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Effects of 469 E/K polymorphism of ICAM1 gene in ischemic stroke and its association with stroke severity and outcome

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ABSTRACT

Background: Stroke is the second leading cause of death globally and it is a major cause of long-term, physical, psychological, and social disability among the elderly. Increasing evidence shows that ischemic injury and inflammation account for its pathogenic progression. So, we studied the association of Intercellular Adhesion Molecule 1 *(ICAM1)* polymorphism with ischemic stroke, stroke severity, and outcome. **Aims and Objectives:** To compare *ICAM1* 469 E/K polymorphism in ischemic stroke patients with healthy controls, and to study its association with stroke severity and outcome.**Materials and Methods:** Fifty patients of ischemic stroke and hundred healthy individuals were included. The stroke severity was assessed clinically and radiologically. Outcome was measured at three and six months of stroke onset. Genomic DNA was used for Allele-Specific PCR to detect *ICAM1* 469 E/K polymorphism. The subjects were categorized into EE, EK, and KK genotypes. **Results:** The odds of EK genotype to develop stroke was 0.41 (95 % CI; 0.17 - 0.92) (p = 0.07) and of KK genotype was 0.41 (95 % CI; 0.11 - 0.87) (p = 0.04) compared to EE genotype. Subjects with *ICAM1K* allele had significantly reduced risk of stroke compared with those with E allele. (RR: 0.55; 95% CI: 0.35-0.87) (p=0.03). **Conclusion:** Subjects with *ICAM1*K allele had significantly reduced the risk of developing stroke. 469 E/K polymorphism of the *ICAM1* gene does not significantly affect stroke severity, mortality, and outcome.

Key words: Ischemic stroke; Intercellular Adhesion Molecule-1; Polymorphism; Polymerase Chain Reaction

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INTRODUCTION

Stroke is the second leading cause of death globally,[1] and is the major cause of long-term physical, psychological and social disability among the elderly. Broadly, stroke is classified into ischemic stroke and hemorrhagic stroke.[2] Ischemic stroke is characterized by the sudden loss of blood flow to an area of the brain caused by thrombotic or embolic occlusion of a cerebral artery. Clinical diagnosis of acute ischemic stroke is done through history, examination, and neuroimaging.

Pathogenesis of stroke involves multiple mechanisms like ischemic injury and inflammation.[3] ICAM-1 (Intercellular

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Adhesion Molecule 1) also known as CD54 (Cluster of Differentiation 54) is a cell adhesion molecule and a surface glycoprotein of immunoglobulin superfamily,[4] expressed in endothelial cells and cells of immune system. Ligand for ICAM-1 is an integrin superfamily member namely leukocyte function-associated antigen-1 (LFA-1), also known as CD11a/CD18. ICAM-1/LFA-1 interaction which is essential for leukocyte infiltration into tissue, cell proliferation and local inflammatory response; it is an important factor for thrombosis in ischemic stroke. SNP in the sixth exon of *ICAM1* gene (rs5498) is also associated with stroke risk.[5,6] Soluble intercellular adhesion molecule-1 (sICAM-1) is the circulating form of ICAM-1.[7] Plasma sICAM-1 levels have been correlated with the risk of ischemic stroke,[8] peripheral arterial disease[9] and type 2 diabetes mellitus.[10]

Studying the effect of the *ICAMI* gene polymorphism can help to identify genetic profiles that are associated with high risk of stroke, along with the severity and outcome in acute ischemic stroke. Studies relating *ICAM1* 469 E/K polymorphism in ischemic stroke studies on Indian population have been sparse. Therefore, we have devised this study to understand the role of *ICAM1* genetic polymorphism in ischemic stroke in north Indian population.

MATERIALS AND METHODS

This retrospective case control study was conducted in the Department of Biochemistry, Maulana Azad Medical College and the Department of Neurology, Govind Ballabh Pant Institute of Postgraduate Medical Education & Research, New Delhi from September 2012 to December 2014, after obtaining approval from the Institutional Ethical Committee (F.11/IEC/MAMC/2012/130). The investigation conforms to the principles outlined in the Declaration of Helsinki. 50 adult patients (>18 years) of either sex, with history of ischemic stroke diagnosed by history and MRI brain were selected as cases. Patients presenting with hemorrhagic stroke, transient ischemic attack (TIA), cardio embolic stroke, history of fever in the recent past (1 week prior to stroke), rheumatological, autoimmune disease, or any kind of acute or chronic infection were excluded. Patients with impaired hepatic function or renal function or those patients on immunosuppressive therapy or regular analgesic intake were excluded. 100 age and sex matched healthy controls were also selected for the study.

Written informed consent was obtained from all the study subjects. The study group was subjected to structured questionnaire (regarding demographic, medical, lifestyle and reproductive information). A detailed history and baseline National Institutes of Health Stroke Scale/Score (NIHSS)[11] were used for assessment of clinical severity. Radiological severity was assessed by calculation of infarct volume by diffusion weighted magnetic resonance imaging (DWI). The etiological classification of acute ischemic stroke was done by TOAST (Trial of ORG 10172 in Acute Stroke Treatment) criteria.[12]

The outcome was measured at 3 months and 6 months in terms of stroke recurrence, death, disability, functional dependence and cognitive functioning. Disability was assessed by Modified Rankin scale[13] and functional dependence was assessed by Barthel index.[14] Cognitive functioning was assessed by Mini-Mental State Exam (MMSE).[15]

Blood sampling for genetic analysis was done within three months after the onset of stroke. 5 mL of peripheral blood sample was collected in EDTA vial for DNA analysis. It was stored at -80 degree Celsius till further analysis. DNA from peripheral blood samples was extracted by genomic DNA Mini Kit (Genaid, Taiwan) according to the manufacturer's instructions and was stored at -20 ^{0}C.

ICAM1 469 E/K polymorphism was detected by Allele Specific PCR (GeneQ™ Thermal Cycler, Bulldog Bio, Portsmouth, USA)using the following allele specific primers:

G-allele specific forward primer:	5'-CACTCAAGGGGAGGTCAC CCGCG-3'
A-allele specific forward primer:	5'-CACTCAAGGGGAGGTCA CCCGCA-3'
Common Reverse primer:	5'-TTGTAGTCTGTATTTCTT GATCTT-3'

PCR conditions were: initial denaturation at 94°C for 10 minutes for 1 cycle, 35 cycles of denaturation at 94°C for 40 seconds, annealing at 55°C for 1 minute and extension at 72°C for 1 minute followed by final extension 72°C for 10 minutes 1 cycle. The amplified products were resolved using electrophoresis in 1.5% agarose gel. Product length obtained was of 249 bp (Figure 1).

Statistical analysis

The data entered in excel sheet was analysed using Statistical Package for the Social Sciences (SPSS) version 18 and VassarStats.net programs.Shapiro-Wilk test was employed to test for the normality of data; at p-value< 0.05, the null hypothesis that data is normally distributed was rejected. Parametric data were expressed in mean and standard deviation. Nonparametric data were expressed as median and quartiles. The continuous data was subjected to unpaired t-test. Dichotomous variables were analysed by

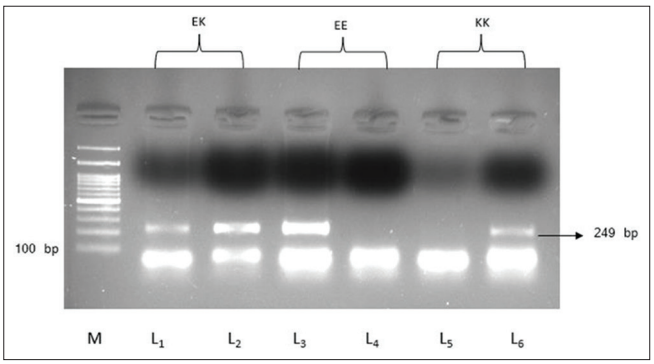

Figure 1: Result of AS PCR results of 3 cases for detection of rs5498. M - 100 bp DNA Ladder. L1, L3, L5: AS PCR for E allele. L2, L4, L6: AS PCR for K allele

Chi-square test. E allele was taken as a reference to compare the frequency of K allele.p-value< 0.05 was considered statistically significant.

RESULTS

Demography

The age range of case population was 17 to 85 years with the mean age of 55.24 ± 15.4 years and that of control population was 8 to 74 years with the mean age of 53.38 ± 14.5 years. The age (p=0.47) and sex (p=0.58) distribution of control group were similar to the cases in our study (Table 1). Among 50 cases included in this study, 40 were males and 10 were females. Control group includes 100 subjects of which 76 were males and 24 were females.

Stroke characteristics

Stroke subtypes: Based on the TOAST criteria, the largest stroke subtype in our study population consisted of patients of large-artery atherosclerosis (40%). Second largest group was patients with small-vessel occlusion (32%). As cardio embolic stroke patients were excluded from our study, we got only 4 categories of patients out of the 5 TOAST categories (Table 2).

Stroke severity: Median value of National Institutes of Health Stroke Scale (NIHSS)score was 4. 72% of cases had mild stroke, 14% had moderate stroke and 14% had severe stroke based on NIHSS score (Table 1). Median Diffusion-weighted imaging (DWI)volume was 1.59 mL (0.04-243.3). Ninety-two percent of patients had mild stroke, 4% had moderate stroke and 4% had severe stroke. Thus these 2 scales highly differ in their quantifying capacity of stroke severity (Table 3).

Outcome

Out of the total 50 patients enrolled in the study, 3 patients died within 3 months and 47 patients completed 6 months follow up. No one had recurrence. Outcome assessed

Table 1: Age and Sex distribution of cases and controls

Parameter	Cases (n = 50)	Controls (n =100)	p value
Age in years (Mean ± SD)	55.24 ± 15.4	53.38 ± 14.5	0.47*
Sex (Male: Female)	40:10	76:24	0.58†

*unpaired t-test
†z-test

Table 2: TOAST categorization of patients

TOAST Category	n %
Large-artery atherosclerosis	20 (40%)
Small-vessel occlusion	16 (32%)
Stroke of undetermined etiology	10 (20%)
Stroke of other determined etiology	4 (8%)
Cardioembolism	0 (0%)*
Total	50 100%)

*Excluded from study

Table 3: Distribution of cases based on stroke severity

NIHSS		
Score	**Severity**	**n (%)**
0-7	Mild	36 (72)
8-13	Moderate	7 (14)
>14	Severe	7 (14)
Diffusion weighted imaging		
Infarct volume (ml)	**Severity**	**n (%)**
0-30	Mild	46 (92)
31-60	Moderate	2 (4)
>60	Severe	2 (4)

Modified Rankin Scale (MRS), Barthel and Mini-Mental State Exam (MMSE) were divided into two groups i.e., good outcome and bad outcome (Table 4).

At 3 months, 46% of patients had MRS with good outcome (0-1) and 54% had bad worse outcome (2-6). Similarly using Barthel index 63.8% had good outcome score (>85%) and 36.2% had bad outcome (Score ≤85). It is evident from the table that these scores improved at 6 months.

At 3 months, 79.5% patients had good cognitive outcome (MMSE Score >22) and 20.5% had bad outcome (MMSE Score ≤22). At 6 months, 86.4% had good cognitive outcome and 13.6% had bad cognitive outcome. Dead patients (n = 3) and aphasic patients (n = 3) were excluded from MMSE.

ICAM1 469 E/K polymorphism

Distribution: The distribution of *ICAM1* genotypes and alleles between cases and controls were studied and were found to be statistically insignificant (Table 5).

Risk of developing stroke: The subjects with *ICAM1* K allele were found to have significantly reduced risk of developing stroke compared to those with *ICAM1* E allele (Table 6).

ICAM1 469 E/K polymorphism and stroke severity & TOAST subtypes: The association of *ICAM1* genotypes with stroke severity and TOAST subtypes were assessed by clinical and radiological means. No statistically significant association was found (Table 7).

Association of ICAM1 469 E/K Polymorphism and long-term outcome: Association of *ICAM1* 469 E/K Polymorphism and long-term outcome measured by MRS, BI and MMSE at 3 months and 6 months were analysed by Fisher Exact probability test. We did not find any association of *ICAM1* 469 E/K genotypes and long-term outcome (Table 8).

Table 4: Stroke outcome

MRS	Range	Good outcome (Score 0-1)	Bad outcome (Score 2-6)
MRS-3 months	0-6	23/50 (46%)	27/50 (54%)
MRS-6 months	0-5	26/47 (55.3%)	21/47 (44.7%)
Barthel	**Range**	**Good outcome (Score >85)**	**Worse outcome (Score ≤85)**
Barthel-3 months	0-100	30/47 (63.8%)	17/47 (36.2%)
Barthel–6 months	0-100	35/47 (74.5%)	12/47 (25.5%)
MMSE	**Range**	**Good outcome (Score >22)**	**Worse outcome (Score ≤22)**
MMSE-3 months	14-30	35/44 (79.5%)	9/44 (20.5%)
MMSE-6 months	18-30	38/44 (86.4%)	6/44 (13.6%)

Association of ICAM1 genotypes with mortality: Out of the 3 patients died, 2 had EK genotype and the other one had EE genotype. Frequency of E allele in dead patients was 4% and frequency of K allele was 2%. When these results were analysed, no statistically significant association was found.

DISCUSSION

The present study was designed to find the effects of 469 E/K polymorphism of ICAM1 gene in ischemic stroke and its association with severity and outcome. We found that the E and K allele frequencies were not statistically significant between cases whereas significant statistical significance was found in genotypes. This can be explained by the high heterozygosity of this polymorphism.[16] The absence of statistically significant association between the 469 E/K polymorphism and stroke severity and outcomecan be attributed to the small sample size of this study.

469 E/K polymorphism is a non-synonymous missense variant leading to alteration in the charge of the amino acid side chain at the fifth immunoglobulin domain which could affect the binding of ICAM-1 to LFA-1. 469 E/K polymorphism has also been shown to affect the mRNA splicing pattern[17] and the level of soluble ICAM-1.[18] This can explain the role of this polymorphism in thedysregulated inflammation andatherosclerosis leading to ischemic stroke.Future in silico modelling and protein analysis studies can shed more light into this area.

In our study group, 14 % of stroke patients were less than 40 years of age (young stroke). This agrees with the finding that the incidence of young stroke is higher in India compared

Table 5: Distribution of *ICAM1*469 E/K polymorphism in cases and controls

Genotype	EE n (%)	EK n (%)	KK n (%)	p value*	E allele frequency (%)	K allele frequency (%)	p value*
Cases (n = 50)	15 (30)	24 (48)	11 (22)	0.0547	54	46	0.0890
Controls (n=100)	14 (14)	54 (54)	32 (32)		41	59	

*Chi-Square test

Table 6: Odds ratio and Relative risk of *ICAM1* 469 E/K genotypes in the development of stroke

Geno type	Cases n (%)	Controls n (%)	OR (95% CI)	RR (95% CI)	p-value*
EE	15 (30)	14 (14)	1 (ref)		
EK	24 (48)	54 (54)	0.41 (0.17-0.92)	0.59 (0.36-0.96)	0.07
KK	11 (22)	32 (32)	0.32 (0.11-0.87)	0.49 (0.26-0.91)	0.04
EK+KK	35 (35)	86 (86)	0.37 (0.16-0.86)	0.55 (0.35-0.87)	0.03

* p value for Odds ratio

Table 7: Association of *ICAM1*469 E/K genotypes with TOAST sub-types

Genotype	Large-artery atherosclerosis vs. Rest	Small-vessel occlusion vs. Rest	Stroke of other determined etiology vs. Rest	Stroke of undetermined etiology vs. Rest
EE				
Present	5	5	1	3
Absent	15	11	3	7
p Value	0.75†	0.74†	> 0.9*	> 0.9*
EK				
Present	12	8	1	3
Absent	8	8	3	7
p Value	0.24†	0.92†	0.61*	0.29*
KK				
Present	3	3	2	4
Absent	17	13	2	6
p value	0.31*	0.72*	0.23*	0.22*

* Fischer exact test
†Pearson's Chi-Square test

Table 8: Association of *ICAM1* 469 E/K genotypes and long-term outcome

Outcome at 3 months by MRS	EE n (%)	EK n (%)	KK n (%)
Good (Score 0-1)	5	11	7
Bad (2-6)	10	13	4
		p = 0.30by Fisher Exact Probability	
Outcome at 6 months by MRS	**EE n (%)**	**EK n (%)**	**KK n (%)**
Good (Score 0-6)	7	14	7
Bad (2-6)	8	10	4
		p = 0.70 by Fisher Exact Probability	
Outcome at 3 months by BI	**EE n (%)**	**EK n (%)**	**KK n (%)**
Good (>85%)	10	10	7
Bad (≤85)	4	12	4
		p = 0.28 by Fisher Exact Probability	
Outcome at 6 months by BI	**EE n (%)**	**EK n (%)**	**KK n (%)**
Good (>85%)	11	13	8
Bad (≤85)	3	9	3
		p = 0.52 by Fisher Exact Probability	
Outcome at 3 months by MMSE	**EE n (%)**	**EK n (%)**	**KK n (%)**
Good (<22)	11	16	8
Bad (≥22)	2	4	3
		p = 0.79 by Fisher Exact Probability	
Outcome at 6 months by MMSE	**EE n (%)**	**EK n (%)**	**KK n (%)**
Good (<22)	12	18	8
Bad (≥22)	1	2	3
		p = 0.44 by Fisher Exact Probability	

to Western countries.[19] Our results of the association of the ICAM1 polymorphism is in accordance with the study by Pola R *et al* in Italian population[20] and in discordance with the study by Wei YS *et al* in Zhuang Chinese population.[21] This suggests that findings of studies done on one ethnic group cannot be extrapolated for another population. Moreover, Indian population is ethnically diverse.

CONCLUSION

In this study, we found that subjects with *ICAM1*K allele had significantly reduced the risk of developing stroke compared to those with *ICAM1*E allele. 469 E/K polymorphism of the *ICAM1* gene does not significantly affect stroke severity, mortality, and outcome.

LIMITATIONS OF THE STUDY

Our results of association of stroke severity and outcome with the ICAM-1 polymorphism may change when the study is done in a large population. This necessitates further studies with large sample.Our study subjects are mainly from the northern parts of India. So, our study results cannot be extrapolated to whole Indian population. We feel the need of a study to cover all ethnic groups of India.

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KP and AS- Carried out the wet lab work under the supervision of MK and PG. KP and AS also drafted the manuscript; **DC and SA**- Assisted in the experiments and provided valuable suggestions. MK mentored the project; **AK and RD-** Provided with valuable inputs and critical comments. **SK**- Overseen the whole work, interpreted the results and helped in communicating the research.

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