygote); age (0 = <50, 1 = >50); Hypertension (0 = normotensive, 1 = hypertensive); Diabetes (0 = non-diabetic, 1 = diabetic); smoker (0 = non-smoker, 1 = smoker); alcohol use (0 = non-alcoholic, 1 = alcoholic).

3. Results

The demographic, as well as clinical data of ischemic stroke patients and controls, have been given in Table 1. The mean age of controls and ischemic stroke patients was 58.08 (9.8) and 58.23 (13.15) years respectively. The ischemic stroke patients and controls had a varying degree of risk factor profile. 57% of patients were hypertensive, 45% had diabetes, 12 % were smokers, 45 % reported alcohol use. Among the controls hypertension was reported in 30 %, 20 % had diabetes, 6 % smokers, and 19 % were alcoholics. The genotypic distribution of ACE I/D variant and their allelic frequencies observed in ischemic stroke patients and controls have been presented in Table 2. The genotypic distribution of the ACE I/D were in agreement with the Hardy-Weinberg law of equilibrium in both cases and controls. Statistical higher frequency of DD genotype was found in ischemic stroke patients in comparison with controls ($p<0.001$, odds ratio= 5.542 (95% CI; 3.041-10.1) (Table 2). Moreover, DD genotype and D allele was found to be significantly associated with the disease using different genotypic models (Table 3). The DD and ID genotype were significantly associated with the disease ($p=<0.001$, odds ratio=3.058 (95% CI;1.906-3.118). On evaluating the association of this polymorphism with stroke subtypes, intracranial large artery as well as extracranial large artery atherosclerosis showed a significant association ($p=<0.001$, odds ratio=2.035 (95% CI;1.465-2.826); $p=<0.001$, odds ratio=4.474 (95% CI;1.967-10.17 respectively) (Table 4). A multiple logistic regression analysis was carried out to confirm these findings using the multiple logistic regression method. The results of this analysis revealed a significant association of variant genotype ID+DD (heterozygous + mutant homozygotes) with disease ($p = .015$) and none of the other confounding risk factors associated significantly with the disease ($p >.05$).

Table 1 Clinical characteristics of stroke patients and controls

Characteristics	Ischemic stroke patients (n=300)	Controls (n=300)	p-value
Age	58.23 (13.15)	58.08 (9.8)	
Male: Female (number of males: females)	200 : 100	200 : 100	
Hypertension (percentage of patients)	57%	30%	<.001
Diabetes (percentage of patients)	45%	20%	<.001
Smoker (percentage of male subjects)	12%	6%	>.05
Alcohol use (percentage of male subjects consuming alcohol)	44%	19%	<.001

Table 2 Distribution of ACE genotypes and allelic frequencies of the study population

Study group	ACE genotypes			Allele frequency			
	II	ID	DD	Total	I	D	Total
Patients n (%)	84	167	49	300	335	265	600
Control n (%)	171	111	18	300	453	147	600

Table 3 Analysis of ACE genotypes and alleles among hemorrhagic stroke patients and controls

	OR (95% CI)	**$\chi 2$**	**p-value**
II vs DD	5.542 (3.041-10.1)	33.72	<0.001
II vs ID	3.063 (2.148, 4.367)	38.21	<0.001
DD vs ID	1.809 (1.002, 3.268)	3.397	0.02
Dominant			
ID +DD vs II	2.138 (1.542–2.964)	20.28	<0.001

Table 4 ACE genotypic and allelic frequencies in stroke patients classified according to TOAST classification.

TOAST classification	No. of patients	Genotype (%)			Allelic frequencies		Odds ratio (95% CI)	p value
		II	ID	DD	I	D		
1. Large artery atherosclerosis subtypes	202							
a. Intracranial large artery (ILA) b. Extracranial large artery (ELA)	166	48	98	20	194	138	2.035 (1.465-2.826)	<0.001
	36	10	18	8	38	34	3.13 (1.52-6.453)	<0.001
2. Small artery occlusions (SAO) (Lacunar)	53	11	30	12	52	54	1.52 (0.88-2.62)	>.05
3. Cardioembolism (CE)	31	10	12	9	32	30	1.377 (0.68-2.76)	>.05
4. Other determined etiology (ODE)	3	-	3	-	3	3	-	-
5. Undetermined etiology (UE)	11	5	6	-	16	6	-	-

4. Discussion

The RAAS system including renin, angiotensinogen, ACE, (AT1R), and aldosterone synthase (CYP11B2) regulates hypertension and also maintains electrolyte and fluid balance (Prasad et al., 2006). The gene encoding human angiotensin converting enzyme (ACE) (kininase II) is an important component in the RAAS, consists of 26 exons, and is located on the long arm of chromosome 17q23 (rs NG011648). ACE is a dipeptidyl carboxylase (rate-limiting enzyme of the renin-angiotensin system (RAS), that catalyzes the conversion of inactive angiotensin I to active angiotensin II, a potent vasoconstrictor and involved in the degradation of bradykinin (a potent vasodilator). Angiotensin II is the peptide involved in the modulation of vascular tone and smooth muscle cell proliferation and acts as an effector molecule that regulates the cell signaling thereby modulating the expression of genes involved in the pathogenesis of vascular diseases including hypertension, atherosclerosis, and stroke (Forrester et al., 2018; Guler, 2021; Mehta & Griendling, 2007). Studies in animal models of stroke and hypertension have suggested that overexpression of Ang II in neurons, glial cells, vascular smooth muscle cells, and endothelial cells resulting in inflammation, hypertension, vascular smooth muscle proliferation, cardiac fibrosis, and hypertrophy (Biancardi, Bomfim, Reis, Al-Gassimi, & Nunes, 2017; Sadhan Das et al., 2018; Husain, Hernandez, Ansari, & Ferder, 2015; Xue et al., 2016) [Fig 1]. Several studies involving various candidate gene studies have established that the ACE gene is an important candidate for the pathogenesis of hypertension and atherosclerosis and stroke. The I/D polymorphism of the ACE gene accounts for half the variance in serum ACE levels. The insertion/deletion (I/D) polymorphism of ACE gene was first reported by Rigat et al (1990) (Rigat et al., 1990). The presence (insertion) or absence (deletion) of a 287-bp AluYa5 portion within intron 16 causes three genotypes (II homozygote, ID heterozygote, and DD homozygote). Although I/D polymorphism is located in an intronic region, several investigators have found that the

D allele is associated with increased activity of ACE in the serum. The highest serum ACE activity has been observed in individuals bearing the DD genotype whereas it is lowest in II genotype bearers (Dai et al., 2019)**.** As the ACE I/D polymorphism is partially associated with the plasma ACE level, the ACE DD genotype increases the plasma ACE concentration and the risk of various cardiovascular and renal diseases like myocardial infarction, cardiomyopathy, stroke, IgA nephropathy, and diabetic nephropathy. Higher level of ACE results in less bradykinin binding to endothelial B2-kinin receptors, resulting in decreased nitric oxide (NO) production and vasodilatory response, which play an important role in the pathophysiology of stroke, vasoconstriction, and atherosclerotic processes. In stroke patients, the increased ACE level in individuals with DD genotype leads to an enhanced level of AngII that eventually adds up to the infarct size and increases the oxidative stress and inflammation worsening the brain damage and also the functional outcome of ischemic stroke (Ferrari, 2004)**.**

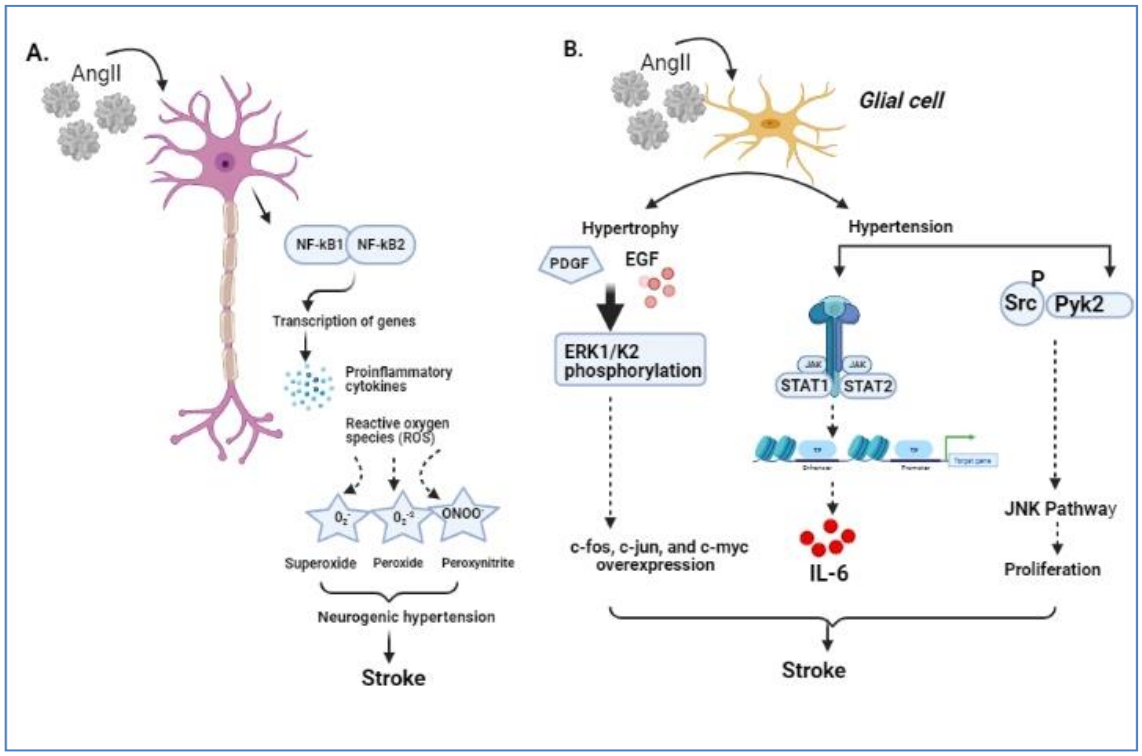

Figure 1 A. In neurons**,** Ang II promotes the nuclear translocation of nuclear factor kappa-B (NFkB) leading to the transcriptional activation of genes implicated in hypertension. It involves overexpression of proinflammatory cytokines, reactive oxygen species (ROS) including superoxide, peroxynitrite, and underexpression of neuronal nitric oxide synthase (nNOS). The increased peroxynitrite affects the Na+ and K+ ion channels of the neuronal cell membrane. It triggers the altered neuronal firing leading to enhanced sympathetic nerve function. This facilitates the hypertensive condition and in turn stroke. **B.** In glial cells, Ang II mediated activation of Angiotensin 1 receptor (AT1) and the membrane-bound tyrosine kinase platelet-derived growth factor (PDGF) receptor, epidermal growth factor (EGF) receptor leads to phosphorylation of ERK1/2. This stimulates astrocyte proliferation and over expression of early response genes, namely c-fos, c-jun, and c-myc. The c-fos, c-jun and c-myc are involved in dysregulation of many other genes implicated in molecular mechanisms that lead to hypertrophy and stroke.
Ang II activates AT1 receptor and triggers the non-receptor tyrosine kinase JAK/STAT (Janus kinases/ signal transducer and activator of transcription proteins) pathway leading to the transcriptional activation of gene encoding of interleukin-6 (IL-6). The increased levels of IL-6 in glial cells further enhances the levels of plasma C-reactive protein (CRP) that initiates a low grade inflammation.
In astrocytes, Ang II induces the phosphorylation of Src and Pyk2. It can leads to hypertension either by activation ERK1/2 causing astrocyte growth or activation of protein serine/threonine kinase c-Jun N-terminal kinase (JNK) leading to astrocyte proliferation. Astrocytes has high levels of the precursor molecule of Ang II i.e. angiotensiogen. The central Ang II levels further increase upon astrocyte proliferation. Hyperactive brain RAAS enhances the sympathetic nervous system activity leading to hypertension (Figure has been prepared using bio render software).

The Malwa region of Punjab has come under focus because of an increased prevalence of many monogenic and multifactorial diseases including ischemic stroke. However, no empirical studies have been carried out to find out significant risk factors including genetic component contributing to development of the disease. We are exploring genes involved in different pathways implicated in the pathogenesis of the disease. Since ACE is a significant enzyme involved in the regulation of hypertension which is one of the most important significant risk factors of the disease. No such studies have been carried to find out the association of ACE I/D polymorphism (most common variation) and ischemic stroke. In addition, the study also explored the significance of this variation as far as the development of various stroke subtypes is concerned. Moreover, previous studies have only focussed on this variation without controlling the significant risk factors. A stepwise multiple logistic regression analysis has been carried out to control the significant risk factors to authenticate the association of this variant with the development of stroke and its subtypes. To the best of our knowledge, this is the first study to evaluate the association between ACE I/D variant with the risk of ischemic stroke and its subtypes in the population from the Malwa region from Punjab. We found a significant difference in the frequency of DD and ID genotypes between ischemic stroke patients and controls. A significant difference was also observed in the allelic frequency between the patients and healthy controls. Moreover, similar findings were observed in our previous study, where we have reported a significant association of ACE I/D with susceptibility to ischemic stroke in patients from Andhra Pradesh, South India (Munshi et al., 2008). In another study on the Indian population by Kalita et al. (2011), the frequency of DD genotype ranged from 37.8% in cases and 11.7% in controls. However, ethnic differences have been reported in the allelic and genotypic frequency of this locus (Kalita, Somarajan, Kumar, Mittal, & Misra, 2011). Some recent studies involving Indian population and Turkish population have also established that DD genotype significantly associated with the development of ischemic stroke (Anisimova et al., 2019; Guler, 2021; Tutāne, 2017).

On the contrary, the D allele of the ACE gene was not found to be associated with the risk of ischemic stroke in a Polish population and subsequent case-control studies conducted on Greece and the Turkish community by Tuncer et al. and Karagiannisa et al. were in consensus with the study carried out in Polish population (Celiker et al., 2009; Tuncer et al., 2006)**.** In a study conducted to assess the association of ACE I/D polymorphism with the disease, no interdependence of D allele with other polymorphism was found (Tascilar et al., 2009; Tutāne, 2017)**.** In an meta-analysis carried out by Wei et al. (2018) involving 99 studies, a significant association of I/D polymorphism with the ischemic stroke development in Caucasian and Asian population was found (Wei et al., 2017). Another study also established a significant association of ACE I/D polymorphism with functional outcome in ischemic stroke patients (Wei et al., 2017).

On evaluating the association of this variant with stroke subtypes, a significant association was observed between the DD genotype and intracranial large artery atherosclerosis and extracranial large artery atherosclerosis. The results of this current study are in conformity with our previous study from South India, where we reported the association of this variant of the ACE gene with intracranial large artery atherosclerosis (Munshi et al., 2008). However, in a Japanese population, a significant effect of ACE polymorphism was observed on lacunar infarction (Mizuno et al., 2003). A significant association between ACE gene polymorphism was observed with thrombotic brain infarction in another Japanese population (Doi et al., 1997). In a case-control study by Kumar et al., D allele was found to be a risk factor for the small vessel disease (SVD) in the North Indian population. A meta-analysis carried out by Zhang et al. (2014) involving 10070 cases of different ethnicity, reported D allele as a low susceptibility penetrance marker, and found its association with 37% higher risk of SVD in Asians and just a borderline significance in Europeans (Zhang et al., 2012)**.** Contrary to the above findings, a study conducted on 200 South Indian cases outlined a positive association of II genotype with large vessel disease (LVD) (Vijayan et al., 2014). The limitations of this study include low sample size and lack of replication in an independent cohort.

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Authors contribution: *Anjana Munshi (AM) and Kanika Vasudeva (KV) designed the study, Kanika Vasudeva (KV) collected blood samples and clinical data from patients. Dr. Sulena (S) helped in recruitment of patients. KV carried out the genotypic analysis. AM and KV prepared the manuscript. AM edited the manuscript.*

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