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Original Article

Association of endothelin-converting enzyme-1 polymorphisms with risk of ischemic stroke in the Chinese Han population

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Abstract: Background: The endothelin-converting enzyme-1 (ECE-1), a membrane bound metalloprotease, is involved in proteolytic processing of precursor big endothelin to activated endothelin-1 (ET-1). ET-1 is implicated in the regulation of vascular function and development of atherosclerosis. As such, ECE-1 may play a crucial role in the pathogenesis of cerebral vascular disease via actions of ET-1. This study aims to investigate whether ECE-1 polymorphisms are associated with ischemic stroke and its subtypes in the Chinese Han population. Methods: We studied 397 ischemic stroke patients and 380 healthy subjects. ECE-1 polymorphisms (C-338A and T-839G) were determined by polymerase chain reaction-ligation detection reaction. Results: 207 patients with atherothrombotic stroke and 190 patients with small artery disease were enrolled in case group. In subgroup of atherothrombotic stroke, the frequency of CA+AA genotype and A allele of C-338A as well as TG+GG genotype and G allele of T-839G was higher in the patient group compared with that in the control group. Logistic regression analysis revealed an increased risk of atherothrombotic stroke in dominant model for both C-338A and T-839G. Conclusions: The present finding demonstrates that ECE-1 polymorphisms are associated with increased risk of developing atherothrombotic stroke in the Chinese Han population.

Keywords: Endothelin-converting enzyme-1, gene polymorphism, ischemic stroke, atherothrombotic stroke, small artery disease

Introduction

Stroke is a major cause of death and disability worldwide [1]. It is estimated that 41% to 79% of all stroke cases are ischemic stroke in China [2]. The significant increase in ischemic stroke burden has led to a series of public health problems [3]. Therefore, effective prevention and treatment for ischemic stroke is an important medical matter. Ischemic stroke is a complex disease caused by both environmental and genetic risk factors. Twin and family history studies provide evidence that genetic factors play an important role in mediating susceptibility to ischemic stroke [4, 5]. Thus, investigations of the genetics of ischemic stroke could be of great value in discovering underlying molecular mechanisms and identifying novel therapeutic strategies for ischemic stroke.

Endothelin-converting enzyme-1 (ECE-1), as a membrane bound metalloprotease, is involved in the proteolytic processing of precursor big endothelin to activated endothelin-1 (ET-1) [6]. Increased expression of ECE-1 could promote ET-1 biosynthesis. ET-1 is an endogenous vasoconstrictor, which is known to take part in regulation of vascular tone and proliferation of smooth muscle cells [7-9]. The previous data demonstrated that overexpression of ET-1 decreased plasma high density lipid (HDL), elevated superoxide generation and inflammatory cell infiltration in atherosclerotic lesions and vascular wall, and further promoted to atherosclerosis progression [10-12]. Moreover, expression of ET-1 as well as ECE-1 was increased in human atherosclerotic plaque [13-15]. Consequently, ECE-1 is believed to contribute to the development of atherosclerosis and various vascular diseases by ET-1 mediated pathophysiological events [13, 16-18].

The *ECE-1* gene maps on human chromosome 1p36 band and consists of 19 exons [19].

Table 1. LDR detection probes

SNPs	Probes for LDR
C-338A	RG: TCTCTCGGGTCAATTCGTCCTTTGTCTTGATTGCTCTGGGCAAC
	RT: TGTTCGTGGGCCGGATTAGTTGTCTTGATTGCTCTGGGCGAA
	RP: ATCGAGGGCACCTTCCTGATTTTTTTTT
T-839G	FA: TACGGTTATTCGGGCTCCTGTCTGTCCCAACCAAGAACCACA
	FC: TTCCGCGTTCGGACTGATATCTGTCCCAACCAAGAACCACC
	FP: GAGAGGTCTAACCCAGCAGATTTCCTTTTTTTTTTTT

Table 2. Characteristics of case and control in this study cohort

	Case (n=397)	Control (n=380)	*P* value
Age (years)[a]	62.0±9.86	62.0±9.79	0.971
Gender (male/female)[b]	275/122	263/117	0.986
Hypertension, n (%)[b]	281 (70.8)	221 (58.2)	<0.001
Diabetes mellitus, n (%)[b]	137 (34.5)	72 (18.9)	<0.001
Dyslipidemia, n (%)[b]	180 (45.3)	144 (37.9)	0.035
Smoking, n (%)[b]	138 (34.8)	76 (20.0)	<0.001

[a]Mann-Whitney *U* test; [b]χ^2 test.

Several *ECE-1* gene polymorphisms have been investigated. The most prevalent is the C-338A, which is associated with susceptibility to hypertension, coronary artery disease, carotid atherosclerosis, and ischemic stroke [20-24]. The other functional polymorphic site in the *ECE-1* promoter region, T-839G, has also been identified. The -338A/-839G haplotype showed increased transcriptional activities compared with the wild type (-338C/-839T) [20]. The two *ECE-1* gene polymorphisms presumably act in an additive manner to promote the development of vascular diseases [20]. However, the relationship between T-839G polymorphism and the risk of ischemic stroke hasn't been investigated so far.

Therefore, the purposes of our study were to determine whether the two polymorphisms (C-338A and T-839G) of *ECE-1* gene affected the susceptibility to ischemic stroke and whether the role differed between certain pathogenic subtypes of ischemic stroke in a cohort of Chinese Han population.

Materials and methods

Study population

397 cases hospitalized in the Department of Neurology at The First Affiliated Hospital of China Medical University (Shenyang, China) between October 2011 and March 2013 was enrolled in this study. The diagnosis of ischemic stroke was made according to clinical history and neurological examination and confirmed by radiological imaging. The subtype classification of ischemic stroke was based on Korean TOAST (Trial of ORG 10172 in Acute Stroke Treatment) criteria [25]. Only patients with atherothrombosis (AT) and small artery disease (SAD) subtypes were included in this study. Patients with cerebral embolism, transient ischemic attack, cerebrovascular malformations, hemorrhagic stroke, coagulation disorders or other chronic diseases were excluded from this study.

During the same period, 380 unrelated controls matched by age and sex were selected from the Physical Examination Center of the Red Cross Hospital (Shenyang, China). The subjects with cerebrovascular disease, ischemic heart diseases, peripheral vascular disease, tumor or severe underlying diseases were excluded from the control group.

All subjects were from Han population living in northern China. The demographic data and clinical parameters, including gender, age, medical history, drug history, blood pressure, blood glucose, lipid profile and smoking status, were collected. Dyslipidemia was defined as total cholesterol level (TC) ≥ 5.72 mmol/L and/or triglycerides (TG) level ≥ 1.70 mmol/L or on drugs. The study was approved by the ethics committees of both hospitals, and all study subjects signed informed written consents.

Genotyping

Fasting venous blood of all participants was sampled in ethylene diamine tetraacetic acid (EDTA) tube. Genomic DNA was extracted from peripheral blood leukocytes using a DNA purification kit from Promega (Madison, WI, USA). Genotype was determined by polymerase chain reaction-ligation detection reaction (PCR-LDR) assay. The primer sequences used for PCRs were as following: for C-338A, forward 5'-GGTCCCCAGTGGCAGATAACAA-3', reverse 5'-TTCATCCCGTGTCAGGGAG TTC-3'; and for T-839G, forward 5'-ATTGCTCCAGGGGGAGTCAGAA-3', re-

Table 3. Genotypic distribution and allelic frequency between case and control

		Case (n=397)	Control (n=380)	*P* value	OR (95% CI)
C-338A					
Genotype	CC	121 (30.5)	128 (33.7)	Reference	
	CA	204 (51.4)	190 (50.0)	0.432	1.136 (0.827-1.560)
	AA	72 (18.1)	62 (16.3)	0.338	1.228 (0.807-1.871)
Allele	C	446 (56.2)	446 (58.7)	Reference	
	A	348 (43.8)	314 (41.3)	0.317	1.108 (0.906-1.355)
Dominant model	CA+AA vs. CC	276/121	252/128	0.338	1.159 (0.857-1.566)
Recessive model	AA vs. CA+CC	72/325	62/318	0.502	1.136 (0.782-1.650)
T-839G					
Genotype	TT	121 (29.0)	135 (35.5)	Reference	
	TG	209 (52.6)	186 (48.9)	0.159	1.254 (0.915-1.718)
	GG	67 (16.9)	59 (15.5)	0.277	1.267 (0.826-1.943)
Allele	T	451 (56.8)	456 (60.0)	Reference	
	G	343 (43.2)	304 (40.0)	0.201	1.141 (0.932-1.396)
Dominant model	TG+GG vs. TT	276/121	245/135	0.135	1.257 (0.931-1.696)
Recessive model	GG vs. TG+TT	67/330	59/321	0.61	1.105 (0.754-1.619)

verse 5'-CGCCTTGCTAGAAGCGGAGAGT-3'. PCRs were performed as the following condition: 95°C for 2 min; followed by 11 cycles of 94°C for 20 sec, 65°C-0.5°C/cycle for 40 sec, 72°C for 90 sec; 24 cycles of 94°C for 20 sec, 59°C for 30 sec, 72°C for 90 sec; and 72°C for 2 min at the final extension reaction. The probes for LDR are listed in **Table 1**. The ligation reactions were performed in a total volume of 10 μl, containing 6 μl of double distilled H_2O, 2 μl of PCR product, 1 μl of 10× ligation buffer, 0.4 μl of each discriminating probe, and 0.25 μl of Taq DNA ligase (New England Biolabs, USA). The LDR cycling conditions were as follows: 38 cycles of 94°C for 60 sec and 56°C for 4 min, 4°C forever. Then, 0.5 μl of purified product was mixed with 0.5 μl of Liz500 SIZE STANDARD and 9 μl of Hi-Di followed by denaturation at 95°C for 5 min. The purified final products of PCR-LDR were sequenced by the ABI Prism 3730XL DNA sequencer (ABI, Foster city, CA, USA) and the data was analyzed by GeneMapper 4.1 (Applied Biosystems, USA).

Statistical analysis

The data were presented as mean ± standard deviation or percent frequency. Differences in clinicopathological characteristics between patients and controls were evaluated by using Mann-Whitney U test for continuous variables and χ^2 test for categorical variables. Genotype frequency for each single nucleotide polymorphism (SNP) in two groups was checked for Hardy-Weinberg equilibrium (HWE) by χ^2 test. Differences of the distributions of genotypes and alleles between cases and controls were estimated by χ^2 test. The relation between ECE-1 gene SNPs and susceptibility to ischemic stroke was analyzed by computing the odds ratios (OR) and 95% confidence intervals (CI) from binary logistic regression analysis. A *P* value <0.05 was considered statistically significant. Statistical analyses were carried out using SPSS version 17.0 software.

Results

The baseline characteristics of patients (n=397) and controls (n=380) are shown in **Table 2**. Age and gender were matched in this case-control study. Compared to controls, the percentage of subjects with hypertension, diabetes mellitus, dyslipidemia, or tobacco smoking was significantly higher in the case group.

Genotypic distributions of *ECE-1* C-338A and T-839G polymorphism in both groups followed HWE. For C-338A, *P*=0.384 and 0.544 between case and control, respectively; whereas, for T-839G, *P*=0.147 and 0.700 between case and control, respectively.

Specifically, in the analyses of total study population, for both two SNPs, there was no statistically significant difference found in either geno-

Table 4. Genotypic distribution and allelic frequency in patients with AT and controls

		AT (n=207)	Control (n=380)	*P* value	OR (95% CI)
C-338A					
Genotype	CC	48 (23.2)	128 (33.7)	Reference	
	CA	119 (57.5)	190 (50.0)	0.012	1.670 (1.116-2.500)
	AA	40 (19.3)	62 (16.3)	0.039	1.720 (1.025-2.887)
Allele	C	215 (51.9)	446 (58.7)	Reference	
	A	199 (48.1)	314 (41.3)	0.026	1.315 (1.033-1.673)
Dominant model	CA+AA vs. CC	159/48	252/128	0.008	1.683 (1.143-2.477)
Recessive model	AA vs. CA+CC	40/167	62/318	0.358	1.229 (0.792-1.906)
T-839G					
Genotype	TT	49 (23.7)	135 (35.5)	Reference	
	TG	121 (58.5)	186 (48.9)	0.004	1.792 (1.203-2.671)
	GG	37 (17.9)	59 (15.5)	0.04	1.728 (1.022-2.921)
Allele	T	219 (52.9)	456 (60.0)	Reference	
	G	195 (47.1)	304 (40.0)	0.019	1.336 (1.049-1.700)
Dominant model	TG+GG vs. TT	158/49	245/135	0.003	1.777 (1.211-2.606)
Recessive model	GG vs. TG+TT	37/170	59/321	0.462	1.184 (0.754-1.859)

Table 5. Logistic regression analysis

SNPs	Genetic model	Genotype contrasts	*P* value	Adjusted OR (95% CI)[a]
C-338A	Dominant model	CA+AA vs. CC	0.008	1.726 (1.153-2.585)
T-839G	Dominant model	TG+GG vs. TT	0.003	1.821 (1.221-2.716)

[a]Adjusted OR and 95% CI were adjusted for hypertension, diabetes mellitus, dyslipidemia, smoking as confounding variables between patients with AT and controls.

typic distribution or allelic frequency between patients and controls (**Table 3**).

While, in the analyses of AT subgroup, consisted of 207 AT cases and 380 controls, we found some meaningful results (**Tables 4**, **5**). For *ECE-1* C-338A, genotypic frequencies found among patients with AT and controls were 23.2% CC, 57.5% CA, 19.3% AA and 33.7% CC, 50.0% CA, 16.3% AA respectively, a significant increased risk of atherothrombotic stroke was observed in both CA (OR: 1.670, 95% CI=1.116-2.500, *P*=0.012) and AA (OR: 1.720, 95% CI= 1.025-2.887, *P*=0.039) genotypes. Allelic frequency of A was significantly higher in patients with AT than in the controls (OR=1.315, 95% CI=1.033-1.673, *P*=0.026). There was also a positive correlation between C-338A polymorphism and atherothrombosis subtype in the dominant model analysis (CA+AA vs. CC: OR= 1.683, 95% CI=1.143-2.477, *P*=0.008) (**Table 4**). Logistic regression analysis revealed that the CA+AA genotype of C-338A polymorphism was still significantly associated with an increased risk of ischemic stroke compared to the CC genotype in AT subgroup (adjusted OR= 1.726, 95% CI=1.153-2.585, *P*=0.008), after adjustment for confounding variables (**Table 5**). For the other SNP, *ECE-1* T-839G, the frequencies of TG and GG genotype were significantly higher in AT subgroup than in control group (OR: 1.792, 95% CI= 1.203-2.671, *P*=0.004; OR=1.728, 95% CI= 1.022-2.921, *P*=0.040, respectively). The incidence of G allele was significantly more frequent in atherothrombotic stroke patients than in control subjects (47.1% vs. 40%; OR=1.336, 95% CI=1.049-1.700, *P*=0.019). Significant association was observed in the dominant model for T-839G (TG+GG vs. TT: OR=1.777, 95% CI=1.211-2.606, *P*=0.003). Moreover, after adjustment for confounding variables, the TG+GG genotype group still exhibited an increased risk of atherothrombotic stroke compared to the TT genotype group (adjusted OR=1.821, 95% CI=1.221-2.716, *P*=0.003) (**Tables 4**, **5**).

However, in the analyses of SAD subgroup, both the C-338A and T-839G variations were not related to the risk of SAD (**Table 6**).

Table 6. Genotypic distribution and allelic frequency in patients with SAD and controls

		Case (n=190)	Control (n=380)	*P* value	OR (95% CI)
C-338A					
Genotype	CC	73 (38.4)	128 (33.7)	Reference	
	CA	85 (44.7)	190 (50.0)	0.216	0.784 (0.534-1.153)
	AA	32 (16.8)	62 (16.3)	0.704	0.905 (0.541-1.514)
Allele	C	231 (60.8)	446 (58.7)	Reference	
	A	149 (39.2)	314 (41.3)	0.495	0.916 (0.712-1.178)
Dominant model	CA+AA vs. CC	117/73	252/128	0.265	0.814 (0.567-1.169)
Recessive model	AA vs. CA+CC	32/158	62/318	0.873	1.039 (0.651-1.658)
T-839G					
Genotype	TT	72 (37.9)	135 (35.5)	Reference	
	TG	88 (46.3)	186 (48.9)	0.539	0.887 (0.605-1.300)
	GG	30 (15.8)	59 (15.5)	0.858	0.953 (0.564-1.611)
Allele	T	232 (61.1)	456 (60.0)	Reference	
	G	148 (38.9)	304 (40.0)	0.732	0.957 (0.744-1.231)
Dominant model	TG+GG vs. TT	118/72	245/135	0.579	0.903 (0.630-1.295)
Recessive model	GG vs. TG+TT	30/160	59/321	0.935	1.020 (0.632-1.646)

Discussion

In this study, we investigated the association between *ECE-1* polymorphisms (C-338A and T-839G) and ischemic stroke in a cohort of Chinese Han population. Our results suggested that the genotypic distribution and allelic frequency of C-338A and T-839G were associated with increased risk of atherothrombotic stroke, but there was no association found in small artery disease subgroup or total cohort population.

Functional analyses suggested that the -338A allele of *ECE-1* gene had increased transcriptional activity than -338C allele by increasing transcription factor binding affinity to *ECE-1* gene promoter [20]. A further research reported that in human prefrontal neocortex, carriers of the -338A allele showed higher level of *ECE-1* mRNA expression than non-carriers [26]. A number of genetic association studies demonstrated that C-338A polymorphism of *ECE-1* gene was associated with diversified vascular diseases. Funke-Kaiser et al. discovered that -338A allele was associated with higher blood pressure values in hypertensive women without medication [20]. Wang et al. demonstrated an association between this functional polymorphic site and coronary artery disease in Chinese population, the A allele was associated with an increased risk of coronary artery disease [27]. Zhao and colleagues found that A allele was associated with increased risk of carotid atherosclerosis, an early marker for generalized atherosclerosis [22]. Two subsequent studies demonstrated that *ECE-1* C-338A polymorphism may contribute to increased ischemic stroke susceptibility [23, 24]. While, ischemic stroke is a complex disease caused by various potential etiologies. The underlying mechanisms of different subtypes are distinguishing. Studies targeted on ischemic stroke subtypes could raise the possibility to reveal underlying different genetic background. However, the previous studies haven't elucidated whether the role of *ECE-1* C-338A polymorphism differs between certain subtypes of ischemic stroke. The epidemiological studies have validated an increased genetic effect on atherothrombosis and small artery disease subtypes. Therefore, we selected patients with atherothrombosis or small artery disease subtype according to Korean TOAST criteria into our present study, while other subtypes were excluded. We found that the associations between C-338A and risk of ischemic stroke were dependent on disease subtypes. In the AT subgroup, patients with CA and AA genotype and A allele had a significantly increased risk of ischemic stroke than those with CC genotype and C allele. Multivariate analyses showed that CA+AA genotype was a risk factor for atherothrombotic stroke.

Besides C-338A, we selected the other polymorphic site T-839G. It has also been reported that the T-839G polymorphism is significantly associated with congenital heart disease in the Chinese population and blood pressure levels in females in the Germany population [20, 28]. The results were similar to those obtained from the C-338A study. The -839G allele carriers had a significantly increased risk for atherothrombotic stroke.

However, our data showed no association between these two *ECE1* gene SNPs and risk of small artery disease subtype.

Atherothrombotic stroke is typically caused by atherosclerotic plaque formation in large arteries [25]. In contrast, small artery disease subtype is predominantly caused by small vessel sclerosis and lipohyalinosis related to hypertension [29, 30]. ECE-1, as the key enzyme to promote formation of ET-1, is implicated in the pathogenesis of vascular diseases via actions of ET-1. Especially, it has been shown that ECE-1 is closely associated with atherosclerosis. Previous studies demonstrated that the levels of both ET-1 and ECE-1 were increased in human atherosclerotic plaque [31]. The upregulation of the ECE-1/ET-1 system may promote vasoconstriction, smooth muscle cell proliferation and inflammatory cell infiltration in early stages of atherosclerosis, and may lead to plaque unstable in advanced stages of atherosclerosis [17, 31]. In addition, it was reported earlier that polymorphisms in other genes of endothelin system (*ET, ET type A receptor and ET type B receptor*) were not the determinants of genetic risk of cerebral small vessel disease [32]. Hence, ECE-1 seems to affect large artery atherosclerosis but not small artery disease. This study indicates that *ECE-1* gene variants confer the susceptibility to atherothrombotic stroke, but not to small artery disease subtype in the Chinese Han population. Our results are therefore in line with these previous observations.

As expected, it is of note that this study was conducted in a relative small sample size. Furthermore, the study did not determine the functional studies of these mutations. Therefore, the suggested relationship between tested SNPs and atherothrombotic stroke still need validation study in large population, and functional investigation should be conducted in future studies to explore the potential etiology of ischemic stroke.

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Disclosure of conflict of interest

None.

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