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Original Article

Epoxyeicosatrienoic Acids are Mediated by *EPHX2* Variants and may be a Predictor of Early Neurological Deterioration in Acute Minor Ischemic Stroke

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***Aim*: To investigatethe association of plasma epoxyeicosatrienoic acids (EETs) with early neurologic deterioration (END), and whether EETs are mediated by *EPHX2* variants in patients with minor ischemic stroke (MIS).**

***Method*: This was a prospective, multi-center observational study in patients with acute MIS in the Chinese population.Plasma EETs levels were measured on admission. Single nucleotide polymorphisms (SNPs) of *EPHX2*rs751141 were genotyped using mass spectrometry. The primary outcome was END within 10 days after admission. END was defined as an increase in NIHSS of 2 or more points. The degree of disability was assessed using the modified Rankin Scale (mRS) at 3 months after admission.**

***Results*: A total of 322 patients were enrolled, of which 85 patients (26.4%) experienced END. The mean EETs level was 64.1±7.5 nmol/L. EETs levels were significantly lower in patients with END compared to patients without END. Frequency of *EPHX2* rs751141 GG was higher in patients with END than in patients without END, and *EPHX2* rs751141 GG genotype was associated with lower EETs levels. Low level (<64.4 nmol/L) of EETs was an independent predictor of END (first and second quartiles) in multivariate analyses. END was associated with a higher risk of poor outcome (mRS scores 3−6) at 3 months.**

***Conclusion*: END is fairly common and associated with poor outcomes in acute MIS. *EPHX2* variants may mediate EETs levels, and low levels of EETs may be a predictor for END in acute MIS.**

Key words: Early neurological deterioration, Epoxyeicosatrienoic acids, Minor ischemic stroke, Outcome, *EPHX2* variants

Introduction

One third of patients with acute ischemic stroke develop early neurological deterioration (END), which in turn is associated with increased mortality and long-term functional disability[1, 2]. END is a common occurrence in patients with minor ischemic stroke (MIS)[3, 4]. Studies have shown that 21% to 50% of MIS patients suffer from END during the 7−10 days after stroke onset[4, 5], and approximately 15% to 30% of these patients were dead or disabled at the time of 3-month follow-up, despite mild symptoms at presentation[6]. The underlying mechanisms of this association are not completely understood, although biochemical factors associated with END have been reported[7, 8].

Arachidonic acid (AA) is a major membrane fatty acid that can be metabolized by cytochrome P450 (CYP) epoxygenases into four epoxyeicosatrienoic acids (EETs), which can then be metabolized by soluble epoxide hydrolase (sEH) to yield less biologically-active dihydroxyeicosatrienoic acids (DiHETEs)[9]. EETs play an important role in cerebral blood flow regulation and neuroprotection after brain injury[10, 11]. Previous

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Table 1. EETs levelsand *EPHX2rs*751141 genotype distribution in patients with or without END

Variables	Patients with END ($n=85$)	Patients without END ($n=237$)	*P* value
EETs (nmol/L)	60.3 ± 7.3	68.4 ± 8.1	< 0.001
DiHETEs (nmol/L)	84.1 ± 7.5	75.3 ± 7.2	< 0.001
*EPHX2rs*751141			
GG, n (%)	66 (77.6)	123 (51.9)	< 0.001
AG, n (%)	17 (20.0)	100 (42.2)	
AA, n (%)	2 (2.4)	14 (5.9)	

END, earlyneurological deterioration; EET, epoxyeicosatrienoic acids; DiHETEs, dihydroxyeicosatrienoic acids.

studies from our lab have shown that low plasma EETs levels were associated with plaque stability or carotid stenosis in ischemic stroke patients, and independently associated with high risk of ischemic stroke[12-14]. However, whether EETs are a risk factor for END after acute MIS has not been well studied.

SEH is a key enzyme in the metabolic conversion and degradation ofEETs[9]. Increasing EETs levels, by inhibiting the sEH enzyme, decreases cerebral damage following stroke. This improved outcome following cerebral ischemia is a consequence of improving cerebral vascular structure or function and protecting neurons from cell death[10, 11]. Thus, sEH is a potential novel therapeutic target for cardiovascular diseases and ischemic stroke[11]. Genetic variations in the sEH gene —*EPHX2*— are associated with ischemic stroke risk[15]. In experimental studies, sEH inhibition and gene deletion reduced infarct size after focal cerebral ischemia in mice[16, 17]. However, the relationship between *EPHX2* variant, EETs levels, and risk of END in patients with acute MIS has not been well investigated. Clarifying this relationship is critical for understanding the mechanisms of END, and for preventing and treating END within the context of stroke. Therefore, the aim of the present study was to evaluate the potential associations between EETs levels, *EPHX2* variants, and END in Chinese patients with MIS.

Materials and Methods

Study Population

This prospective multi-center study of MIS was conducted in the People's Hospital of Deyang City, the Second and Third Affiliated Hospital of Wenzhou Medical University. The study protocol was approved by the Ethics Committees at the participating hospitals. Written informed consent was obtained from each patient prior to study enrollment. We consecutively enrolled 322 patients with MIS who had their first strokes and were admitted to the participating hospitals within 24 h of stroke onset between March 2013 and June 2015. All enrolled patients underwent computed tomographic angiography or magnetic resonance angiography of the brain, as well as color duplex ultrasound investigation of the carotid arteries. Common electrocardiogram (ECG), 24-h Holter electrocardiogram (ECG) and echocardiogram were performed to reveal any possible cardio-embolic stroke. The inclusion criteria were: (1) age ≥ 40 years old; (2) diagnosis of MIS with National Institutes of Health Stroke Scale (NIHSS) score ≤ 3 points at admission[18]; (3) no history of carotid endarterectomy or carotid stent therapy; (4) the etiology of stroke in MIS was due to atherothrombotic or small artery disease, according to the Trial of Org 10172 in Acute Stroke Treatment criteria[19]; and (5) no history of clopidogrel or aspirin treatment for at least seven days prior to admission. The exclusion criteria were: (1) allergy to clopidogrel and aspirin; (2) patients who declined participation in the study; (3) cardiac or any other etiology of stroke (determined or undetermined)[19]; (4) cerebellar infarction or multiple infarction; (5) previous myocardial infarction (MI); (6) usage of warfarin or heparin in the preceding 2 weeks or within 10 days after admission; (7) blood platelet count $< 100 \times 10^9$/L or $> 450 \times 10^9$/L; (8) fever, hypoxia, or hemodynamic compensation at admission; (9) other conditions such as asthma or severe cardiovascular, liver, or renal disease. All patients received standard therapy based on the practice guidelines[20]. Detailed background information on the patients was described in our previous article[21].

Data on various risk factors, including age, gender, current smoking, history of diabetes mellitus and hypertension, were recorded. Fasting blood samples were tested for blood sugar, hemoglobin A1c (HbA1c), triglycerides (TG), total plasma cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C).

Measurement of Plasma EETs Levels

Whole blood (4 ml) was drawn from each patient at admission and transferred into a sterile tube con-

Table 2. Baseline characteristics and clinical outcomes between low EETs and high EETs groups

	Low EETs (≤64.3 nmol/L) (*n*=161)	High EETs (>64.4 nmol/L) (*n*=161)	*P* value
Age (years)	70.1±11.8	66.8±12.6	0.016
Males (n, %)	105 (65.2)	96 (59.6)	0.292
Diabetes mellitus (n, %)	77 (47.8)	64 (39.8)	0.152
Hypertension (n, %)	133 (82.6)	114 (70.8)	0.014
Current smoker (n, %)	63 (39.1)	68 (42.2)	0.579
NIHSS score at enrollment	1.7±0.8	1.8±0.9	0.426
TG (mmol/L)	1.7±0.7	1.6±0.6	0.224
TC (mmol/L)	5.7±1.7	5.5±1.6	0.327
LDL-C (mmol/L)	3.1±0.9	2.9±1.1	0.077
HDL-C (mmol/L)	1.2±0.6	1.3±0.7	0.226
Fasting glucose (mmol/L)	6.7±2.6	6.5±2.3	0.442
Hemoglobin A1c (%)	6.6±2.2	6.7±2.8	0.721
Systolic blood pressure	152.4±20.8	141.8±22.6	<0.001
Diastolic blood pressure	91.6±14.6	88.4±15.7	0.063
Stroke subtype			
Atherothrombotic (n, %)	92 (57.1)	85 (52.8)	0.436
Small artery disease (n, %)	69 (42.9)	76 (47.2)	–
Intracranial artery stenosis ≥50% (n, %)	92 (57.1)	72 (44.7)	0.027
Carotid ultrasoundfindings			
Stenosis ≥50% (n, %)	54 (33.5)	36 (22.4)	0.028
Echolucentplaque (n, %)	64 (36.5)	48 (29.8)	0.068
Onset to admission time (h)	17.8±5.2	18.2±6.2	0.514
tPA administered	14 (8.7)	12 (7.5)	0.711
END (n, %)	61 (37.9)	24 (14.9)	<0.001
HT during 10 days (n, %)	0 (0.0)	0 (0.0)	–
RIS during 10 days (n, %)	3 (1.9)	2 (1.2)	0.682
MI during 10 days (n, %)	1 (0.6)	1 (0.6)	0.998
Deathduring 10 days (n, %)	0 (0.0)	0 (0.0)	–
mRS at 3 months	2.0±1.1	1.5±1.2	<0.001

EETs, epoxyeicosatrienoic acids; NIHSS, National Institutes of Health Stroke Scale; TC, total cholesterol; TG, triglycerides; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; tPA, tissue plasminogen activator; END, earlyneurological deterioration; HT, hemorrhagic transformation; RIS, recurrent ischemic stroke; MI, myocardial infarction; mRS, modified Rankin Scale.

taining ethylene diaminetetraacetic acid/butylated hydroxytoluene/glutathione. Plasma was isolated following centrifugation and samples were stored at −80℃ until analysis. Total plasma EETs and DiHETEs levels were measured using a stable isotope dilution gas chromatography/mass spectrometer following base hydrolysis and separation on high performance liquid chromatography, as described previously[12]. Validity and reproducibilityof plasma EETs and DiHETEs levels have been established[13, 14].

Genotyping of *EPHX2* Variants

The single nucleotide polymorphisms (SNPs) of *EPHX2* rs751141 were selected according to the following criteria: (i) SNPs with minor allele frequency >0.05; (ii) SNPs that have been assessed in our previous studies[12-15]; (iii) SNPs leading to amino acid changes.

Genotypes of the *EPHX2* rs751141 were performed using the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry method, according to our previous study[12-15]. Genotype calling was performed in real time with MassARRAY RT software version 3.0.0.4 and analyzed using the MassARRAY Typer software version 3.4 (Sequenom Inc., San Diego, CA).

Table 3. Clinical characteristics among *EPHX2* rs751141 genotypes

	EPHX2 re751141 genotypes			*P* value
	GG ($n=189$)	AG ($n=117$)	AA ($n=16$)	
Age (years)	78.8 ± 12.7	69.7 ± 12.5	70.2 ± 14.3	0.892
Males (n, %)	117 (61.9)	73 (62.4)	11 (68.7)	0.436
Diabetes mellitus (n, %)	84 (44.4)	50 (42.7)	7 (43.7)	0.658
Hypertension (n, %)	157 (83.1)	79 (67.5)	11 (68.7)	0.006
Current smoker (n, %)	77 (40.7)	48 (41.0)	6 (37.5)	0.589
TG (mmol/L)	1.7 ± 0.9	1.6 ± 0.7	1.7 ± 0.6	0.623
TC (mmol/L)	5.8 ± 1.9	5.7 ± 1.7	5.7 ± 1.5	0.856
LDL-C (mmol/L)	3.0 ± 1.5	3.1 ± 1.2	2.9 ± 1.2	0.427
HDL-C (mmol/L)	1.2 ± 0.7	1.2 ± 0.6	1.3 ± 0.5	0.893
Fasting glucose (mmol/L)	6.6 ± 2.9	6.8 ± 2.5	6.7 ± 2.1	0.796
Hemoglobin A1c (%)	6.7 ± 2.4	6.6 ± 2.6	6.5 ± 2.5	0.801
Systolic blood pressure	155.6 ± 21.7	140.6 ± 20.5	141.7 ± 15.4	< 0.001
Diastolic blood pressure	91.7 ± 15.7	87.2 ± 15.3	88.1 ± 14.7	0.217
Stroke subtype				
Atherothrombotic (n, %)	106 (56.1)	62 (53.0)	9 (56.3)	0.522
Small artery disease (n, %)	83 (43.9)	55 (47.0)	7 (43.7)	–
Intracranial artery stenosis ≥ 50% (n, %)	109 (57.7)	48 (41.0)	7 (43.7)	0.018
Carotid ultrasoundfindings				
Stenosis ≥ 50% (n, %)	64 (33.9)	23 (19.6)	3 (18.7)	0.022
Echolucentplaque (n, %)	74 (39.2)	34 (29.1)	4 (25.0)	0.165

TC, total cholesterol; TG, triglycerides; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol.

Assessment of Clinical Outcomes

The primary outcome of this study was END, which was defined as an increase in NIHSS score of ≥ 2 points within 10 days after admission, excluding for hemorrhagic transformation (HT) of an infarct or a new infarct in another vascular territory (recurrent ischemic stroke, RIS)[4, 21]. For each patient, an NIHSS assessment was performed by a member of the stroke team upon presentation to the emergency department, and subsequently on a daily basis throughout the period of hospitalization. An additional NIHSS assessment was performed whenever a patient's condition deteriorated. Upon notification of deterioration, a stroke team member reassessed the patient and performed the additional NIHSS evaluation. The secondary outcome was a composite of HT, RIS, MI and death during the first 10 days after admission. RIS was defined as a new focal neurologic deficit of vascular origin lasting at least 24 h, diffusion weighted imaging (DWI)-positive lesion(s) which corresponded to their clinical symptom(s) and was proven to be non-hemorrhagic. Death was defined as vascular mortality due to MI, ischemic stroke, or other vascular causes. The degree of disability was measured using the modified Rankin Scale (mRS) at 3 months after admission by a certified stroke team member. A good outcome was defined as mRS ≤ 2 points, while mRS > 2 points was considered a poor outcome. During the 3-month treatment, interviews were conducted every month by investigators who were blinded to EETs levels of the patients.

Statistical Analysis

Previous studies have reported the prevalence of END to be approximately 21%−50% in patients with MIS[4, 5]. Using this estimate, we calculated a minimum sample-size requirement of 310 patients for determining the true incidence rate within ± 15% with 95% confidence.

We examined total EETs by quartiles of decreasing levels to evaluate for possible threshold effects. Baseline and clinical characteristics were compared using χ^2 test or Fisher exact test (categorical variables) and the Student *t*-test (continuous variables). Deviation of Hardy-Weinberg equilibrium for genotype frequencies was also analyzed by χ^2-test. Difference of genotype frequencies between patients with or without END was compared by χ^2-test, while plasma EETs and DiHETEs levels were compared between patients with or without END using Student's *t*-test. Differences of plasma EETs and DiHETEs levels among *EPHX2* genotypes were compared using analysis of variance. Variables that showed a significant associa-

tion ($p<0.1$) with END on univariate analysis were included in the multivariate logistic regression model for evaluation of possible contributing factors for END. A Cox proportional hazard model was used to assess the probability of END according to EETs levels. The association of EETs levels with mRS score at 3 months was analyzed using a Spearman rank-order correlation. All tests were two-sided, and the threshold of $p<0.05$ was used to denote statistical significance. All statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

Results

A total of 322 patients (201 men; mean age, 68.3 years) were enrolled in this study, the average duration of hospital stay was 12.4 days. No patients were discharged within 10 days. Based on the defined criteria, 85 patients (26.4%) experienced ENDwithin 10 days after admission. The median (interquartile range [IQR]) time to deterioration from first NIHSS score collected was 22 (5.3−29) hours. The median (IQR) increase in total NIHSS score was 3 (2−6) at the time of deterioration. Baseline characteristics of patients with and withoutENDwere described in our previous article[20]. Univariate analyses revealed that old age, DM, fasting plasma glucose, HbA1c were associated with END. There was no significant difference in prevalence of END between patients with atherothrombotic and small artery disease (27.7% [49/177] vs. 24.8% [36/145], $p=0.57$)[20].

The mean EETs and DiHETEs level were 60.3 ± 7.3 nmol/L and 84.1 ± 7.5 nmol/L in patients with END, and 68.4 ± 8.1 nmol/L and 75.3 ± 7.2 nmol/L in patients without END, respectively. Plasma EETs levels were significantly lower, and DiHETEs levels were significantly higher in patients with END compared to patients without END (**Table 1**).

The mean EETs level was 64.1 ± 7.5 nmol/L in patients with MIS, with quartiles as follows: 31.5 to 51.3 nmol/L (first quartile); 51.4 to 64.3 nmol/L (second quartile); 64.4 to 75.8 nmol/L (third quartile); and 75.9 to 99.6 nmol/L (fourth quartile). The incidence of END for patients in the first quartile, second quartile, third quartile and fourth quartile was 46.2% (24/52), 33.9% (37/109), 16.4 (18/110) and 11.8% (6/51), respectively. The incidence of END in patients in the first and second quartiles was significantly higher than for patients in the third and fourth quartiles ($p<0.001$). Decreased EETs level was significantly associated with age, hypertension, systolic blood pressure, intracranial artery stenosis or carotid stenosis, END, and mRS score at 3 months. However, there was no significant association between EETs levels and secondary outcome (**Table 2**). There was no significant difference in rate of tissue plasminogen activator treatment between the low EETs and high EETs groups (**Table 2**). There wasalso no significant difference of EETs levels betweenpatients with atherothrombotic and those with small artery disease (63.7 ± 7.6 nmol/L vs. 64.5 ± 7.8 nmol/L, $p=0.322$).

Table 4. Association of *EPHX2* genotypes with EETs and DiHETEs levels

Variables	EETs (nmol/L)	DiHETEs (nmol/L)
*EPHX2rs*751141		
GG ($n=189$)	59.6 ± 7.8	83.9 ± 9.1
AG ($n=117$)	67.9 ± 8.2	75.2 ± 7.3
AA ($n=16$)	68.8 ± 3.2	74.8 ± 4.4
P*	<0.001	<0.001

*Statistical significance was based on analysis of variance, compared among genotypes.
EETs, epoxyeicosatrienoic acids; DiHETEs, dihydroxyeicosatrienoic acid.

Genotype distributions for *EPHX2 rs*751141 were in accordance with Hardy−Weinberg equilibrium ($p>0.05$). Genotype frequencies for *EPHX2 rs*751141GG, AG, and AA patients were 58.7% ($n=189$), 36.3% ($n=117$), and 5.0% ($n=16$), respectively. The *EPHX2 rs*751141GG was significantly associated with hypertension, systolic blood pressure, intracranial artery stenosis or carotid stenosis (**Table 3**). Frequency of *EPHX2* rs751141 GG was significantly higher in patients with END than in patients without END (**Table 1**). We did not detect a significant difference in genotype distributions for*EPHX2* between patients with atherothrombotic and small artery disease ($p>0.05$). Stratified analyses revealed that patients carrying *EPHX2* rs751141 GG genotype had statistically lower plasma EETs levels and higher plasma DiHETEs levels compared to patients carrying *EPHX2* rs751141 AA/AG genotypes (**Table 4**). A multiple logistic regression analysis found that *EPHX2* rs751141 GG genotype was significantly associated with lower EETs levels (OR, 0.66, 95% CI 0.42−0.95, $p=0.017$) after adjusting for age, diabetes mellitus, fasting glucose, hypertension, intracranial artery stenosis or carotid stenosis, and HbA1c.

After multiple logistic regression analyses, 3 factors (EETs, Diabetes mellitus, and *EPHX2* rs751141 GG) emerged as independent predictors of END. The odds ratio for END increased with decreasing quartile of EETs levels, using the highest quartile (fourth quartile) as the reference value. The first and second quartiles of EETs levels were identified as independent pre-

Table 5. Logistic Regression Model of Independent Predictors of END and Odds Ratio according to EETs Quartiles

Factor	OR*	95% CI	*P* value
EETs Quartile, pmol/L			
fourth quartile (reference)			
third quartile	1.36	0.57−3.67	0.413
second quartile	2.46	1.06−6.89	0.028
first quartile	2.96	1.18−8.77	0.018
Age	0.87	0.79−1.28	0.694
Hypertension	1.28	0.83−1.96	0.185
Diabetes mellitus	1.42	1.04−2.13	0.031
Intracranial artery or carotid stenosis	1.38	0.98−2.01	0.072
DiHETEs	1.22	0.77−1.75	0.264
*EPHX2*rs751141 GG	2.12	1.03−6.24	0.022

END, early neurological deterioration; EET, epoxyeicosatrienoic acids; DiHETE, dihydroxyeicosatrienoic acid; CI, confidence intervals; OR, odds ratio.
*OR for Age, and DiHETEs meansper 1-Standard Deviationincrease

dictors of END (first quartile OR, 2.96; 95% CI, 1.18−8.77, $p=0.02$; second quartile OR, 2.46; 95% CI, 1.06−6.89, $p=0.03$; **Table 5**).

A Cox proportional hazard curve showed an increase in the risk for END with lower levels of EETs (**Fig. 1**). Spearman rank-order correlations showed a significant association between EETs levels and mRS score at 3 months (correlation coefficient = −0.67, $p<0.001$). Patients with END had a higher risk for poor outcome (mRS scores 3−6; relative risk: 1.82; 95% CI: 1.46−2.35; $p=0.02$) after adjusting for age, diabetes mellitus, fasting glucose, hypertension, intracranial artery stenosis or carotid stenosis, and HbA1c.

Discussion

In this prospective, multi-center observational study, the incidence of END was 26.4% in acute MIS. EETs levels were significantly lower in patients with END than patients without END. *EPHX2* rs751141 GG genotype was independently associated withlower EETslevels. Low levels (<64.4 nmol/L) of EETs was independently associated with END, and the risk of END tended to increase with decreasing EETs level quartiles. END was associated with a higher risk of poor outcome (mRS scores 3−6) at 3 months.

Previous research has suggested an association between EETs levels and stroke risk[22]. Our previous work also showed that EETs levels were associated with plaque stability or carotid stenosis, and independently associated with a high risk of ischemic stroke[12-14]. EETs have been shown to play an important role in the regulation of cerebrovascular tone; they protect against cerebral ischemia[23], and are associated with outcomes in patients with aneurysmal subarachnoid hemorrhage[24]. However, the relationship between EETs levels and END after acute MIS has not been well investigated. The present study is the first to identify a positive relationship between EETs levels and END in acute MIS. The mechanisms underlying these associations are not yet understood. EETs exert vascular relaxation effects, and have diverse protective roles in the cardiovascular system against stroke, including vasodilation, neuroprotection, promotion of angiogenesis and suppression of platelet aggregation, oxidative stress and post-ischemic inflammation[10, 11, 25]. EETs can be metabolized by sEH to yield less biologically active DiHETEs. Pharmacological inhibition or genetic deletion of sEH has been shown to increase EETs levels, reduce infarct size after focal cerebral ischemia, and protect from stroke-induced brain injury[16, 17]. This protective effect of EETs has also been demonstrated in an animal model[26]. All these findings suggest a potential molecular mechanism that links low EETs with risk of END.

Our data revealed that *EPHX2* rs751141 GG was independently associated with END and EETs levels. One study showed that patients with at least one copy of the variant *EPHX2* had lower mean EETs levels[24], and this is consistent with our current findings. Przybyla-Zawislak and colleagues[27] reported that the*EPHX2* variant increases sEH enzyme activity and results in reduction in EETs levels. They also found that *EPHX2* rs751141 GG genotype is associated with EETs levels in Black, Asian, and White healthy populations. Our previous study showed that the mean EETs level was 61.76 ± 4.52 nmol/L in ischemic stroke patients and 73.68 ± 4.88 nmol/Lin healthy controls. The EETs lev-

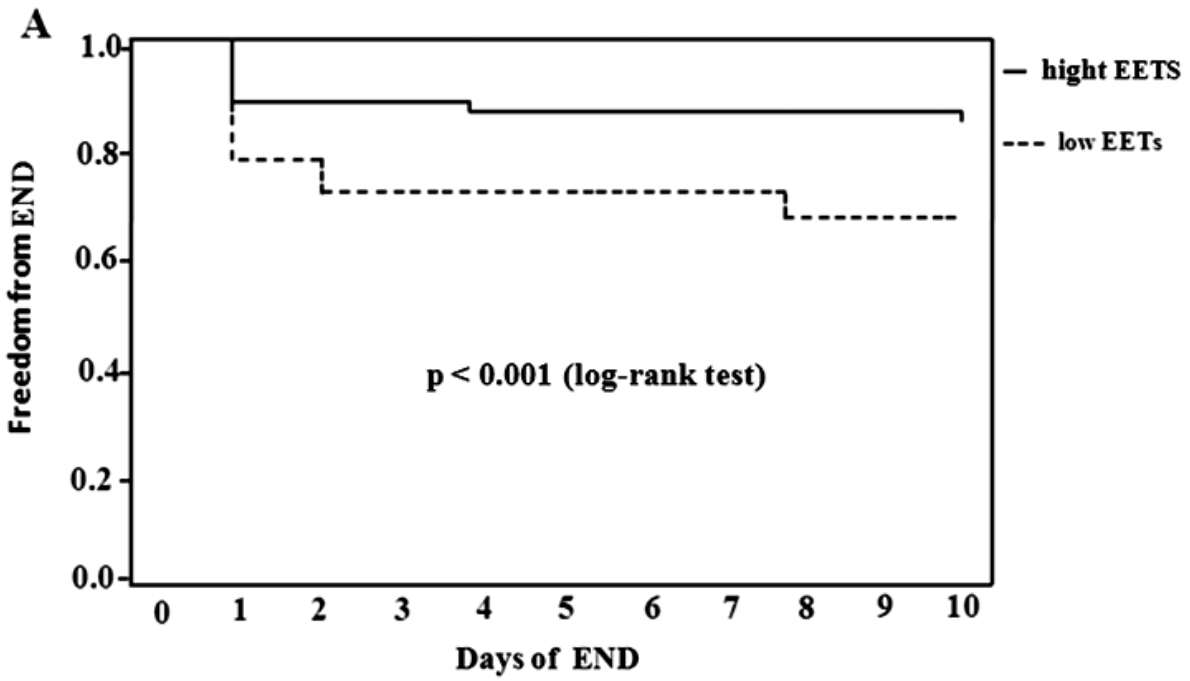

Fig. 1. Kaplan-Maier analysis of cumulative freedom from END associated with EETs level. END indicates early-neurological deterioration; EETs indicates epoxyeicosatrienoic acids

els were significantly lower in ischemic stroke patients compared with healthy controls[12]. Furthermore, the *EPHX2* rs751141 GG, and the genotype combination of *EPHX2* rs751141 GG, *CYP2C8*rs17110453CC, *CYP4A11* rs9333025 GG was significantly associated with EETs levels whether in ischemic stroke patients or healthy controls[12]. Rat neuronal cell cultures and cardiomyocytes from sEH knockout mice transduced with the EPHX2 enzyme reduced 14,15-DiHETE levels after administration of excess 14,15-EET and reduced ischemic cell death[28]. Moreover, clinical studies report that the *EPHX2* variant was associated with reduced EETs metabolism and higher cholesterol and triglyceride levels in plasma[29, 30]. These findings suggest that the *EPHX2* variant is a loss-of-function SNP associated with reduced EETs metabolism and ischemic cell death. Therefore, it is expected that patients with-*EPHX2* rs751141 GG would have decreased EET levels, increased DiHETEs levels, and less favorable outcomes.

In some studies, the degree of carotid stenosis as well as middle cerebral artery occlusion have been shown to be associated with END[31]. In the present study, we did not observe such an association. Our results were consistent with one recent study in the literature[5]. However, these findings must be validated in larger, multi-center studies in the future.

In spite of these novel findings, several limitations of this study should be noted. First, some studies have shown the association of biomarkers such as high-sensitive C reaction protein, inflammatory cytokines and brain natriuretic peptide with END. However, these biomarkers were not measured in this study and we did not account for the effect of these biomarkers on END. Second, the time interval from onset to admission was variable. Therefore, patients who had already deteriorated within that time prior to enrollment may not have been recognized as END. This could have underestimated the incidence of END. Third, although this study examined the association of *EPHX2* variant with EETs levels and END, some functional genetic variants in the EET metabolic pathway, such as *CYP2C8* were not genotyped, we did not eliminate effects of other genetic variants on EETs levels and END. Thus, future studies involving a larger set of genetic variants could be conducted. Fourth, EETs levels may change dynamically after ischemic stroke[22], and maybe affected by ischemia itself. In this study, plasma EETs levels were measured on admission. We did not investigate effects of acute ischemia itself on EETs levels. Further studies are needed to evaluate the association between dynamic changes in EETs levels and END. Finally, the lack of a control group in the present study is a limitation. Thus, well-designed studies are needed to validate our findings in future.

In conclusion, END is fairly common in acute MIS in the Chinese population, and is associated with poor outcomes. Decreased EETs levels and *EPHX2* rs751141 GG were significantly associated with END. *EPHX2* rs751141 GG genotype may mediate EETs levels. Low level of EETs may be a predictor for END in acute MIS. A detailed understanding of the basic mechanisms could provide valuable insights into potential prevention and treatment of END.

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Conflict of Interest

The authors declare no conflicts of interest.

References

1) Tei H, Uchiyama S, Ohara K, Kobayashi M, Uchiyama Y, Fukuzawa M: Deteriorating ischemic stroke in 4 clinical categories classified by the Oxfordshire Community Stroke Project. Stroke, 2000; 31: 2049-2054
2) Dávalos A, Toni D, Iweins F, Lesaffre E, Bastianello S, Castillo J: Neurological deterioration in acute ischemic stroke: potential predictors and associated factors in the European Cooperative Acute Stroke Study (ECASS) I. Stroke, 1999; 30: 2631-2636
3) Kim JT, Heo SH, Yoon W, Choi KH, Park MS, Saver JL, Cho KH: Clinical outcomes of patients with acute minor stroke receiving rescue IA therapy following early neurological deterioration. J Neurointerv Surg, 2016; 8: 461-465
4) Yi X, Wang C, Liu P, Fu C, Lin J, Chen Y: Antiplatelet drug resistance is associated with early neurological deterioration in acute minor ischemic stroke in the Chinese population. J Neurol, 2016; 263: 1612-1629
5) Vahidy FS, Hicks WJ 2nd, AcostaI, Hallevi H, Peng H, Pandurengan R, Gonzales NR, Barreto AD, Martin-Schild S, Wu TC, Rahbar MH, Bambhroliya AB, Grotta JC, Savitz SI: Neurofluctuation in patients with subcortical ischemic stroke. Neurology, 2014; 83: 398-405
6) Smith EE, Fonarow GC, Reeves MJ, Cox M, Olson DM, Hernandez AF, Schwamm LH: Outcomes in mild or rapidly improving stroke not treated with intravenous recombinant tissue-type plasminogen activator: findings from Get With The Guidelines-Stroke. Stroke, 2011; 42: 3110-3115
7) Kwon HM, Lee YS, Bae HJ, Kang DW: Homocysteine as a predictor of early neurological deterioration in acute ischemic stroke. Stroke, 2014; 45: 871-873
8) Llombart V, Dominguez C, Bustamante A, Rodriguez-Sureda V, Martín-Gallán P, Vilches A, García-Berrocoso T, Penalba A, Hernández-Guillamon M, Rubiera M, Ribó M, Eschenfelder C, Giralt D, Molina CA, Alvarez-Sabín J, Rosell A, Montaner J: Fluorescent molecular peroxidation products: a prognostic biomarker of early neurologic deterioration after thrombolysis. Stroke, 2014; 45: 432-437
9) Zeldin DC: Epoxygenase pathways of arachidonic acid metabolism. J Biol Chem, 2001; 276: 36059-36062
10) Imig JD, Simpkins AN, Renic M, Harder DR: Cytochrome P450 eicosanoids and cerebral vascular function. Exp Rev Mol Med, 2011; 13: e7
11) Iliff JJ, Alkayed NJ: Soluble Epoxide Hydrolase Inhibition: Targeting Multiple Mechanisms of Ischemic Brain Injury with a Single Agent. Future Neurol, 2009; 4: 179-199
12) Yi X, Wu L, Liao D, Wang C, Zhang B: Interactions Among CYP2C8, EPHX2, and CYP4A11 Variants and CYP Plasma Metabolite Levels in Ischemic Stroke. J Atheroscler Thromb, 2016; 23: 1286-1293
13) Yi X, Liao D, Wu L, Chen H, Li J, Wang C: CYP Genetic Variants, CYP Metabolite Levels, and Symptomatic Carotid Stenosis in Ischemic Stroke Patients. J Atheroscler Thromb, 2016; 23: 621-631
14) Yi X, Liao D, Wang C, Cheng W, Fu XQ, Zhang B: Cytochrome P450 genetic variants and their metabolite levels associated with plaque stability in ischemic stroke patients. J Atheroscler Thromb, 2016; 23: 330-338
15) Yi X, Zhang B, Wang C, Liao D, Lin J, Chi L: CYP2C8 rs17110453 and EPHX2 rs751141 two-locus interaction increases susceptibility to ischemic stroke. Gene, 2015; 565: 85-89
16) Zuloaga KL, Zhang W, Roese NE, Alkayed NJ: Soluble epoxide hydrolase gene deletion improves blood flow and reduces infarct size after cerebral ischemia in reproductively senescent female mice. Front Pharmacol, 2015; 5: 290
17) Dorrance AM, Rupp N, Pollock DM, Newman JW, Hammock BD, Imig JD: An epoxide hydrolase inhibitor, 12- (3-adamantan-1-yl-ureido) dodecanoic acid (AUDA), reduces ischemic cerebral infarct size in stroke-prone spontaneously hypertensive rats. J Cardiovasc Pharmacol, 2005; 46: 842-848
18) Wang Y, Wang Y, Zhao X, Liu L, Wang D, Wang C, Li H, Meng X, Cui L, Jia J, Dong Q, Xu A, Zeng J, Li Y, Wang Z, Xia H, Johnston SC; CHANCE Investigators: Clopidogrel with aspirin in acute minor stroke or transient ischemic attack. N Engl J Med, 2013; 369: 11-19
19) Han SW, Kim SH, Lee JY, Chu CK, Yang JH, Shin HY, Nam HS, Lee BI, Heo JH: A new subtype classification of ischemic stroke based on treatment and etiologic mechanism. Eur Neurol, 2007; 57: 96-102
20) Kernan WN, Ovbiagele B, Black HR, Bravata DM, Chimowitz MI, Ezekowitz MD, Fang MC, Fisher M, Furie KL, Heck DV, Johnston SC, Kasner SE, Kittner SJ, Mitchell PH, Rich MW, Richardson D, Schwamm LH, Wilson JA: Guidelines for the prevention of stroke in patients with stroke and transient ischemic attack: a guideline for healthcare professionals from the American Heart Association/American Stroke Association. Stroke, 2014; 45: 2160-2236
21) Yi X, Han Z, Zhou Q, Lin J, Liu: 20-Hydroxyeicosatet-

raenoic Acid as a Predictor of Neurological Deterioration in Acute Minor Ischemic Stroke. Stroke, 2016; 47: 3045-3047
22) Ward NC, Croft KD, Blacker D, Hankey GJ, Barden A, Mori TA, Puddey IB, Beer CD: Cytochrome P450 metabolites of arachidonic acid are elevated in stroke patients compared with healthy controls. Clin Sci (Lond), 2011; 121: 501-507
23) Alkayed NJ, Birks EK, Hudetz AG, Roman RJ, Henderson L, Harder DR: Inhibitionof brain P-450 arachidonic acid epoxygenase decreases baseline cerebral blood flow. Am J Physiol, 1996; 271: H1541-H1546
24) Donnelly MK, Conley YP, Crago EA, Ren D, Sherwood PR, Balzer JR, Poloyac SM: Genetic markers in the EET metabolic pathway are associated with outcomes in patients with aneurysmal subarachnoid hemorrhage. J Cereb Blood Flow Metab, 2015; 35: 267-276
25) Larsen BT, Gutterman DD, Hatoum OA: Emerging role of epoxyeicosatrienoic acids in coronary vascular function. Eur J Clin Invest, 2006; 36: 293-300
26) Liu M, Alkayed NJ: Hypoxic preconditioning and tolerance via hypoxia inducible factor (HIF) 1alpha-linked induction of P450 2C11 epoxygenase in astrocytes. J Cereb Blood Flow Metab, 2005; 25: 939-948
27) Przybyla-Zawislak BD, Srivastava PK, Vazquez-Matias J, Mohrenweiser HW, MaxwellJE, Hammock BD, Bradbury JA, Enayetallah AE, Zeldin DC, Grant DF: Polymorphisms in human soluble epoxide hydrolase. Mol Pharmacol, 2003; 64: 482-490
28) Merkel MJ, Liu L, Cao Z, Packwood W, Young J, Alkayed NJ, Van Winkle DM: Inhibition of soluble epoxide hydrolase preserves cardiomyocytes: role of STAT3 signaling. American journal of physiology. Heart Circ Physiol, 2010; 298: H679-H687
29) Lee JP, Yang SH, Kim DK, Lee H, Kim B, Cho JY, Yu KS, Paik JH, Kim M, Lim CS, Kim YS: In vivo activity of epoxidehydrolase according to sequence variation affects the progression of human IgAnephropathy. Am J Physiol, 2011; 300: F1283-F1290
30) Sato K, Emi M, Ezura Y, Fujita Y, Takada D, Ishigami T, Umemura S, Xin Y, Wu LL, Larrinaga-Shum S, Stephenson SH, Hunt SC, Hopkins PN: Soluble epoxidehydrolase variant (Glu287Arg) modifies plasma total cholesterol and triglyceride phenotype in familial hypercholesterolemia: intrafamilial association study in an eight-generation hyperlipidemic kindred. J Hum Genet, 2004; 49: 29-34
31) Cuadrado-Godia E, Jimena S, Ois A, Rodriguez-Campello A, Giralt-Steinhauer E, Soriano-Tarraga C, Jiménez-Conde J, Martínez-Rodríguez JE, Capellades J, Roquer J: Factors associated with early outcome in patients with largevessel carotid strokes. J Neurol Neurosurg Psychiatry, 2013; 84: 305-309