Association of the *APOE*, *MTHFR* and *ACE* genes polymorphisms and stroke in Zambian patients

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Abstract

The aim of the present study was to investigate the association of *APOE*, *MTHFR* and *ACE* polymorphisms with stroke in the Zambian population. We analyzed 41 stroke patients and 116 control subjects all of Zambian origin for associations between the genotype of the *APOE*, *MTHFR* and *ACE* polymorphisms and stroke. The *APOE* ε2ε4 genotype showed increased risk for hemorrhagic stroke (P<0.05) and also a high risk for ischemic stroke (P=0.05). There was complete absence of the *APOE* ε2ε2 and the *MTHFR* TT genotypes in the Zambian population. The difference between cases and controls was not significant for the other genetic variants when analyzed for relationship between stroke, stroke subtype and genotype. We show that genetic variation at the *APOE locus* affects susceptibility to stroke. No detectable association were observed for the *MTHFR* and *ACE* genotypes and stroke in the Zambian population.

Introduction

While stoke incidence has declined in developed countries over the last decade, its incidence rose during the same period in low- and middle- income countries.[1] Although stroke is an ever-increasing problem in developing countries, little is known about its incidence and outcomes in sub-Saharan Africa (SSA).[2] The INTERSTROKE study showed the commonality of the main risk factors for stroke worldwide, including SSA: hypertension, obesity, diabetes mellitus (DM), smoking, alcohol abuse, hypercholesterolemia, diet and physical activity.[3] In addition, genetic factors may act either by predisposing to vascular risk factors, such as hypertension, heart disease, DM, hyperlipidemia or by a direct independent effect on stroke risk.[4,5]

Association studies have nominated a number of candidate genes associated with stroke risk.[4,5] These genes include apolipoproteinE (*APOE*), plasminogen activator inhibitor 1 (*SERPINE1*), platelet receptor *GP1BA*, angiotensin-converting enzyme (*ACE*), and methylenetetrahydrofolate reductase (*MTHFR*).[4] Case-control studies of stroke candidate genes in different ethnic groups have produced contrary results. A meta-analysis of Chinese, Japanese, and Korean ancestry reported significant association of polymorphisms in the *APOE*, *MTHFR* and *ACE* genes with ischemic stroke.[6] However, in other studies, only minor or non-significant risk associations were observed,[7] and it was concluded that it is a combination of several genotypes interacting which increases the odds ratio for risk of ischemic stroke.[8]

Studies on stroke conducted within African populations mainly focus on the prevalence, risk factors, subtypes and outcome of stroke.[9,10] To date, there are only a handful of studies examining genetic association of *APOE*, *MTHFR*, *ACE* or other candidate genes with cardiovascular disease in African populations.[11,12] These candidate genes provide some of the most likely genetic variants which are involved in stroke risk and have been among the most studied. Common *APOE* alleles has been shown to affect risk of cardio- and cerebrovascular disease and is considered an established genetic risk factor, however common variation in the *MTHFR* gene (related to folate metabolism) and the *ACE* gene although implicated in disease risk are not confirmed.

The aim of the present study therefore, was to investigate association of the *APOE*, *MTHFR* and *ACE* gene polymorphisms with stroke in Zambian patients.

Materials and Methods

Study population

Forty one consecutive patients with mean age 54±16 years admitted to the University Teaching Hospital (UTH) Lusaka, Zambia, between June and December 2010 with a diagnosis of stroke, were enrolled. We included only patients who had stroke confirmed by neurological examination and brain CT scan. Patients who had no brain imaging done and those with head injury were excluded. Daily physical checks/reviews were made of emergency room, admission ward and in-patient wards for stroke patients.

Clinical procedure

Within 24 hours of admission, we obtained medical history, conducted general and neurological examination and brain CT scan was performed. The patient's demographic details and risk factors for stroke were documented on a data collection sheet by study physician. Risk factors for stroke included: hypertension, DM, elevated cholesterol, heart disease, tobacco smoking/sniffing, alcohol consumption, history of transient ischemic attack, and a family history of stroke. As a risk factor of stroke hypertension was defined as blood pressure of ≥140/90 mmhg and current use of antihypertensive medication. DM was diagnosed when patient was taking antidiabetic drugs prior to stroke. Elevated cholesterol was considered if serum fasting cholesterol was >5.2 mmol/L or prestroke treatment with a cholesterol lowering agent. Other risk factors included HIV infection. The severity of stroke was evaluated on admission using the National Institutes of Health Stroke Scale (NIHSS). Data on risk factors and stroke severity in our study population was published in our previous article.[13] As a control population, 116 subjects (68 women, 48 men) matched for age, sex, free of cerebrovascular diseases and other risk factors including hypertension, DM and HIV, were recruited from the department of internal medicine of UTH. Only indigenous Zambian patients and control subjects were included in the study. All participants gave written informed consent and the study was approved by the Biomedical Research Ethics Committee of the University of Zambia.

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Key words: Sub-Saharan Africa, Zambia, ischemic stroke, intra-cerebral hemorrhage, outcome, risks factors.

Acknowledgements: the authors would like to thank Dr P. Kelly, Ms N. Shawa, and the Department of Internal Medicine UTH for supporting this study.

Contributions: the authors contributed equally.

Conflict of interests: the authors declare no potential conflict of interests.

Received for publication: 28 January 2013.
Revision received: 16 July 2013.
Accepted for publication: 22 July 2013.

Neurology International 2013; 5:e20
doi:10.4081/ni.2013.e20

Genetic analysis

Whole blood was collected in 4 mL EDTA tubes and stored at 4 degrees Celsius prior to DNA extraction which was performed using silica-gel-based membrane with vacuum technology QIAamp DNA Mini Kit (250) (QIAgen, London, UK). The common APOE epsilon alleles defined by the p.C112R (rs429358) and p.R158C (rs7412) substitutions were selected for analysis, as well as the common *MTHFR* C677T (p.A222V; rs1801133) variant and the ACE exonic synonymous variant p.T202T (rs4343; A/G); which acts as a surrogate marker for the common intronic AluI insertion/deletion (I/D; rs4646994) polymorphism.[14] Genotyping was performed with ABI predesigned TaqMan probes and chemistry (Applied Biosystems, Foster City, CA) with analysis performed using SDS 2.2.2 software on an ABI 7900 for TaqMan. Positive and negative controls where included on all TaqMan assay plates. Positive or ambiguous results in the TaqMan assay were also resolved with direct sequencing.

Statistical analysis

All calculations were done using Graphpad Prism version 6.0 software. The Fishers test was used to calculate for P-value and the two tailed probability levels for statistical significance with P<0.05 being considered significant. An odds ratio (OR) was calculated as a measure the strength of association

Results

The genotype and allele frequencies in the study and control groups are shown in Table 1. There was no significant difference between the two groups in the frequencies of *APOE*, *MTHFR* and *ACE* genotypes and alleles. The *APOE*, *MTHFR* and *ACE* genotype frequencies for the case group were compatible with the Hardy-Weinberg equilibrium. The APOE ε2ε2 and the *MTHFR* TT genotypes were absent in the Zambian population. However, in the control group, the *APOE, MTHFR* genotype frequencies were compatible with the Hardy-Weinberg equilibri*um (HWE), while the ACE* genotype was not in agreement with HWE. The frequency of control subjects homozygous for APOE ε4 was 7.7% compared to 2.4% for stroke patients. The *MTHFR* T allele frequency was 8.6% in control subjects and was observed at a frequency of 11.0% in patients. The frequency for *ACE* rs4343 G-allele corresponding to the D allele was 13.8% for subjects in the control group compared to 12.2% for patients.

There was significant genotypic variations between cases and controls for APOE ε2ε4 and ε2ε3 genotypes, statistical analysis demonstrated a higher risk for hemorrhagic stroke in *APOE* ε2ε4 carriers [OR of 4.45 (P<0.05)] while the presence of *APOE* ε2ε3 genotype seemed to significantly lower the risk for hemorrhagic stroke (OR 0.097; 0.006-1.67, P<0.05) (Table 2).

APOE ε2ε2 and *MTHFR* 677TT polymorphisms were absent in both ischemic and hemorrhagic stroke. Though not significant, the frequency of the *MTHFR* 677T allele was higher (double) in ischemic stroke (17.4%) compared to hemorrhagic stroke (8.3%). The *ACE* genotypes showed no significant frequency differences between cases and controls.

Discussion and Conclusions

Studies conducted over the last decade has shown that among blacks, stroke is more common, more severe, and carries higher mortality when compared with other races; blacks also have increased frequency of risk factors such as DM, hypertension, and obesity.[15] In this study we have analyzed for the first time common polymorphisms of the *APOE*, *MTHFR* and *ACE* genes in Zambian stroke patients and in controls. This constitutes an approach to start characterization of the genetic risk architecture of Zambian patients with cerebrovascular disease. The study also describes the prevalence of *APOE*, *MTHFR* and *ACE* polymorphisms corresponding to stroke in the Zambian population.

We observed a relatively high frequency of the *APOE* ε4 allele and a complete absence of the *APOE* ε2ε2 genotype in both patients and controls. We did not find any association between the *APOE* ε4 allele and stroke in our patients. However, there was an increased risk to hemorrhagic stroke by the *APOE* ε2ε4 genotype with OR at 4.45 P<0.05. This may suggest that Zambian carriers of *APOE* ε2 and ε4 have an increased risk of hemorrhagic stroke. Of note, in our previous study,[13] the prevalence of hemorrhagic stroke was observed to be higher than in reported Western cohorts. A similar high frequency for hemorrhagic stroke has been reported for other African countries with estimates up to 52% in Democratic Republic of Congo,[10] up to 60% in Ghana,[16] and Tanzania.[17] According to the literature, carriers of *APOE* ε2 and ε4 have an increased risk of intracerebral hemorrhage perhaps because of the effects of these gene variants on risk of cerebral amyloid angiopathy.[18]

Other studies have found conflicting results regarding association of *APOE* alleles and stroke. Some studies report an increased risk of stroke for *APOE* ε4 carriers. A recent meta-analysis in Caucasian populations found *APOE* ε2 genotypes to be associated with increased risk of hemorrhagic stroke.[19] We found the effect of *APOE* ε2 on carriers to be inconclusive as it increased hemorrhagic stroke risk in

Table 1. Genotypes and alleles of stroke patients and controls.

Alleles	Cases n=41 (%)	Control n=116 (%)	OR	P
		APOE		
ε2 2	0	0		
ε2 3	6 (14.6)	25 (21.6)	0.62 (0.24-1.65)	0.494
ε2 4	4 (9.8)	7 (6)	1.68 (0.466-6.080)	0.479
ε3 3	15 (36.6)	38 (32.8)	1.184 (0.562-2.494)	0.703
ε3 4	15 (36.6)	37 (31.9)	1.232 (0.584-2.597)	0.700
ε4 4	1 (2.4)	9 (7.7)	0.297 (0.036-2.423)	0.456
ε2	10 (12.2)	32 (13.8)	0.868 (0.406-1.855)	0.851
ε3	51 (62.2)	138 (59.5)	1.121 (0.668-1.881)	0.696
ε4	21 (25.6)	62 (26.7)	0.944 (0.531-1.678)	0.885
		MTHFR		
CC	32 (76.2)	96 (82.8)	0.740 (0.306-1.791)	0.492
CT	9 (22.0)	20 (17.2)	1.35 (0.558-3.264)	0.492
TT	0	0		
C	73 (89.0)	212 (91.4)	0.765 (0.333-1.756)	0.512
T	9 (11.0)	20 (8.6)	1.307 (0.570-2.999)	0.512
		ACE		
AA (II)	32 (76.2)	90 (77.6)	1.03 (0.44-2.43)	0.951
AG (ID)	8 (19.5)	20 (17.2)	1.16 (0.47-2.89)	0.744
GG (DD)	1 (2.4)	6 (5)	0.40 (0.05-3.43)	0.673
A (I)	72 (87.8)	200 (86.2)	1.152 (0.539-2.462)	0.851
G (D)	10 (12.2)	32 (13.8)	0.868 (0.405-1.855)	0.851

Table 2. The genotype and allele data of ischemic and hemorrhagic cases and controls.

Allele	Ischemic			Hemorrhagic			P
	Cases (%)	Control (%)	OR	Cases (%)	Control (%)	OR	
APOE	n=23	n=116		n=18	n=116		
ε2ε2	0	0	0.7663 (0.239-2.459)	0	0		
ε2ε3	4 (17.4)	25 (21.6)	2.336 (0.557-9.804)	0	25 (21.6)	0.097 (0.006-1.67)	≤0.05
ε2ε4	3 (13.0)	7 (6)	1.32 (0.524-3.321)	4 (22.2)	7 (6)	4.45 (1.15-17.1)	≤0.05
ε3ε3	9 (39.1%)	38 (32.8)	0.934 (0.354-2.465)	6 (33.3)	38 (32.8)	1.03 (0.358-2.95)	
ε3ε4	7 (30.4)	37 (31.9)	0.24 (0.014-4.286)	7 (38.9)	37 (31.9)	1.36 (0.487-3.79)	
ε4ε4	0	9 (7.7)	1.122 (0.462-2.724)	1 (5.6)	9 (7.7)	0.699 (0.083-5.88)	
ε2	7 (15.2)	32 (13.8)	1.162 (0.604-2.234)	4 (11.1)	32 (13.8)	0.781 (0.259-236)	
ε3	29 (63.0)	138 (59.5)	0.762 (0.357-1.627)	19 (52.8)	138 (59.5)	0.761 (0.376-1.54)	
ε4	10 (21.7)	62 (26.7)		13 (36.1)	62 (26.7)	1.55 (0.740-3.25)	
MTHFR	n=23	n=116		n=18	n=116		
CC	15 (65.2)	96 (82.8)	0.39 (0.146-1.045)	15 (83.3)	96 (82.8)	1.04 (0.275-3.94)	
CT	8 (34.8)	20 (17.2)	2.56 (0.957-6.85)	3 (16.7)	20 (17.2)	0.96 (2.54-3.63)	
TT	0	0		0	0		
C	38 (82.6)	212 (91.4)	0.448 (0.184-1.091)	33 (91.7)	212 (91.4)	1.04 (0.292-3.69)	
T	8 (17.4)	20 (8.6)	2.23 (0.917-5.43)	3 (8.3)	20 (8.6)	0.964 (0.271-3.42)	
ACE	n=23	n=116		n=23	n=116		
AA (II)	18 (81.0)	90 (77.6)	1.04 (0352-3.07)	15 (70.0)	90 (77.6)	0.542 (0.207-1.42)	
AG (ID)	5 (19.0)	20 (17.2)	1.33 (0.443-4.01)	5 (20.0)	20 (17.2)	1.33 (0.443-4.01)	
GG (DD)	0	6 (5)	0.362 (0.0197-6.65)	3 (10.0)	6 (5)	2.75 (0.635-11.9)	
A (I)	41 (90.5)	200 (86.2)	1.31 (0.482-3.57)	35 (80.0)	200 (86.2)	0.509 (2.35-1.10)	
G (D)	5 (9.5)	32 (13.8)	0.762 (0.280-2.07)	11 (20.0)	32 (13.8)	1.96 (0.906-4.26)	

APOE ε2ε4 subjects while this risk seemed to be reversed in the *APOE* ε2ε3 subjects. These conflicting results may be due to a possible synergistic interaction effect of the ε2 allele towards the ε4 or simply the low sample size and low frequency of these alleles.[20,21] Corbo *et al*.[22] found the frequency of ε2 allele to be generally higher among sub-Saharans (0.116) compared to Europeans with the range of 0.044-0.108 (with the exception of Swedish with frequency 0.119). Hence, it would be plausible to hypothesize that an increase in the *APOE* ε2 allele frequency (0.122, Table 1) increases the risk for development of hemorrhagic stroke in a population with a generally high ε4 allele frequency (0.256 in the Zambian population). We observed low minor allele frequencies for the *MTHFR* C677T and *ACE* I/D polymorphisms in the Zambian population. No significant association were found between the *MTHFR* and *ACE* genotypes and stroke risk in the Zambian population. Association between these polymorphisms and stroke have, however, been demonstrated in other populations including Chinese population,[7] Hungarian population,[8] and the Northern Irish population.[23] This may reflect population heterogeneity or specificity in disease risk. To clarify the role of these variants in stroke risk in Sub-Saharan populations larger studies are warranted. It is important to note that the major limitation of this study is the sample size which does not yet allow exclusion of *APOE*, *MTHFR* and *ACE* polymorphisms as a risk factor in Zambian patients with stroke, and therefore, large scale prospective studies are needed to confirm these findings. However, as genetic technologies become more available to African countries and in particular those between the Sahara and South Africa we will gain a better understanding of how genetics will determine health outcomes. These data will help ease health-related social and economic burdens within these developing nations.

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