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Association between *ECE1* gene polymorphisms and risk of intracerebral haemorrhage

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Abstract

Objective: To determine whether endothelin converting enzyme-1 (*ECE1*) gene polymorphisms contribute to susceptibility to intracerebral haemorrhage (ICH) by influencing blood pressure.

Methods: This case–control study enrolled patients with ICH and healthy control subjects from a Southern Han Chinese population. The *ECE1* gene polymorphisms rs212528 and rs213045 were genotyped. The association between the genotypes and the risk of ICH was assessed. The effects of these two *ECE1* gene polymorphisms on blood pressure were also analysed.

Results: A total of 389 patients with ICH and 404 healthy control subjects participated in the study. There was no significant association between the *ECE1* rs212528 and rs213045 polymorphisms and ICH even after adjusting for different confounding variables. In patients with ICH, the systolic blood pressure of patients with the rs212528 AA genotype was significantly lower than that of patients with the AG/GG genotypes.

Conclusions: These results indicated that the *ECE1* rs212528 and rs213045 polymorphisms had no major role to play in the genetic susceptibility to ICH, although rs212528 might influence blood pressure in patients with ICH.

Keywords

Endothelin converting enzyme-1, *ECE1*, intracerebral haemorrhage, polymorphisms, case–control study

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Introduction

Endothelins are well known to be highly powerful vasoconstrictor peptides.[1] Endothelin-1 (ET-1), the most abundant and important endothelin isoform, is generally produced by endothelial cells and has a diverse range of effects.[1] Creation of the active form of ET-1 requires multiple steps of enzymatic cleavages.[2] The final step in this process involves the conversion of the relatively inactive big ET-1 to its physiologically active form by endothelin converting enzyme-1 (ECE-1).[3,4] ECE-1, which is a membrane-bound metalloendopeptidase,[5,6] is positioned at a crucial point in the production of ET-1.

Two common polymorphisms of the *ECE1* gene, rs212528 and rs213045 (C338A), were found to be associated with blood pressure regulation.[7–9] Hypertension is the most common risk factor for stroke.[10] The *ECE1* rs213045 polymorphism was demonstrated to be associated with an increased risk of ischaemic stroke (IS) in a Han Chinese population.[11] A subsequent study established that *ECE1* rs212528 may contribute to susceptibility to IS in Han Chinese, but it failed to duplicate the results of the previous study.[11,12] IS and haemorrhagic stroke have a shared genetic background.[13] Moreover, soluble and catalytically active ECE-1 has been found in the cerebrospinal fluid (CSF) of patients with subarachnoid haemorrhage (SAH).[14]

To the best of our knowledge, data have not been published in relation to the association between *ECE1* gene polymorphisms and intracerebral haemorrhage (ICH). This study evaluated the relationship between the *ECE1* gene polymorphisms rs212528 and rs213045 and the risk of ICH, and their influence on blood pressure in a Southern Han Chinese population.

Patients and methods

Study population

This case–control study recruited consecutive Han Chinese patients with acute ICH diagnosed at the Department of Neurology, Xiangya Hospital, Central South University, Changsha, Hunan Province, China between January 2006 and December 2011 using ICD-10.[15] Brain computed tomography (CT) and/or magnetic resonance imaging scans were performed on all patients using a clinical dual source CT scanner (SOMATOM® Definition Flash; Siemens Healthcare, Erlangen, Germany) and/or a clinical 3.0 Telsa Signa™ Excite system (GE Healthcare, Waukesha, WI, USA), respectively. No antiplatelet, thrombolytic, anticoagulant drugs or traditional Chinese medicines had been used during the 2 weeks prior to enrolment in the study. Patients with ICH related to trauma, neoplasms, coagulation disorders or thrombolytic therapy, aneurysms, or other vascular malformations and lobar ICH were excluded. Age- and sex-matched healthy control subjects who were genetically unrelated Han Chinese living in Southern China, diagnosed healthy after having a routine physical check-up for the purpose of health maintenance at Xiangya Hospital by ICD-10,[15] were enrolled in the study. Control subjects had no symptoms or history of stroke, coronary artery disease, autoimmune disease, peripheral atherosclerotic disease, or haematological disease.

Information about age, sex, body mass index (BMI; weight [kg]/height $[m]^2$), systolic blood pressure (SBP), diastolic blood pressure (DBP), smoking and alcohol drinking status, hypertension (SBP $\geq$ 140 mmHg or DBP $\geq$ 90 mmHg, or a history of high blood pressure or hypotensive drug use), type 2 diabetes mellitus (fasting blood glucose $\geq$ 7.0 mmol/l, random blood glucose $\geq$ 11.1 mmol/l or a history of type 2 diabetes mellitus, or hypoglycaemic agent use), ischaemic heart disease history, as well as

serum lipid levels (total cholesterol, triglycerides, high-density lipoprotein cholesterol and low-density lipoprotein cholesterol) and fasting blood glucose (FBG) were recorded. A peripheral venous blood sample was collected after a 12-h overnight fast and serum levels of lipids and FBG were determined immediately with standard laboratory techniques using a Roche Hitachi 912 chemistry analyser (Roche Diagnostics, Branchburg, NJ, USA). Casual blood pressure was calculated as the mean of two sets of three supine brachial blood pressure readings 10 min apart measured using a mercury sphygmomanometer when subjects were enrolled in the study.

The study was approved by the Ethics Committee of Xiangya Hospital, Central South University (no. 201003225) and all study participants provided written informed consent.

Genotyping

Genomic DNA was extracted from a peripheral whole blood sample from each study participant as described below. A peripheral venous blood sample (5 ml) was collected after a 12-h overnight fast. Genomic DNA was extracted immediately from ethylenediaminetetra-acetic acid (20 g/l) anticoagulated peripheral blood using a QIAamp® DNA Blood Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions and stored at −80°C until analysis. The polymerase chain reaction (PCR) primers and extended probe primers for multiplex SNaPshot® were designed using Primer 3 online software version 0.4.0 (Table 1).[16] PCR was carried out in a final volume of 10 µl containing 1x HotStarTaq® buffer (QIAGEN), 3.0 mM Mg^{2+}, 0.3 mM dNTP, 1 U HotStarTaq® polymerase (QIAGEN), 0.1 µM multiplex PCR primers and 1µl DNA sample. PCR amplification was carried out using an Applied Biosystems® GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The cycling programme involved preliminary denaturation at 96°C for 1 min, followed by 28 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and elongation at 60°C for 30 s, followed by a final elongation step at 60°C for 1 min. The PCR products were stored at 4°C until further analysis. 1 U shrimp alkaline phosphatase (SAP) (Promega, Madison, WI, USA) and 2 U exonuclease I (Epicentre, Madison, WI, USA) were added to 10 µl of PCR products, and incubated at 37°C for 1 h, and then at 75°C for 15 min. The multiplex SNaPshot® technique was performed following purification of the PCR products in order to determine the genotypes. The extension PCR was in a final volume of 10 µl containing 5 µl of SNaPshot® Multiplex Kit (Applied Biosystems), 2 µl of purified PCR products, 1 µl of probe primers and 2 µl of ultrapure water. The cycling programme involved preliminary denaturation at 96°C for 1 min, followed by 28 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and elongation at 60°C for 30 s, followed by a final elongation step at 60°C for 1 min. 1U SAP was added to 10 µl of the extension products and incubated at 37°C for 1 h and 75°C for 15 min. The purified extension products were run on an ABI 3130XL genetic analyser (Applied Biosystems) for data collection and GeneMapper® Software version 4.0 (Applied Biosystems) was used to analyse the data. A random sampling was conducted to verify the accuracy of the SNaPshot® technique for single nucleotide polymorphism genotyping through forward and reverse sequencing.

Statistical analyses

All statistical analyses were performed using the SPSS® statistical package, version 11.5 (SPSS Inc., Chicago, IL, USA) for

Table 1. Polymerase chain reaction (PCR) primers and extended primers used for multiplex SNaPshot® analysis of two polymorphisms (rs212528 and rs213045) of the endothelin converting enzyme-1 gene used in a study to investigate their contribution to susceptibility to intracerebral haemorrhage.

Polymorphism	PCR primers	Multiplex SNaPshot® primers
rs212528		TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
Forward	TCCCAGAGGCACAATTGAATTTATGT	GGCACAATTGAATTTATGTTTTGAT
Reverse	TTGGTCTTCATCGGGGGTTTC	
rs213045		CTTGATTGCTCTGGGCCA
Forward	AGCCCCTAGCAGGCAGGAAAG	
Reverse	GGGGAACTGAAAGGGGACACC	

Windows®. The Hardy–Weinberg equilibrium was assessed using χ^2-test. χ^2-test was also used to investigate whether there was any significant difference in genotype frequencies between the patients with ICH and the control subjects. The associations between the genotypes and risk of ICH were estimated by odds ratios (ORs) and their 95 % confidence intervals (CIs) using binary logistic regression analysis, with adjustment for age, sex, ischaemic heart disease, hypertension, type 2 diabetes mellitus, smoking and alcohol drinking status, the SBP and DBP level, and FBG. Student's *t*-test was used to compare blood pressure levels between the patients with ICH and the control subjects. A P-value < 0.05 was considered statistically significant.

Results

The present study enrolled 389 patients with acute ICH and 404 healthy control subjects. Their demographic and clinical characteristics are shown in Table 2. No significant differences were found between the patients with ICH and the control subjects in terms of age and sex distribution. Similarly, there were no significant differences in smoking status, BMI, serum total cholesterol level, serum triglyceride level, serum high-density lipoprotein cholesterol level, and serum low-density lipoprotein cholesterol level between the two groups. There were significant differences in the alcohol drinking status ($P < 0.001$), ischaemic heart disease ($P < 0.001$), hypertension ($P < 0.001$), SBP ($P = 0.003$), DBP ($P < 0.001$), type 2 diabetes mellitus ($P < 0.001$), and FBG ($P < 0.001$).

A total of 793 samples (389 patients with ICH and 404 healthy control subjects) were genotyped. The observed genotypes and allele frequencies for the patients with ICH and the healthy control subjects are shown in Table 3. The genotypic frequencies were in Hardy-–Weinberg equilibrium. The frequency of the minor allele G of rs212528 was lower in patients with ICH (17.7%) than in the control subjects (20.3%). The frequency of the minor allele T of rs213045 was increased in patients with ICH (44.0%) compared with the control subjects (41.3%). However, there was no significant difference in the allele frequencies between the patients with ICH and the control subjects for both rs212528 and rs213045.

Student's *t*-test was used to determine if there was an association between *ECE1* gene polymorphisms and blood pressure. The effect of different genotypes of the *ECE1* gene on blood pressure are shown in Table 4. The *ECE1* rs212528 AA genotype was associated with a significantly lower SBP compared with the rs212528 AG/GG genotypes in the patients with ICH

Table 2. Demographic and clinical characteristics of patients with intracerebral haemorrhage (ICH) and healthy control subjects.

Characteristic	Patients with ICH $n = 389$	Control subjects $n = 404$	Statistical significance[a]
Age, years	58.42 ± 11.19	54.96 ± 10.42	NS
Sex, male/female	258/131	259/145	NS
Ischaemic heart disease, yes/no	33/356	0/404	$P < 0.001$
Hypertension, yes/no	194/195	86/318	$P < 0.001$
Type 2 diabetes mellitus, yes/no	32/357	0/404	$P < 0.001$
Smoker, yes/no	37/352	45/359	NS
Alcohol drinker, yes/no	272/117	59/345	$P < 0.001$
BMI	24.35 ± 4.03	23.42 ± 4.24	NS
SBP, mmHg	158.47 ± 28.06	123.22 ± 19.67	$P = 0.003$
DBP, mmHg	91.25 ± 20.53	79.53 ± 11.68	$P < 0.001$
TC, mmol/l	2.06 ± 4.91	1.65 ± 1.60	NS
TG, mmol/l	4.53 ± 1.04	4.85 ± 1.18	NS
HDL-C, mmol/l	1.35 ± 0.41	1.64 ± 0.38	NS
LDL-C, mmol/l	2.51 ± 0.86	2.49 ± 0.88	NS
FBG, mmol/l	6.83 ± 4.20	5.39 ± 1.20	$P < 0.001$

Data presented as mean ± SD or *n* of patients.
[a]Student's *t*-test for continuous variables and χ^2-test for categorical variables.
BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; FBG, fasting blood glucose; NS, no statistically significant between-group difference ($P \geq 0.05$).

Table 3. Distribution of *ECE1* gene polymorphism genotypes and their associations with risks of intracerebral haemorrhage (ICH) in patients with ICH and healthy control subjects.

Polymorphism	Genotypes	Patients with ICH $n = 389$	Control subjects $n = 404$	Statistical significance[a]	Adjusted OR[b] (95% CI)
rs212528	AA	265 (68.1)	258 (63.9)		1.00 (reference)
	AG	110 (28.3)	128 (31.7)	NS	1.361 (0.374, 4.362)
	GG	14 (3.6)	18 (4.5)	NS	1.173 (0.301, 3.687)
	A allele	640 (82.3)	644 (79.7)	NS	
	G allele	138 (17.7)	164 (20.3)		
rs213045	GG	118 (30.3)	145 (35.9)		1.00 (reference)
	GT	200 (51.4)	184 (45.5)	NS	0.915 (0.432, 1.746)
	TT	71 (18.3)	75 (18.6)	NS	1.259 (0.594, 2.361)
	G allele	436 (56.0)	474 (58.7)	NS	
	T allele	342 (44.0)	334 (41.3)		

Data presented as *n* of patients (%) or *n* of alleles (%).
[a]Two-sided χ^2-test test for distribution between the patients and control subjects.
[b]Adjusted for age, sex, ischaemic heart disease, hypertension, type 2 diabetes mellitus, smoking and alcohol drinking status, systolic blood pressure, diastolic blood pressure, and fasting blood glucose in the binary logistic regression analysis.
OR, odds ratio; CI, confidence interval; NS, no statistically significant difference ($P \geq 0.05$).

($P = 0.014$). No other associations between *ECE1* polymorphisms and blood pressure were found, even when compared in male or female subgroups (data not shown).

Discussion

Intracerebral haemorrhage is a complex disease influenced by genetic as well as environmental factors.[17] The detection of polymorphisms that affect gene function or expression and contribute to ICH susceptibility is important as it may help to predict individual and population risk and clarify pathophysiological mechanisms related to ICH.

Elevated blood pressure is found in up to 90% of patients with ICH,[18] and both excessively high and low admission SBP and DBP levels are associated with mortality in patients with acute ICH.[19] ET-1 is a highly potent vasoconstrictor peptide that is associated with vascular tone and blood pressure regulation.[20] ECE-1 is the main enzyme responsible for ET-1 generation.[9] ECE-1 protein has been found in the endothelial cells of the aorta, lung, kidney, liver, heart, ovary, epididymis, and certain endocrine cells.[21–23] A recent study reported for the first time the presence of the catalytically active, soluble form of ECE-1 in the CSF of patients with SAH.[14]

The human *ECE1* gene is located on chromosome 1, spanning over 120 kilo base pairs (bp) and consisting of 20 exons.[24] This single gene encodes four different isoforms of ECE-1 (ECE-1a, 1b, 1c and 1d), each one under the control of a different promoter.[25] The *ECE1* gene polymorphism rs212528 is located in the *ECE1* gene intron region between exons 5 and 6,[12] while the rs213045 polymorphism is located in the *ECE1* gene promoter (338 bp upstream from the translation start site).[26] The *ECE1* rs213045 polymorphism is a functional variant, of which the A allele is associated with increased promoter activity,[8] and two-fold higher mRNA expression levels.[12]

Table 4. Associations between *ECE1* gene polymorphism genotypes and blood pressure in patients with intracerebral haemorrhage (ICH) and healthy control subjects.

	Genotypes	*n*	SBP, mmHg	DBP, mmHg
Patients with ICH				
rs212528	AA	265	154.53 ± 27.78	90.42 ± 20.38
	AG + GG	124	165.17 ± 25.53	92.26 ± 20.77
	Statistical significance[a]		$P = 0.014$	NS
rs213045	GG	118	157.78 ± 24.93	90.78 ± 18.54
	GT + TT	271	158.41 ± 29.37	91.23 ± 21.49
	Statistical significance[a]		NS	NS
Control subjects				
rs212528	AA	258	121.19 ± 20.58	78.68 ± 12.39
	AG + GG	146	126.72 ± 18.14	81.41 ± 10.53
	Statistical significance[a]		NS	NS
rs213045	GG	145	124.43 ± 17.47	79.48 ± 11.66
	GT + TT	259	122.33 ± 21.32	79.69 ± 11.98
	Statistical significance[a]		NS	NS

Data presented as mean ± SD.

[a]Student's *t*-test.

SBP, systolic blood pressure; DBP, diastolic blood pressure; NS, no statistically significant difference ($P \geq 0.05$).

Currently, there is no known change in function connected with the alternative alleles of the *ECE1* rs212528 polymorphism. The association between these two common polymorphisms of the *ECE1* gene and ECE-1 protein level is still not clear, however, they are indicated to be involved in hypertension and IS.[7–9,11,12]

This present molecular epidemiological study investigated whether the *ECE1* rs212528 and rs213045 polymorphisms could have an impact on the susceptibility to ICH by influencing blood pressure. In contrast to what was expected, the present case–control study in a Southern Han Chinese population demonstrated no significant difference in the distribution of the *ECE1* rs212528 and rs213045 polymorphism genotypes between patients with ICH and the control subjects. No significant association emerged between the risk of ICH and *ECE1* rs212528 and rs213045 polymorphisms in the overall statistical analyses. Research has demonstrated the existence of a negative feedback action of ET-1 to regulate *ECE1* gene expression.[25] Plasma ET-1 levels were shown to be increased in patients with acute ICH, which may be positively associated with haematoma volume and severity of ICH.[27] It is possible that elevated ET-1 levels downregulate the association between these two *ECE1* gene polymorphisms with ICH. Moreover, there are other factors negatively effecting *ECE1* expression such as shear stress (the mechanical force exerted on endothelial cells by the flow of blood through vessels), and also positive ones such as hypercholesterolaemia, statin therapy, and high glucose/diabetes.[25] These factors are all possibly involved in patients with ICH. The pathophysiological mechanisms of *ECE1* and its polymorphisms related to ICH need further study.

This present study found that the *ECE1* rs212528 AA genotype was associated with a significantly lower SBP compared with the rs212528 AG/GG genotypes in patients with ICH. There was no other association between the two *ECE1* polymorphisms and blood pressure in this study, even when they were compared in male or female subgroups. These current findings suggest that the *ECE1* rs212528 polymorphism is not associated with ICH by affecting blood pressure in patients with ICH. A study undertaken in 704 hypertensive patients in Germany demonstrated that the *ECE1* rs213045 A allele was significantly associated with ambulatory blood pressure values in untreated hypertensive women.[8] This finding was then confirmed by a study undertaken in 1189 subjects participating in the Etude du Vieillissement Artériel study in France.[9] Another study of 1873 individuals from a general Japanese population reported that the SBP in women with the *ECE1* rs212528 CC genotype was higher than that in those with the TT genotype.[7] There were no significant differences between *ECE1* rs212528, rs213045, SBP, and DBP in males or females in 381 patients with IS and 366 control subjects in a study of Northern Han Chinese.[12] These discrepancies may be due to ethnic variations between the populations that were studied.

The present study had several limitations. First, this study was only carried out in a Southern Han Chinese population and the stratified analyses were relatively limited by the sample size. Further studies in other ethnic groups with larger samples sizes are needed to validate this current result. Secondly, blood pressure is highly variable in patients with ICH and many factors can influence the recorded blood pressure values. Although the present study used the standard method for measuring and recording blood pressure as used by a large-scale blood pressure study in patients with ICH,[28] the possibility of measuring and recording bias cannot been eliminated.

In conclusion, to the best of our knowledge, this is the first study to investigate the association between *ECE1* gene

polymorphisms and the risks of ICH in a Southern Han Chinese population. The present study demonstrated that the *ECE1* rs212528 and rs213045 polymorphisms played no major role in the genetic susceptibility to ICH, although rs212528 might influence blood pressure in patients with ICH.

Declaration of conflicting interest

The authors declare that there are no conflicts of interest.

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