Association of apolipoprotein E 4 polymorphism with cerebral infarction in Chinese Han population[1]

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ABSTRACT

AIM: To study the association between *APOE* polymorphisms and cerebral infarction through a case-control study among the Chinese *Han* population. **METHODS**: First-ever cerebral infarction patients (*n*=226) whose ages ranged from 40 to 60 years old were recruited from Department of Neurology, Zhongshan Hospital, Shanghai, and Zhejiang Chinese Traditional Medicine Hospital, Zhejiang, China. Unrelated healthy controls (*n*=201) were selected from the general population in the same area with similar age and sex distribution. *APOE* was amplified by one-stage PCR using the forward primer: 5'-GGC ACG GCT GTC CAA GGA GCT-3' and reverse primer: 5'-GAT GGC GCT GAG GCC GCG CT-3'. The PCR product was digested directly with 5 U of *Cfo*I and separated by a 20 % polyacrylamide (acrylamide: *bis*-acrylamide=29:1) nondenaturing gel. **RESULTS**: Both cerebral infarction patient and control groups were in Hardy-Weinberg equilibrium. The allele frequency of *APOE*2, APOE*3*, and *APOE*4* was 4.6 %, 81.9 %, and 13.5 % respectively in the patients with cerebral infarction; 5.7 %, 87.3 %, and 7.0 % respectively in the healthy control group. Compared with *APOE*3/3 subjects, *APOE*4/4 carriers had a 2.1-fold risk of cerebral infarction (odds ratio 2.1, 95 % confidence limits 1.3 to 3.4). The allele frequency of *APOE*4* in the cerebral infarction patient group was significantly higher than that in the control group (13.5 % *vs* 7.0 %; *P*=0.002). **CONCLUSION:** *APOE* 4 is a risk factor for cerebral infarction among the Chinese *Han* population.

INTRODUCTION

Stroke is one of the three leading causes of morbidity and mortality in both developing and developed countries[1]. Genetic and environmental risk factors relating to stroke have been studied in the past. The principal risk factors for stroke (including age and hypertension) are well defined, and genetic factors may account for some of this risk. For example, twin studies[2,3], animal studies[4], epidemiologic data[5], and monogenic stroke disorders[6] have provided the most clear evidence for genetic influences in stroke. It is believed that genes may play a role in the pathogenesis of stroke.

Apolipoproteins E (ApoE) is one of the major protein constituents in very low-density lipoprotein and plays a central role in lipid metabolism. It is the prod-

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uct of a single gene on chromosome 19 with the molecular weight of 34000 kDa glycoprotein which is composed of 299 amino acid residues[7].

Although the association between *APOE* polymorphism, in particular the relative proportion of *APOE*4,* and stroke-related pathologies have been investigated in Western populations, few publication can be found in international journals on the study of relationship between *APOE* polymorphism and cerebral infarction among the Chinese *Han* population, the largest population in the world, which easily offers a large sample size. The current study focused on the detailed studies of *APOE* polymorphism and cerebral infarction in the Chinese *Han* population.

In order to search for genetic influences on cerebral infarction, a kind of subtypes of stroke in Chinese *Han*, we studied a candidate gene, apolipoprotein E (*APOE*) which was controversial in the Western population.

MATERIALS AND METHODS

Subjects First-ever cerebral infarction patients (97 female and 129 male, total 226) whose ages ranged from 40 to 60 years old were recruited from Department of Neurology, Zhongshan Hospital, Fudan University, Shanghai, China and Zhejiang Traditional Hospital, Zhejiang College of Traditional Chinese Medicine, Hangzhou, Zhejiang, China, 147 km away from Shanghai. All the patients were systematically evaluated within 24 h of the symptom onset. The neurologist completed a detailed clinical questionnaire including information of vascular risk factors and physical examination of each patient. Electrocardiography, cardiac and carotid ultrasonography, and MR angiography are performed routinely for the cerebral infarction patients. The final diagnosis of cerebral infarction patients was confirmed by serial CT or MRI findings[8].

Unrelated healthy controls (92 female and 109 male, total 201) were selected from the general population in the same area. They must meet the following criteria: good physical and mental health confirmed by clinical, radiological, and biological examinations.

A brief cognitive test[9] was conducted to exclude any subject having potential cognitive impairment in both cerebral infarction patients and controls.

Peripheral blood samples (2 mL each) were collected in acid citrate dextrose (ACD)[10] tubes after 12-h overnight fasting from all the control subjects. All blood samples were obtained within 24 h of stroke onset from cerebral infarction patients.

The study was started from September 1998 to August 2002 and was approved by the Hospital Ethics Committee and informed consent was obtained from all subjects.

A blinded *APOE* genotyping study was conducted including both cerebral infarction patients and control subjects.

Amplification of *APOE* gene from genomic DNA for restriction isotyping

Amplification of *APOE* gene from genomic DNA The protocol for isolation of genomic DNA from peripheral blood samples in this study was the same as that described by Russell[11]. The amplification of *APOE* gene region encoding common *APOE* isoforms from genomic DNA was conducted in a DNA Thermal Cycler (Perkin Elmer Cetus 9600) using oligonucleotide primers (forward primer: 5'-GGC ACG GCT GTC CAA GGA GCT-3' and reverse primer: 5'-GAT GGC GCT GAG GCC GCG CT-3'). Each amplification reaction contained 100 ng of genomic DNA, 8 pmol of each primer, 5 mL of 10×PCR reaction buffer [0.67 mol/L Tris-HCl (pH 8.8), 0.166 mol/L $(NH_4)_2SO_4$, 0.067 mol/L $MgCl_2$, 0.72 % (v/v) β-mercaptoethanol (Sigma)], 10 % (v/v) dimethylsulfoxide (Sigma), 200 μmol/L of dATP, dTTP, dCTP, and dGTP (Merck), 2 U of *Taq* polymerase in a final volume of 50 mL. Each reaction mixture was denatured at 94 ºC for 2 min and subjected to five cycles of amplification by primer annealing (67 ºC for 10 s), extension (72 ºC for 80 s) and denaturation (94 ºC for 10 s). Immediately after completing this mini-cycle, the reaction mixture was continuously subjected to 30 cycles of amplification by primer annealing (60 ºC for 5 s), extension (72 ºC for 80 s), and denaturation (94 ºC for 10 s). The length of PCR product was 262 bp.

Analysis of amplified APOE product with RFLP After PCR amplification, 12 mL of each unpurified PCR product was directly digested with 5 U of *Cfo*I at 37 ºC for overnight. The digested product was loaded onto a 20 % 1-mm thick and 7-cm long nondenaturing polyacrylamide gel (acrylamide:*bis*-acrylamide=29:1) for 2.5 h under constant voltage (150 V). After electrophoresis, the gel was treated with ethidium bromide (0.5 mg/L) for 6 min and DNA fragments were visualized by UV illumination. The sizes of *Cfo*I fragments were determined by the marker[12,13] (*Msp*I-digested pUC18 DNA).

Although the total length of PCR product was

different from that obtained by Hixon and Vernier due to the difference of reverse primer, the length for *APOE* typing was the same as Hixon since the surplus of the base pair was cut off at another *Cfo*I cleavage site contained in the reverse primer.

Statistical analysis Allele frequencies were calculated by allele counting. Hardy-Weinberg equilibrium [14] was tested by a χ^2 goodness-of-fit test. The other data were compared by a χ^2 test. The computer software of Epi Info 6.04 edition was also used in the analysis. The Mantel-Haenszel[15] method was used to calculate the adjusted odds ratio (OR) with 95 % confidence limits (CI).

RESULTS

The allele frequency of *APOE*2, APOE*3*, and *APOE*4* was 4.6 %, 81.9 %, and 13.5 % respectively in the cerebral infarction patient group; 5.7 %, 87.3 %, and 7.0 % respectively in the healthy control group. Compared with *APOE*3/3 subjects, 4 carriers had a 2.1-fold risk of cerebral infarction (OR 2.1, 95 % CI 1.3 to 3.4). The allele frequency of *APOE*4* in the cerebral infarction patient group was significantly higher than that in the control group (13.5 % *vs* 7.0 %; *P*=0.002; Tab 1).

Tab 1. Distribution (*n*, subjects number)of *APOE* allele genotype in both cerebral infarction patient and control groups. [b]*P*<0.05 *vs* control.

APOE Geno-type	Cerebral Infarction Observed	Expected	χ^2	Control Observed	Expected	χ^2
2/2	2	0.4878	4.688	2	0.6580	2.737
2/3	14	17.19	0.5920	17	20.08	0.4724
2/4	3	2.834	0.0097	2	1.602	0.0989
3/3	152	151.4	0.0036	156	153.2	0.0512
3/4	52	49.93	0.0095	22	24.45	0.2455
4/4	3	4.116	0.3026	2	0.9751	1.077
Total	226	226.0	5.605	201	201.0	4.682

The allele and genotype frequencies were in Hardy-Weinberg equilibrium by comparison with that of the corresponding theoretical distribution. Both cerebral infarction patient and control groups were in Hardy-Weinberg equilibrium (*P*>0.05 respectively; df=5; Tab 2).

Tab 2. Check for Hardy-Weinberg equilibrium in both cerebral infarction patient and control groups. χ^2=5.605 and 4.682, respectively, *P*>0.05. df=5. Age, Mean±SD.

	Cerebral infarction (*n*=226)	Control (*n*=201)
Sex (F/M)	97/129	92/109
Age/a	48.5±3.4	47.1±2.4
E2/2	2	2
E2/3	14	17
E2/4	3	2
E3/3	152	156
E3/4	52	22
E4/4	3	2
E*2	21 (4.6 %)	23 (5.7 %)
E*3	370 (81.9 %)	351 (87.3 %)
E*4	61 (13.5 %)[b]	28 (7.0 %)

DISCUSSION

The *APOE* allele frequencies have been found to be associated with some kind of diseases. Three common alleles of *APOE* are *APOE*2*, *APOE*3,* and *APOE*4*[16,17], which can be distinguished by the variation of positions at 112 and 158 in the receptor-binding region of ApoE[18]. If positioned with 112 cys and 158 arg, it forms isoform *APOE*3* which is the commonest isoform; If the positioned with 112 arg and 158 arg, it forms isoform *APOE*4* which is associated with Alzheimer's disease[19] , vascular dementia[20], cognition in the very old[21], artery disease[22], gallstones[23], diabetes, and atherosclerosis[24], colon cancer[25], etc; If positioned with 112 cys and 158 cys, it forms isoform *APOE*2* which is a risk factor for type III hyperlipidemia[26].

Our data shows significantly higher frequency of *APOE*4* allele in the Chinese *Han* patient with cerebral infarction when compared with the control group; and this is similar to the results in the studies of ischemic stroke by Pedro-Botet *et al*[27] in 100 males in the Spanish and Margaglione *et al*[28] in the Italian population, Peng *et al*[29] in the Chinese population, Kessler *et al*[30] in the German population. However, our results do not show the significantly higher frequency of *APOE*2* allele as evidenced in a Japanese rural population[31], Couderc *et al*[32], Ferrucci *et al*[33] in a cohort study. Basun *et al*[34], Catto *et al*[35], Kuusisto *et al*[36], MacLeod *et al*[37], Morrison *et al*[38], and Nakata *et al*[39] found no association between *APOE* and cerebral infarction.

Surprisingly a study by McCarron and colleagues found a favourable effect of *APOE4* on stroke outcome[40]. All these controversial data, firstly, may be mainly due to the ethnic difference; secondly, might be the different criteria for selecting the unrelated healthy subjects as controls since many diseases in the control group could not be easily ruled out especially for those early cognitive impaired healthy subjects. For this reason, all the healthy subjects were given a brief cognitive test in our study.

Interestingly, the case-control study showed inconsistency data, but the animal study presented promising results. Laskowitz *et al*[41] utilized apolipoprotein E-deficient mice as a model, and found that apolipoprotein E had increased susceptibility to focal cerebral ischemia. Furthermore, Sheng H *et al*[42] used transgenic mice model, and detected that apolipoprotein E3 was favorable for focal ischemia when it was compared to apolipoprotein E4 in transgenic mice. These data suggested that the epsilon 4 allele could increase infarct size and functional impairment; and they also supported that APOE might be a key molecule for the clearance of infarction tissue after ischemia in the brain.

The study of gene-environment and gene-gene interactions, as well as genotype to phenotype links, is critical in order to gain insight into complex traits. The recent advances in the human genome project[43,44] and the identification of multiple single-nucleotide polymorphisms within the human genome[45], combined with bioinformatics, will facilitate the study of multifactorial, complex disorders such as stroke. Finally, the identification of causative genes will facilitate early diagnosis, prevention, and a more effective treatment of ischemic stroke.

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