A Preliminary Study of the Association between *Apolipoprotein E* Promoter Methylation and Atherosclerotic Cerebral Infarction

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Aim: To investigate association of *Apolipoprotein E* (*ApoE*) gene promoter methylation with atherosclerotic cerebral infarction (ACI) in the Han Chinese population. *Methods:* Twenty-six ACI patients (the case group) and 26 healthy (the control group) were recruited randomly from Henan Han nationality population, from April 2016 to August 2016. Bisulfite pyrosequencing technology was used to examine the role of the aberrant gene promoter methylation in ACI in Han Chinese population. Especially, we used the odds ratio and 95% confidence interval (95% CI) method to elevate the correlation between *ApoE* Promoter Methylation and ACI. *Results:* The case group was significantly more likely to have hypertension and carotid atherosclerotic plaques (Table 1). The case group had significantly lower levels of high-density lipoprotein cholesterol (HDL-C), folate, and higher levels of homocysteine (Table 2). We observed that ACI patients (n = 26) had significantly higher methylation levels at cytosine-phosphate-guanine (CpG) 14 and CpG16 compared with controls (n = 26) (Table 3). Importantly, our results indicated a significant association between *ApoE* promoter methylation and ACI (OR = 16.146; 95% CI, 1.154-225.832; $P^* < .05$; P^*: adjusted for age, gender, carotid atherosclerotic plaque, hypertension, HDL-C, homocysteine, and folate) (Table 4). *Conclusions:* Our study indicates that *ApoE* promoter methylation may be associated with ACI in Han Chinese people.

Key Words: Atherosclerotic cerebral infarction—*ApoE* promoter methylation—epigenetic—carotid atherosclerotic plaque—folate

Introduction

Atherosclerotic cerebral infarction (ACI), a complex disease caused by environmental and genetic factors, has been a leading cause of disability and mortality worldwide. Factors such as ACI family history, smoking habit, hypertension, diabetes, and dyslipidemia are associated with ACI, and dyslipidemia has a major influence on ACI. Several studies have suggested that a low level of high-density lipoprotein cholesterol (HDL-C) and an elevated level of total cholesterol and low-density lipoprotein cholesterol are associated with the occurrence and prognosis of ACI. The *Apolipoprotein E* (*ApoE*) gene and its protein play a crucial role in lipid metabolism and antiatherosclerosis. Linton et al proposed that a small amount of *ApoE* was enough to correct dyslipidemia and prevent atherosclerosis.[1] The lack of *ApoE* would result in the accumulation of sphingomyelin-enriched remnants, inducing macrophages to accumulate more cholesterol.[2] *ApoE* also exerts an effect on high-density lipoprotein metabolism, by taking part in hepatic uptake and the formation and maturation of high-density lipoprotein.[3] *ApoE* deficiency leads to abnormal high-density lipoprotein maturation.[4] In 1992, *ApoE* knockout mice ($ApoE^{-/-}$) were created. Compared with wild-type mice, the most

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significant characteristic of *ApoE*$^{-/-}$ mice is that they suffer from serious hyperlipidemia and atherosclerosis, irrespective of a normal or high-fat diet. In addition, *ApoE* variants are associated with ACI and coronary heart disease (CHD) because *ApoE* functions in lipid metabolism and anti-atherosclerosis and in the pathogenesis and progression of ACI. Deoxyribonucleic acid (DNA) methylation is a stable epigenetic marker, occurring at cytosine-phosphate-guanine (CpG) dinucleotides.[5] Recent studies have suggested that many human diseases, including cancers and atherosclerosis, are associated with abnormal DNA methylation.[6,7] It is well established that DNA methylation has links to transcriptional silencing, while demethylation (the loss of methylation) promotes, if not directly activates, gene expression.[8] Several studies have indicated that promoter methylation of certain genes increases the risk of ACI. Therefore, we attempted to establish whether the DNA methylation of selected CpG dinucleotides in the *ApoE* gene promoter region contributed to the risk of ACI.

Methods

Study Participants

Twenty-six ACI patients (11 females, 15 males) and 26 healthy (13 females, 13 males) were enrolled at the First Affiliated Hospital of Zhengzhou University of Henan province, China, from April 2016 to August 2016. They were diagnosed and reviewed by 2 independent neurologists and no subject had atrial fibrillation, or liver or renal diseases. Patients in the case group had 1 or more major neurological impairments that were confirmed by nuclear magnetic resonance imaging. The control group is constituted of nonvascular neurological disorder patients. Bisulfite pyrosequencing technology was applied to test the DNA methylation levels of 17 CpG dinucleotides in the *ApoE* promoter of the study subjects. The study was approved by the First Affiliated Hospital of Zhengzhou University Ethics Committee. Written informed consent was obtained from all participants.

Clinical Data and Collection of Samples

Each patient's basal and clinical characteristics were measured. Carotid atherosclerotic plaques were examined through carotid duplex ultrasound, recording echo, size, number, and location. Fasting blood samples were collected in the morning from the antecubital vein and stored at −80°C. Biochemical characteristics, such as alanine aminotransferase, blood urea nitrogen, creatinine, low-density lipoprotein cholesterol, homocysteine, triglyceride, cholesterol, HDL-C, glucose, folate, and vitamin B12 were measured and recorded.

DNA Methylation Analysis

DNAwas extracted from total blood cells using a commercially available DNA extraction kit (SK8224, Sangon Biotech, Shanghai, China). DNA concentrations were quantified using an ultraviolet spectrophotometer (U-3010, Hitachi, Tokyo, Japan), and were determined to be more than 50 ng/μL. A fragment consisting of 17 CpG dinucleotides from the *ApoE* gene promoter, upstream from the first exon of the *ApoE* gene (*ApoE* CpG1-CpG17; Additional file 1) was selected for its enrichment of CpG dinucleotides. Bisulfite pyrosequencing technology was applied to measure the methylation levels of the 17 CpG dinucleotides. DNA was modified under specific conditions, and the *ApoE* promoter regions were amplified (Verity 96-well, ABI, Carlsbad, CA). Polymerase chain reaction (PCR) products were then purified using a purification recovery kit (SK1141, Sangon Biotech, Shanghai, China), cloned and sequenced. Sequence data were analyzed using Chromas software.

Statistical Analysis

Statistical analyses were carried out using SPSS 17.0 software. Data according with normal distribution were described as the mean ± standard deviation (mean ± SD), otherwise data were analyzed with the Rank-Sum test. Enumeration data were assessed with the chi-square test, if the expectation value was less than 5, as analyzed by Fisher's exact test. A stepwise logistic regression analysis was applied to assess whether DNA methylation levels at the 17 selected CpG dinucleotides might be associated with ACI occurrence. For the correlation of enumeration data, we used the odds ratio (OR) and 95% confidence interval (95% CI) method. A 2-tailed *P* value less than .05 indicated significance.

Results

The basic characteristics of the cases and controls were summarized in Table 1. The case group was significantly more likely to have hypertension and carotid atherosclerotic plaques ($P = .005$, $P = .001$, respectively). The biochemical characteristics of the cases and controls were described in Table 2. Compared with the control group, the case group had significantly lower levels of HDL-C and folate, and higher levels of homocysteine. The methylation levels of the 17 selected CpG dinucleotides in the cases and controls were described in Table 3. It should be noted that each sample was cloned 5times, and if methylation at 1 site was detected in all 5 clones, then the site was considered to be methylated. We observed that ACI cases (n = 26) showed significantly higher methylation levels at CpG14 and CpG16 compared with subjects without ACI (n = 26) ($P = .026$, $P = .020$, respectively). The potential relationship between the promoter methylation markers and clinical data with the risk of ACI was assessed in Table 4. Confounding factors regarding ACI risk include age and gender, and levels of carotid atherosclerotic plaques, hypertension, HDL-C, homocysteine, and folate;

Table 1. *Basic characteristics comparison between ACI and non-ACI*

Variables	Cases (n = 26)	Controls (n = 26)	t/χ^2	*P* value
Age (y), mean ± SD	56.38 ± 10.35	53.85 ± 10.97	.858	.395
Gender (male), n (%)	15 (57.69)	13 (50)	.310	.578
Systolic pressure (mm Hg), mean ± SD	124.69 ± 12.052	118.62 ± 10.944	1.903	.063
Diastolic pressure (mm Hg), mean ± SD	81.19 ± 7.79	77.81 ± 7.67	1.579	.121
Family history of stroke, n (%)	6 (23.08)	4 (15.38)	.495	.482
Hypertension, n (%)	15 (57.69)	6 (23.08)	7.879	.005*
Diabetes, n (%)	4 (15.38)	2 (7.69)	.754	.668
Smoking, n (%)	6 (23.08)	4 (15.38)	.495	.482
Alcohol, n (%)	6 (23.08)	2 (7.69)	2.364	.248
Carotid atherosclerostic plaque, n (%)	22 (84.62)	10 (38.46)	11.700	.001*
Height (cm), mean ± SD	163.77 ± 7.40	163.62 ± 7.44	.075	.941
Weight (kg), mean ± SD	64.85 ± 6.44	64.27 ± 6.48	.322	.749
BMI (kg/m^2), mean ± SD	24.16 ± 1.51	23.92 ± 1.39	.574	.569

Abbreviations: ACI, atherosclerotic cerebral infarction; BMI, body mass index; SD, standard deviation.
*The *P* value is less than .05.

therefore, the relationship between methylation and the risk of ACI was analyzed using logistic regression. Our analysis indicated a significant association of DNA methylation at CpG16 with the increased risk of ACI (OR = 16.146; 95% CI, 1.154-225.832; $P^* < .05$; P^*: adjusted for age, gender, carotid atherosclerotic plaque, hypertension, HDL-C, homocysteine, and folate). Carotid atherosclerotic plaques were positively associated with the risk of ACI (OR = 27.194; 95% CI, 2.266-326.362; $P^* < .05$; P^*: adjusted for age, gender, hypertension, HDL-C, homocysteine, and folate). In contrast, folate was negatively associated with ACI occurrence (OR = .586; 95% CI, .355-.968; $P^* < .05$; P^*: adjusted for age, gender, hypertension, high-density lipoprotein, cholesterol, and homocysteine). However, we found no convincing association between DNA methylation at CpG14 and ACI risk.

Discussion

ACI is a multifactorial disease, consisting with a large number of conventional risk factors, such as smoking, hypertension, hypercholesterolemia, and diabetes. In the current study, we furnish evidence that hypertension, carotid atherosclerotic plaques, and HDL-C are statistically different in ACI patients compared with non-ACI patients.

Accumulating evidence indicates that hyperhomocysteinemia is a major risk factor for atherosclerotic and thrombotic disease, occurring in about 5%-7% of the general population.[9] Many studies consistently suggest that homocysteine has a strong association with atherosclerosis.[10-12]Homocysteine is an important and independent risk factor for arteriosclerosis and ACI. In the current study, we provide confirmatory evidence that a higher

Table 2. *Clinical characteristics comparison between ACI and non-ACI*

Variables	Cases (n = 26)	Controls (n = 26)	t/Z	*P* value
ALT (U/L), mean ± SD	24.73 ± 9.15	21.85 ± 8.288	1.191	.239
BUN (mmol/L), mean ± SD	5.0258 ± 1.361	5.0415 ± 1.5075	−.04	.969
CREA (mmol/L), mean ± SD	62.081 ± 11.11	61.37 ± 10.51	.236	.814
LDL-C (mmol/L), mean ± SD	2.716 ± .688	2.495 ± .8588	1.021	.312
HCY (umol/L), mean ± SD	21.278 ± 6.709	16.718 ± 4.198	2.038	.005*
TG (mmol/L), M (P25-P75)	1.58 (1.21-1.79)	1.23 (0.91-1.49)	−1.95	.051
CHOL (mmol/L), M (P25-P75)	3.88 (3.47-4.34)	3.65 (3.20-4.27)	−1.016	.309
HDL-C (mmol/L), M (P25-P75)	0.92 (0.91-1.00)	1.00 (0.93-1.19)	−2.549	.011*
GLU (mmol/L), M (P25-P75)	4.56 (4.15-5.00)	4.36 (4.04-4.80)	−.97	.332
Folate (ng/mL), M (P25-P75)	4.10 (3.13-5.33)	5.05 (3.90-9.05)	−1.978	.048*
VitB12 (pg/mL), M (P25-P75)	264.00 (235.0-286.00)	273.00 (266.00-278.00)	−.979	.327

Abbreviations: ACI, atherosclerotic cerebral infarction; ALT, alanine aminotransferase; BUN, blood urea nitrogen; CHOL, cholesterol; CREA, creatinine; GLU, glucose; HDL-C, high-density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; TG, homocysteine, triglyceride.
*The *P* value is less than .05.

Table 3. ApoE *gene promoter CpG sites DNA methylation comparison between ACI and non-ACI*

Variables	Cases (n = 26)	Controls (n = 26)	χ^2	*P* value
CpG1, n (%)	8 (30.77)	4 (15.4)	1.73	.188
CpG3, n (%)	10 (38.46)	10 (38.46)	0	1
CpG4, n (%)	8 (30.77)	8 (30.77)	0	1
CpG5, n (%)	4 (15.4)	6 (23.1)	.495	.482
CpG6, n (%)	2 (7.69)	2 (7.69)	0	1
CpG7, n (%)	4 (15.4)	0 (0)	4.333	.110*
CpG9, n (%)	2 (7.7)	0 (0)	2.08	.490*
CpG10, n (%)	12 (46.2)	8 (30.8)	1.3	.254
CpG11, n (%)	12 (46.2)	18 (69.2)	2.836	.092
CpG12, n (%)	24 (92.31)	24 (92.31)	0	1
CpG13, n (%)	14 (53.8)	8 (30.8)	2.83	.092
CpG14, n (%)	18 (69.23)	10 (38.46)	4.952	.026
CpG15, n (%)	22 (84.62)	20 (76.9)	.495	.482
CpG16, n (%)	24 (92.30)	14 (53.8)	9.774	.020
CpG17, n (%)	26 (100.0)	26 (100.0)	0	1

Abbreviations: ACI, atherosclerotic cerebral infarction; *ApoE*, *Apolipoprotein E*; CpG, cytosine-phosphate-guanine.
*The Fisher's exact test.

level of homocysteine, which can lead to insufficient blood supply by causing injury to the vascular endothelium, is independently associated with ACI.

Folic acid and vitamin B12 have a great influence on modulating the metabolic process of homocysteine, as a cosubstrate and cofactor, respectively.[13] Current studies have indicated that supplying folic acid and vitamin B12 to patients with hyperhomocysteinemia could decrease homocysteine levels.[14] Some studies suggest low serum levels of folic acid and vitamin B12 are significantly correlated with ACI. However, we only found a significant discrepancy in folic acid levels between the case and control groups.

The present study provides evidence that *ApoE* is the major enzyme responsible for homocysteine-induced atherogenesis. Homocysteine in foam cells derived from monocytes may induce *ApoE* to be expressed,[15] and the *ApoE* promoter can be hypomethylated after homocysteine treatment.[16] Folate also exerts effects on the homocysteine-induced *ApoE* response; folate suppressed *ApoE* promoter methylation induced by homocysteine in cultured human monocytes.[16,17] In fact, alteration of epigenetic modifications, particularly DNA methylation status, is increasingly recognized as an important factor in the pathogenesis of many diseases, including atherosclerosis.[18] DNA methylation is a nontraditional and stable heritable factor, occurring at cytosines located upstream of a guanine (CpG dinucleotides), and its levels can be modulated by several environmental factors.[19] Promoter CpG island hypermethylation is closely associated with gene silencing and inactivation, leading to X-chromosome inactivation and the loss of expression of tumor suppressor genes.[20,21] Sharma et al provided evidence that global DNA hypomethylation and locus-specific hypermethylation are potentially associated with the process of atherosclerosis, but this relationship has yet to be explored.[22] Many studies indicate that abnormal DNA methylation has a predominant influence on the pathogenesis of atherosclerosis.[23] Aberrant promoter region methylation of certain genes is associated with ACI. *ApoE* is closely associated with dyslipidemia, CHD, ACI, and Alzheimer disease. Many investigations pay attention to the correlation

Table 4. *Logistic regression analysis model*

Variables	B	S.E.	Waldχ^2	*P* value	OR	95%CI
CpG14	.629	1.024	.377	.539	1.875	(.252, 13.964)
GpG16	2.782	1.346	4.271	.039	16.146	(1.154, 225.832)
Age	−.017	.053	.099	.753	.983	(.886, 1.091)
Gender	−1.172	1.349	.755	.385	.310	(.022, 4.359)
Carotid athero-sclerostic plaque	3.303	1.268	6.787	.009	27.194	(2.266, 326.362)
Hypertension	1.507	1.206	1.562	.211	4.513	(.425, 47.925)
HDL-C	−6.250	5.484	1.299	.254	.002	(.000, 89.860)
HCY	.033	.098	.115	.735	1.034	(.853, 1.254)
folate	−0.535	.256	4.358	.037	.586	(.355, .968)
Constant	4.483	5.136	.762	.383	88.515	

Abbreviations: CI, confidence interval; CpG, cytosine-phosphate-guanine; HDL-C, high-density lipoprotein cholesterol; OR, odds ratio.
B, the coefficient for each independent variable in the logistic regression equation; S.E., the standard errors associated with B coefficients.

of *ApoE* and Alzheimer disease. For example, a study proposed that the epsilon 2, epsilon 3, and epsilon 4 *ApoE* alleles are related with sporadic and familial Alzheimer's disease.[24] Several studies have separately shown that methylation of CpG islands in CHD risk genes has a significant role in the development of CHD.[23] However, although *ApoE* variants are associated with ACI, the effects that *ApoE* promoter methylation may exert on ACI are yet to be determined. As far as we know, this is the first study exploring the correlation between *ApoE* promoter methylation and ACI and we provide the first evidence that a higher DNA methylation level at CpG14 and CpG16 is independently associated with ACI.

Aberrant methylation of the *ApoE* promoter could contribute to ACI risk via modifying gene expression, influencing blood lipid levels, or even participating in the pathogenesis of atherosclerosis. We evaluated factors associated with ACI risk, using logistic regression analysis with confounding factors. We show statistically significant evidence to show that methylation of the *ApoE* promoter (at CpG16) increases the risk of ACI (OR = 16.146; 95% CI, 1.154-225.832; $P^* < .05$). At the same time, we observed that 2 clinical characteristics were significantly related to ACI. Carotid atherosclerotic plaques might also contribute to the risk of ACI (OR = 27.194; 95% CI, 2.266-326.362; $P^* < .05$). In contrast, folate is a protective factor for ACI occurrence (OR = .586; 95% CI, .355-.968; $P^* < .05$).

There are some limitations to be addressed in our study. First, to the best of our knowledge, this is the first analysis of the association between *ApoE* gene promoter methylation and ACI. The sample size is comparatively small; only 26 ACI patients and 26 control subjects were recruited. Hence, further investigation with larger samples is required to verify the association between *ApoE* promoter methylation and ACI. Second, from the whole *ApoE* gene promoter, we only selected 1 fragment containing 17 CpG dinucleotides, but there might be other fragments related to ACI occurrence. Third, unknown confounding factors might exist that affect our conclusions, although a Stepwise logistic regression analysis was applied. It is necessary to further screen out case groups, control groups with the same risk factors and verify the association between *ApoE* promoter methylation and ACI.

Conclusions

ACI is a complex disease caused by environmental and genetic factors and is a leading cause of disability and mortality worldwide. Epigenetic modifications, particularly DNA methylation status, is increasingly recognized as an important factor in the pathogenesis of many diseases, including atherosclerosis.[18] This study indicates that *ApoE* promoter methylation and carotid atherosclerotic plaques are highly likely to increase the risk of ACI, while folate may be a protective factor. These findings could help our understanding of the molecular mechanisms involved in the pathophysiological processes leading to ACI and to the development of new therapeutic strategies. It is also suggested that the relationship between *ApoE* promoter methylation and ACI deserves further exploration and research.

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