Original Article

Association of *APOE* Gene Polymorphism with Stroke Patients from Rural Eastern India

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Abstract

Context: Studies from the different ethnic regions of the world have reported variable results on association of APOE gene polymorphism in stroke. **Aim:** The aim of this study is to find out the possible association of APOE polymorphism in stroke patients in ethnic Bengali population. **Settings and Design:** A prospective case–control study was undertaken in the Department of Neurology, Burdwan Medical College, Burdwan, West Bengal, India, over a period of 3 years. **Methods:** We collected 10 ml venous blood samples from 148 clinically and radiologically diagnosed acute stroke patients (80 of ischemic stroke and 68 of intracerebral hemorrhage) and consecutive 108 ethnic age- and sex-matched controls, in ethylenediaminetetraacetic acid vials after informed written consent. Genomic DNA was prepared at S.N. Pradhan Centre of Neurosciences, University of Calcutta, Kolkata, India. Exotic single-nucleotide polymorphisms (rs429358, rs 7412) were analyzed by polymerase chain reaction-restriction fragment length polymorphism for genotype of APOE. **Results:** The frequencies of different *APOE* allele among 80 ischemic stroke patients were 5.6% (n = 9) for E2, 75.68% (n = 121) for E3, and 18.7% (n = 30) for E4. The E3 allele is significantly over-represented (P = 0.004) in controls compared to the patients (88% in controls vs 75.6% ischemic stroke patients and 80% hemorrhagic patients). A significantly high frequency of APOE4 allele was observed in ischemic (18.7%) and hemorrhagic patients (11%) compared to controls (8%). The E4 allele plays a major risk for developing ischemic stroke [odds ratio (OR) = 2.744; 95% confidence interval (CI): 1.43–5.10] and E3 plays a protective role for hemorrhagic stroke (OR = 0.53; 95% CI: 0.29–0.96), while E4 allele plays a nonsignificant (P = 0.31) increase in trend in hemorrhage stroke (OR = 1.4). **Conclusions:** There is significant association of APOE gene polymorphism in stroke patients of ethnic Bengali population. The E4 allele increases significant risk for development of ischemic strokes, and it also plays nonsignificant increase in trend in hemorrhagic strokes.

Keywords: Apolipoprotein E, hemorrhage stroke, ischemic stroke, polymorphisms, stroke

Introduction

Stroke is the second most common cause of death and disability worldwide.[1,2] It is a multifactorial disease influenced by both environmental and genetic factors.[3] With its heterogeneous etiopathogenesis, the exact role of genetics in causing stroke remains controversial; family history studies, twin studies, and studies adopting candidate gene approaches have produced diverse results.[4] Nonetheless, there is a definite suggestion that susceptibility to stroke is influenced by genetic factors and various mendelian stroke syndromes have been identified in humans.[5,6]

The human apolipoprotein E (ApoE) is a serum glycoprotein consisting of 299 amino acids and is located on chromosome 19q13.2 and consists of four exons.[7] Exon 4 of *APOE* harbors two polymorphic sites at codon 112 and 158, respectively, thereby yielding three isoforms: ApoE2 (T/T, Cys112/Cys158), ApoE3 (T/C, Cys112/Arg158), ApoE4 (C/C, Arg112/Arg158) and six APOE genotypes (E2/2, E2/3, E2/4 E3/3, E4/4, and E3/4). Different populations worldwide inherit variable frequencies of the *APOE* alleles and genotypes, with the most frequent allele being E3.[8] Since the first demonstration of *APOE* gene polymorphism in dysbetalipoproteinemia,[9] numerous studies have undertaken to investigate the significant correlation between *APOE* genotypes with cholesterol metabolism, atherosclerosis, ischemic heart disease, and cerebral amyloid angiopathy and stroke. Studies from the different ethnic regions of the world have reported variable results on association of *APOE* gene polymorphism in stroke, both for intracerebral hemorrhage and ischemic strokes. With this background, the purpose of the present study is to find out the possible association of *APOE* gene polymorphisms with stroke subtypes in ethnic Bengali population.

Methods

A prospective hospital-based case–control study was undertaken in the Department of Neurology Burdwan Medical College, Burdwan, West Bengal, India over a period of 3 years

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from September 2012 onwards after Ethical Committee clearance. A total of 148 consecutive patients (80 patients of ischemic stroke and 68 patients of hemorrhagic strokes) and 108 age-matched controls without any history of stroke or other neurological dysfunction were recruited in this study. A written informed consent was taken from the participants, including cases and controls. Patients who were admitted as acute stroke cases in the Department of Neurology were selected randomly after clinical and radiological diagnosis was confirmed. A diagnosis of ischemic stroke was made clinically and verified by noncontrast computed tomography (NCCT) brain, CT angio brain and neck vessels, and many cases by MRI [diffusion weight images (DWI)]. Hemorrhagic stroke case was diagnosed by NCCT brain. All the patients included in the study represented ethnic Bengali population, and all were ≥18 years of age. Ischemic stroke patients due to cardioembolic, infective, vasculitis, and cortical venous thrombosis etiology were excluded. Transient ischemic attack patients were also excluded. The National Institutes of Health Stroke Scale (NIHSS) was used as a clinical parameter in ischemic strokes cases.

Similarly, acute hemorrhagic strokes secondary to arteriovenous malformation, cavernomas, subarachnoid hemorrhage, cortical venous thrombosis, and anticoagulation were excluded from the study. Controls were taken from the same geographical area and were age- and sex-matched to the cases. The controls did not suffer any stroke or coronary artery diseases. The controls were subjects who were attending the outpatient department for minor neurological or nonneurological problems. Sometimes controls were the normal healthy attendants of stroke patients.

Approximately 10 ml of peripheral blood sample in ethylenediaminetetraacetic acid (EDTA) tubes were collected from patients and controls, and written and informed consent from patients and from normal individuals as control was taken making sure about adequate understanding by the donors. The experiments were conducted in accordance with the Helsinki Declaration. The internal review committee on research using human samples cleared the project after proper review as per regulation of Indian Council of Medical Research. Genomic DNA was prepared from fresh whole blood by using conventional salting out method using sodium perchlorate followed by isopropanol precipitation.[10] Genomic DNA was dissolved in TE (10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0).

APOE genotypes were determined by polymerase chain reaction (PCR) and restriction fragment length polymorphism techniques [Figure 1]. The primer sequences were forward: 5′-TCGGAACTGGAGGAACAACT-3′ and reverse: 5′-CCTGCTCCTTCACCTCGT-3′. PCR mixtures contained 150 ng of each primer and 50–80 ng DNA in the presence of 2.5 mM magnesium, 2.5 mM dNTPs, and 1 U of TaqTM DNA polymerase (Roche Diagnostis, Indianapolis, IN, USA). The cycle conditions were initial denaturation of 10 min at 94°C followed by 35 cycles (94°C for 30 s; 68°C for 30 s; 72°C for 30 s) with final extension of 10 min at 72°C. The PCR

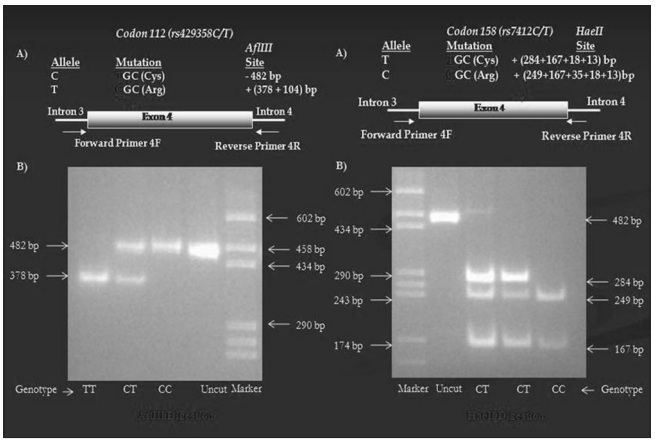

Figure 1: Representation picture of Restriction Fragment Length Polymorphism (RFLP) Analysis

product was digested for 3 h at 37°C with the use of 1 U of the restriction enzymes (AflIII and HaeII). The digested products were separated on a 6% polyacrylamide gel in 1× Tris–borate–EDTA buffer with a peptide binding site/HaeIII DNA marker. The gel was stained with ethidium bromide and visualized in a Gel documentation system (BIORAD).

Significant deviation of the genotype frequency from Hardy-Weinberg equilibrium at each polymorphic variant was tested by χ^2 with 1 degree of freedom. For the association study, the data were evaluated for *P* value, odds ratio (OR), and 95% confidence interval (CI) by using the "Online Statistical Calculations" (statpages.org/ctab2×2.html) by John C. Pezzullo, Georgetown University, Washington, DC, USA.

Results

A total of 148 patients, comprising 80 patients with ischemic stroke and 68 with intracerebral hemorrhage, and 108 age- and gender-matched healthy controls from the same geographical location and ethnic background were recruited in this study. Demographic details of the study subjects are summarized in Table 1.

Out of ischemic stroke patients, 31% (n = 25) were having lacunar stroke and 69% (n = 55) were having large vessel strokes. The most common large arterial occlusion was middle cerebral artery or its branches in 70.9% (n = 39), posterior circulation (basilar and PCA occlusion) in 21.8% (n = 12) patients, anterior cerebral artery territory in 5.4% (n = 3) patients, and 1.8% (n = 1) had internal carotid artery tandem occlusion. The mean NIHSS score of ischemic strokes present in the study was 12 ± (4–26). In the ischemic stroke group, various subtypes were: cortical in 31, subcortical periventricular white matter in 11, basal ganglion in 10, thalamus in 7, internal capsule in 8, cerebellar in 8, and brain stem in 5 patients. In the intracranial hemorrhage (ICH) group, basal ganglion hemorrhage was most commonly seen in 54.4% (n = 37) patients, followed by thalamic hemorrhage in 17.7% (n = 10), lobar hemorrhages in 11.8 (n = 8)

patients, cerebellum in 7.4% (n = 5) patients, brain stem in 8.8% (n = 6) patients, and primary intraventricular hematoma in 2.9% (n = 2) patients. Out of 68 patients having intracranial hemorrhage, 21 patients had associated intraventricular extension. The details of ischemic strokes, hemorrhagic strokes, control group, vascular risk factors, and univariate analysis of risk factors for both ischemic and hemorrhagic stroke patients compared with controls, respectively, are described in Table 1. We have not found any significant relation of other vascular risk factors in the ischemic stroke cohort compared with the controls.

Genetic analyses were performed to identify the frequency of distribution of three alleles and six possible *APOE* genotypes, among patients and controls. The E3/E3 genotype was identified as a predominant one in both patient groups (ischemic and hemorrhagic) and controls groups. No individual from either group was found to harbor E4/E4 genotype [Tables 2 and 3]. Our findings are that E3 allele frequency is followed by E4 and E2, respectively. Our case–control association study identified E3 and E4 alleles as protective factor (P = 0.004; OR = 0.469; 95% CI: 0.272–0.806) and risk factor (P = 0.029; OR = 2.015; 95% CI: 1.059–3.863), respectively, in statistically significant manner [Table 4]. Further, total patients were subdivided into ischemic and hemorrhage groups with an aim to identify the differential involvement of *APOE* genotype and phenotype in particular disease pathogenesis [Tables 2 and 3].

In case of ischemic stroke group (n = 80), the frequency of E2 allele was 9 (5.6%). E3 was 121 (75.6%) and E4 was 30 (18.7%). The E4 allele also plays a major risk for developing ischemic stroke (OR = 2.74; 95% CI: 1.4–5.10). The relative risk for causation of ischemic stroke was 2.74 compared to controls. Comparing E3 and E4 allele in ischemic stroke group and control group, we found that E4 had significant association in causation of ischemic stroke (P = 0.001) and E3 has significant association in prevention of ischemic stroke (P = 0.001). The E2 allele does not have significant association with ischemic stroke patients as controls in our cohorts (P = 0.37).

The frequency and percentage of APO genotype in ischemic and hemorrhage is given in Tables 2 and 3. As E3/E3 genotype is considered neutral genotype in our Asian population, E3/E3 genotype is highly significant in prevention of ischemic stroke (P = 0.005) and E3/E4 genotype has significant association in causing ischemic stroke (P = 0.011). In ischemic stroke group, E2/E4 genotype also shows significant association (P = 0.021) in causing stroke, but it was present in very few subjects. We did not find any association with E2/E3 genotype in ischemic stroke patients (P = 0.41).

In hemorrhagic group (n = 68), the frequency ApoE E2 allele was 12 (9%), E3 was 109 (80%), and E4 was 15 (11% patients) [Table 3]. The E3 allele plays a protective for hemorrhagic stroke (P = 0.033) (OR = 0.528; 95% CI: 0.28–0.995) among our patient cohort. The relative risk of E4 for causation hemorrhagic stroke was 1.4%, but was not significant (P = 0.31). In others words, we can say that APO E4 allele shows nonsignificant but increased trends toward causation of hemorrhagic stroke. APOE E2 allele (P = 0.44) was significant in causation hemorrhagic stroke compared to controls but it was less frequently present. Out of 68 hemorrhagic stroke patients, E3/E3 genotype was present in 47 (69%), and E3/E4 was present in 10 (14.7%), E2/E4 in 5 (7.5%), E2/E3 in 5 (7.5%), and E2/E2 in 1 patient (1.4%). The rest of the frequency and percentage distribution of the APOE genotype in various groups is given in Tables 2 and 3. In our study group, we did not find association in E3/E3, E3/E4, and E2/E3 genotypes in hemorrhagic stroke. Only E2/E4 showed significant association in hemorrhagic patients (P = 0.022) but represented a small number. There is significant association of E3/E4 genotype in the lobar hematoma subgroup (P = 0.0001) as compared to deep bleed [Table 5].

A statistical significant negative association was found for *APOE3* allele with disease pathogenesis when patients from both groups were compared with controls separately

Table 1: Details of Stroke Patients (Ischemic and hemorrhagic controls and risk factors in the study. Table 1 also shows univariate analysis of various risk factors in both ischemic and hemorrhagic strokes patients compared to controls respectively

Parameters	Ischemic (n=80)	P	Hemorrhagic (n=68)	P	Controls (n=108)
Age (yrs), mean, SD	54.4 (24-78±14.5)	0.358	55.8 (28-80±13.5)	0.871	56.1 (30-85±10.8)
Male	57 (71.2%)	0.59	43 (63%)	0.550	73 (68%)
Female	23 (27.8%)		25 (37%)		35 (32%)
Rural	77 (96.2%)		66 (97.1%)	-	108 (100%)
Smoking	46 (59%)	0.128	32 (47%)	0.920	50 (46%)
Hypertension	56 (70%)	0.313	60 (88%)	0.002	66 (63%)
Diabetes	16 (20%)	0.276	5 (7.3%)	0.001	29 (30%)
Past history of stroke	18 (23.4%)	-	9 (13%)	-	0 (0%)
Family history of stroke	24 (29.5%)	0.627	14 (21%)	0.067	36 (33%)
Total cholesterol (mg/dl), mean	166.7 (82-280±45.0)	0.520	165 (65-293±44.4)	0.830	163 (95-293±40.3)
LDL-C (mg/dl), mean	98.2 (34-179±35)	0.501	96.5 (36-180±36)	0.930	96.1 (43-206±31)
HDL-C (mg/dl), mean	34.4 (19-71±9)	0.100	41.3 (14-140±19)	0.320	96.1 (22-93±10)
LDL-C (mg/dl), mean	147.5±82.8	0.100	147.7±60.0	0.100	128.3±66.2

Table 2: Allele and Genotype frequency distribution of APOE among ischemic Stroke patients & Controls and their significance

Allele	Ischemic	Controls	*P*	Odds ratio
E2	9 (5.6%)	8 (4%)	0.37	1.55 (0.58-4.11)
E3	121 (75.6%)	191 (88%)	0.001	0.41 (0.23-0.70)
E4	30 (18.7%)	17 (8%)	0.001	2.7 (1.43-5.10)
Total	160	216		
Genotype	**Ischemic**	**Controls**		
E2/E2	0	0		
E2/E3	3 (3.8%)	7 (6.5%)	0.41	0.56 (0.14-2.24)
E2/E4	6 (7.5%)	1 (0.9%)	0.02	8.6 (1.2-73.57)
E3/E3	47 (58.8%)	84 (77.8%)	0.005	0.41 (0.22-0.77)
E3/E4	24 (30%)	16 (14.8%)	0.011	2.4 (1.21-5.03)
E4/E4	0	0		
Total	80	108		

Table 3: Allele and Genotype frequency distribution of APOE among hemorrhagic Stroke patients & Controls and significance

Allele	Hemorrhagic	Controls	*P*	Odds ratio
E2	12 (9%)	8 (4%)	0.04	2.52 (1.0-6.3)
E3	109 (80%)	191 (88%)	0.033	0.53 (0.29-0.96)
E4	15 (11%)	17 (8%)	0.31	1.4 (0.7-3.1)
Total	136	216		
Genotype	**Hemorrhagic**	**Controls**		
E2/E2	1 (1.5%)	0	-	-
E2/E3	5 (7.4%)	7 (6.5%)	0.82	1.15 (0.35-3.76)
E2/E4	5 (7.4%)	1 (0.9%)	0.22	8.49 (0.97-74.34)
E3/E3	47 (69%)	84 (77.8%)	0.20	0.64 (0.31-1.27)
E3/E4	10 (14.7%)	16 (14.8%)	0.98	0.99 (0.42-2.33)
E4/E4	0	0		
Total	68	108		

($P = 0.001$; OR = 0.411; 95% CI: 0.23–0.77 for ischemic and $P = 0.033$; OR = 0.53; 95% CI: 0.29–0.96 for hemorrhage). The *APOE4* allele was found to be risk factor only in the ischemic stroke patient group ($P = 0.001$; OR = 2.74; 95% CI: 1.43–5.6) but not in the hemorrhage group. The E3/E3 genotype was found to be a protective ($P = 0.012$; OR = 0.405; 95% CI: 0.191–0.855) and E3/E4 as risk factor ($P = 0.016$; OR = 2.588; 95% CI: 1.122–5.989] among the ischemic stroke group.

Discussion

The genetic contribution to common multifactorial stroke is polygenic, and identification of individual causative mutations is problematic due to the complexity of such a condition and probably there are many alleles with small effects. Significant research is being conducted to establish the relationship between the functional variants of a variety of genes and the risk of stroke in different ethnic groups across the world. Very few attempts have been made to study the role of genetic variation in development of stroke in Indian population.

Table 4: Association of APOE gene polymorphisms with different Stroke patients

Association	*P*	Odds ratio
Ischemic/Controls		
E3 vs others	0.004	0.411 (0.217-0.777)
E4 vs others	0.004	2.74 (1.322-5.699)
E3/E3 vs others	0.012	0.405 (0.191-0.855)
E3/E4 vs others	0.016	2.588 (1.122-5.989)
Hemorrhagic/Controls		
E3 vs others	0.044	0.528 (0.28-0.995)
E4 vs others	0.344	1.451 (0.659-3.188)
E3/E3 vs others	0.218	0.639 (0.305-1.342)
E3/E4 vs others	1.000	0.991 (0.387-2.511)

Table 5: Distribution of APOE genotype in numbers with Location of hematoma

Location of hematoma	APOE genotypes					Total
	E2/E2	E2/E3	E2/E4	E3/E3	E3/E4	
Lobar hematoma	0	1	1	0	8	10
Nonlobar/deep	1	4	4	47	2	58
Total	1	5	5	47	10	68

$P<0.0001$

Since the first demonstration of APOE gene polymorphism on dysbetalipoproteinemia,[9] numerous studies have undertaken to investigate the significant correlation between APOE genotypes with cholesterol metabolism, atherosclerosis, ischemic heart disease, and cerebral amyloid angiopathy and stroke. Studies from the different ethnic regions of the world have reported variable results on association of APOE gene polymorphism in stroke both for intracerebral hemorrhage and ischemic strokes. Luthra in 2002[11] published first report that examined the association of APOE gene polymorphism with stroke in the Asian Indians. Very few studies have been done since then to show the association of APOE gene with causation of stroke in Indian populations.

In this study we report the *APOE* E3 and E4 alleles as a protective and risk factors, respectively, for developing stroke among the ethnic Bengali population of West Bengal. The homozygous E3 genotype was most common in controls (77.8%), followed by E3/E4 (14.7%) and E2/E3 (6.5%), respectively, which is similar to results reported from studies in India.[12,13] In contrary to the other population of the world, the E4/E4 genotype was absent in our study cohort. This may be due to the small number of sample size. Similar results were drawn from a study from Northern India,[12] where they concluded that homozygous E4 genotype was rare in the studied population, and in another study, it was found to be absent in Koch and Maria Gond population of India.[13]

The *APOE4* allele showed a 2.74-fold odds for developing ischemic stroke, whereas the E3 allele showed protection with OR of 0.411. Our result is consistent with a meta-analysis report described by Gu *et al.* (2013).[14] They found that the

E4 allele carrier had a 2.34-fold increase risk of ischemic stroke (OR = 2.34; 95% CI: 1.92–2.86). Our results are similar to the results in the ischemic stroke patients in Spanish,[15] Italian,[16] Chinese,[17] and German[18] studies. The E3/E4 genotype was found to be a risk for developing ischemic stroke, whereas the homozygote E3 was protective in our study cohort. On the contrary, Duzenli *et al.* (2004) reported a reduced E3/E4 genotype frequency in subjects with stroke, and found that E2/E3 genotype frequency was elevated in patients with previous stroke.[19]

The E3 allele showed protection from developing hemorrhagic stroke with OR 0.528. In a meta-analysis involving 1238 ICH cases and 3575 controls, a significantly higher frequency of E4 allele with OR 1.42 was reported.[20] In contrast to our study, in Asian patients, the relative risk of ICH for E2 and E4 carriers was 2.11, and 1.48 in Europeans. The authors reported that E2 or E4 allele had an increased risk of both incident and recurrent ICH and both cortical and deep ICH; the risk estimates were higher in Asians than Europeans.[21] A study from central India found that E2 and E4 were significantly related to recurrent ICH compared with healthy controls. The E4 allele was also independently related to recurrent compared to nonrecurrent ICH, even after adjustment for stroke risk factors.[22] The *APOE* gene polymorphisms are associated with many other diseases. *APOE* E4 allele appeared to be associated with a higher prevalence of dementia in Parkinson's disease,[23] and increased risk for progression from mild cognitive impairment to Alzheimer's type dementia.[24] It also influence the age of onset of temporal lobe epilepsy and associated with developing hypertension.[25] The exact mechanism of *APOE* polymorphism and risk of ICH remains unclear. It plays a role in distributing lipids among central nervous system cells for normal lipid homeostasis,[26] synaptic plasticity,[27] mitochondrial resistance to oxidative stress,[28] and glucose use by neurons and glial cells.[29]

Conclusion

There is significant association of *APOE* gene polymorphism with ischemic stroke in patients of ethnic Bengali population. Our results are in agreement with the findings from national and international studies that E4 allele is a risk for ischemic stroke. However, for hemorrhagic strokes the E3 allele is a protective factor. The E4 allele increases significant risk for development of ischemic stroke and it plays nonsignificant increase in trend in hemorrhagic stroke. However, further studies need to be conducted for this hypothesis.

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Conflicts of interest

There are no conflicts of interest.

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