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ORIGINAL ARTICLE

The correlation between gene polymorphisms of endothelial nitric oxide synthase and aneurismal subarachnoid hemorrhage

Zhang Zhe[1,2] · Yang Bo[1]

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Abstract

To discuss the association of the T786C and G894T polymorphisms of endothelial nitric oxide synthase (eNOS) with the occurrence and prognosis of aneurismal subarachnoid hemorrhage (aSAH). One hundred sixty-nine patients with aSAH were collected as the case group, which was divided into the good prognosis group and adverse prognosis group according to the condition 3 months after the treatment. One hundred fifty-six healthy volunteers were collected as the control group. The allele and genotype of T786C and G894T polymorphisms of eNOS were detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The genotype and allele frequency of eNOS were compared between different groups. And then, the risk factors of aSAH occurrence and prognosis were analyzed by using the logistic regression model. Both the genotype and allele frequency distributions of T786C and G894T between the case group and control group were significantly different ($P < 0.05$). There were significant differences in the distribution of G894T and T786C allele frequency and G894T genotype between the good prognosis group and adverse prognosis group, and there was no difference in T786C genotype. The results of the logistic regression analysis indicated that T786C and G894T polymorphisms of eNOS were independent influencing factors on the occurrence of aSAH and the G894T polymorphism was also closely related to the prognosis. T786C and G894T polymorphisms of eNOS gene were correlated with the occurrence and prognosis of aSAH, and the G894T polymorphism might be an independent influencing factor.

Keywords eNOS · Gene polymorphism · Aneurismal subarachnoid hemorrhage · Prognosis

Introduction

Aneurismal subarachnoid hemorrhage (aSAH) is caused by ruptured aneurysm and results in the blood to flow to the subarachnoid space, which is taken about 80% of all spontaneous disease [23]. It is one of the most common neurosurgical emergency diseases with extremely high disability and mortality (12.4%) [24]. Currently, it is widely considered that the occurrence, development, and rupture of intracranial aneurysm were caused by multiple environmental factors and genetic factors [3]. And with the development of molecular and genetic technology, related gene of aSAH gradually became the research focus. Endothelial nitric oxide synthase (eNOS) was an important regulatory factor for secretion of vascular endothelial cells, which could effectively regulate vasodilation, maintain normal structure of the vascular wall, and protect cerebral vascular endothelial cells [2]. With the development of molecular biological techniques, eNOS received more and more attention, which was considered as one of the genes related to cerebral vascular diseases [25]. eNOS was the limited enzyme of NO synthesis in endothelial cell, and the two typical SNPs of eNOS: T786C SNP in the promoter region and G894T SNP in the coding region were likely to lead to quantitative differences in expression and in the resultant protein of eNOS, resulting in the change of NO biological activities, which affected the structure of the vascular wall, vasoconstriction, and other pathophysiological changes. In addition, there were many studies about the

✉ Yang Bo

[1] Department of Neurosurgery, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan, China

[2] Department of Neurosurgery, Zhengzhou Peoples' Hospital, Zhengzhou 450052, Henan, China

correlation between the two SNPs and aSAH, but no unanimous conclusion on the correlation between the two SNPs and the development and prognosis of aSAH [5]. So, the correlation between the eNOS polymorphisms and the occurrence and prognosis of aSAH were not clear yet. This study aimed to investigate the association between T786C and G894T polymorphisms of eNOS and the occurrence and prognosis of aSAH.

Material and methods

Cases

The clinical data of 169 patients with aSAH hospitalized in the neurosurgery department of the authors' hospital were retrospectively studied as the case group during January 2015 to May 2017. All patients were diagnosed as aSAH through CTA and DSA, and treated and survived the hemorrhagic event. The secondary SAH caused by other diseases such as trauma, arteriovenous malformations, and other brain tumors was excluded. General data of age, gender, accompanying chronic diseases (hypertension, diabetes, et al), smoking, drinking, Hunt-Hess (H-H) grade, Fisher grade, and therapy methods of all patients were collected. According to the condition, all aSAH patients were treated with surgery ($n = 105$, 62.1%) and conservative treatment ($n = 64$, 37.8%). One hundred fifty-six cases which had a health examination visiting the hospital at the same period were selected as the health control group, who were biologically related to the subjects with aSAH, and were free from hypertensive crisis and any family history of aneurysm, subarachnoid hemorrhage, cerebral hemorrhage, or cerebral infarction. The study was reviewed and approved by the Ethical Inspection Committee of Zhengzhou University and all participants conformed and signed the informed content.

Genotype detection

Five milliliters peripheral blood of each participant was collected, and anticoagulant with EDTA-Na_2. Genome DNA was extracted by using blood genomic DNA isolation kit (Sigma) and stored at the temperature of −20 °C. Genotype of eNOS T786C and G894T was detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The primers were synthesized by Shanghai Bio-Engineering Co. Ltd. For T786C, sense primer: 5′-CAGATGACACAGAA CTACAA-3′ and anti-sense primer: 5′-GAGTCTGACATTAGGGTATCC-3′ were used. For G894T, sense primer: 5′-GAGATGAAGGCAGG AGACAGT-3′ and anti-sense primer: 5′-TCCA TCCCACCC AGTCAAT-3′ were used. Specific fragments of eNOS were amplified by PCR. Ten-microliter amplification product of T786C and G894T was digested by restriction enzyme *NaeI* and *BanII* at 37 °C for 8 h separately. And the restriction fragments were detected by agarose gel with a concentration of 2.5%.

Prognosis evaluation

Disease progression of 169 aSAH patients was surveyed 3 months after the treatment, and the Glasgow Outcome Scale (GOS) was applied to evaluate the prognosis. Scoring criteria of GOS are the following: 1 score stands for death; 2, for vegetative state; 3, for severely disabled state (cannot take care of oneself); 4, for mildly disabled state (able to take care of oneself but with mild disorders in cognition or movement); and 5, for good condition (recovery, able to live normally). According to the GOS evaluation, the 169 patients were divided into two groups: good prognosis group (4–5 scores, $n = 109$, 64.5%) and adverse prognosis group (≤ 3 scores, $n = 60$, 35.5%).

Statistical analysis

All data were analyzed by SPSS 19.0. The chi-square test was used to test whether the genotype distribution was in the line with the Hardy-Weinberg (H-W) equilibrium and $P > 0.05$ was considered the frequency of genotype in the sample conformed to the theory, and the sample was benign. Measurement data were shown as mean ± standard deviation ($\bar{\chi} \pm \sigma$), and the comparison between two groups was performed by t test. The comparison of enumeration data, genotype frequency, and allele frequency was examined with chi-square test. $P < 0.05$ was considered that the difference was statistically significant. Outcome was dichotomized as dependent variable of logistic regression analysis, and logistic regression was conducted to analyze the risk factors of aSAH occurrence and prognosis, and odds ratio (OR) value and 95% confidence interval (95% CI) were used to show the relative risk.

Results

General data

General data of all participants are shown in Table 1. There was no significant difference in age, gender, smoking, drinking, hypertension, and diabetes between the case group and control group ($P > 0.05$). There were significant differences between the good prognosis group and adverse prognosis group in the H-H grade and Fisher grade ($P < 0.05$), while other general data had no difference in the two groups.

Table 1 Comparison of basic data [case (%)]

	n	Age	Gender		Smoking	Drinking	Diabetes	Hypertension
			Male	Female				
Case group	169	53.65 ± 10.85	89 (52.7)	80 (47.3)	61 (36.1)	71 (42.0)	48 (28.4)	76 (44.9)
Control group	156	56.08 ± 14.81	76 (48.7)	80 (51.3)	45 (28.8)	51 (32.7)	32 (20.5)	59 (37.8)
t/χ^2		1.698	0.505		1.939	3.005	2.721	1.708
P		0.090	0.477		0.164	0.083	0.099	0.191
Good prognosis group	109	54.42 ± 11.40	58 (53.2)	51 (46.8)	35 (32.1)	41 (37.6)	26 (23.8)	47 (43.1)
Adverse prognosis group	60	52.41 ± 10.33	31 (51.7)	29 (48.3)	26 (43.3)	30 (50.0)	22 (25.0)	35 (50.0)
t/χ^2		1.136	0.037		2.113	2.437	3.124	3.586
P		0.258	0.847		0.146	0.119	0.077	0.058
			H-H grade		Fisher grade		Therapy	
			I~III	IV~V	0~2	3~4	Surgery	Conservative
Good prognosis group			77 (70.6)	32 (29.4)	77 (70.6)	32 (29.4)	69 (63.3)	40 (36.7)
Adverse prognosis group			33 (55.0)	27 (45.0)	31 (51.7)	29 (48.3)	36 (60.0)	24 (40.0)
χ^2			4.167		6.041		0.179	
P			0.041		0.014		0.672	

The distribution of T786C and G894T genotype and allele frequency

The distribution of eNOS T786C and G894T genotype and allele frequency in the case group and health control group are shown in Table 2. In Table 2, the results of the chi-square test showed that the genotype distribution of T786C and G894T conformed to the Hardy-Weinberg equilibrium ($P > 0.05$). There were significant differences in the distribution of T786C and G894T genotype and allele frequency between the case group and health control group ($P < 0.05$). The CC genotype of T786C and TT genotype of G894T in the case group were higher than those in the health control group ($P < 0.05$), which meant that the 786C allele or 894T allele of eNOS gene was at higher risk for aSAH (OR > 1). The results indicated that both T786C and G894T polymorphisms of eNOS were correlated with the occurrence of aSAH.

The distribution of eNOS T786C and G894T genotype and allele frequency in the good prognosis group and the adverse prognosis group are shown in Table 3. It can be seen that there were significant differences in the distribution of allele frequency of G894T and T786C and genotype of G894T between the two groups ($P < 0.05$), while the genotype of T786C had no difference between the two groups. The frequency of 786C allele and 894T allele in the adverse prognosis group was higher than that in the good prognosis group, and the differences were all significant ($P < 0.05$). The result indicated that T786C and G894T polymorphisms of eNOS might be correlated with the prognosis of aSAH.

Table 2 Comparison in genotype and allele frequency distribution between the case group and control group

Locus		Case group [*n* (%)]	Health control [*n* (%)]	χ^2	*P*	*P*	OR (95% CI)
T786C	TT	82 (48.5)	95 (60.9)	6.146	0.046	0.049	1
	CT	60 (35.5)	47 (30.1)			0.112	1.479 (0.913~2.397)
	CC	27 (16.0)	14 (9.0)			0.026	2.234 (1.099~4.544)
	H-W	$\chi^2 = 2.190$, $P = 0.335$	$\chi^2 = 1.221$, $P = 0.543$	–			
	T	224 (66.3)	237 (76.0)	7.386	0.007		1
	C	114 (33.7)	75 (24.0)			0.007	1.608 (1.140~2.268)
G894T	GG	90 (53.3)	106 (67.9)	9.486	0.009	0.010	1
	TG	50 (29.6)	38 (24.2)			0.090	1.550 (0.934~2.572)
	TT	29 (17.2)	12 (7.7)			0.005	2.846 (1.373~5.901)
	H-W	$\chi^2 = 4.958$, $P = 0.084$	$\chi^2 = 2.340$, $P = 0.310$	–			
	G	230 (68.0)	250 (80.1)	12.260	< 0.001		1
	T	108 (32.0)	62 (19.9)			0.001	1.893 (1.321~2.714)

Table 3 Comparison in genotype and allele frequency distribution between the two groups with different prognoses

Locus		Good prognosis group [*n* (%)]	Poor prognosis group [*n* (%)]	χ^2	*P*	*P*	OR (95% CI)
T786C	TT	56 (51.4)	26 (43.3)	5.643	0.060	0.068	1
	CT	41 (37.6)	19 (31.7)			0.996	0.998 (0.488~2.042)
	CC	12 (11.0)	15 (25.0)			0.029	2.692 (1105~6.558)
	T	153 (71.1)	71 (57.5)	4.203	0.040		1
	C	65 (28.9)	49 (42.5)			0.041	1.624 (1.020~2.588)
G894T	GG	67 (61.5)	23 (38.3)	9.711	0.008	0.010	1
	TG	29 (26.6)	21 (35.0)			0.090	1.550 (0.934~2.572)
	TT	13 (11.9)	16 (26.7)			0.005	2.846 (1.373~5.901)
	G	163 (74.8)	67 (55.8)	127.66	< 0.001		1
	T	55 (25.2)	53 (44.2)			0.001	2.344 (1.462~3.760)

Multi-factor logistic regression analysis

The logistic regression model incorporated the general data and eNOS polymorphism was used to analyze the independent risk factors of aSAH occurrence and prognosis. The results are shown in Tables 4 and 5. In Table 4, it could be seen that hypertension and T786C and G894T polymorphisms of eNOS were independent risk factors for the occurrence of aSAH ($P < 0.05$). In Table 5, the prognosis logistic analysis showed that H-H grade, hypertension, and G894T polymorphism were risk-influencing factors for the prognosis of aSAH, and patients with eNOS 894T allele had a higher risk of adverse prognosis.

Discussion

It is widely considered that the occurrence, development, and rupture of intracranial aneurysm are the result of the interaction of multiple environmental factors and genetic factors. And the genetic factors in the pathogenesis of aneurysm were paid more and more attention in recent years. The eNOS gene was considered as a susceptible gene of carotid atherosclerosis, hypertension, and other vascular diseases [8, 16, 20]. Studies showed that lack of eNOS may lead to cystic expansion in intracranial vascular walls and result in intracranial aneurysms. Although the association between the eNOS gene polymorphism and the risk of SAH caused by intracranial aneurysm rupture has been reported by various studies globally [7, 22], the conclusion has not yet reached consensus. On this basis, this study aimed to discuss whether T786C and G894T polymorphisms of eNOS were correlated with the occurrence and prognosis of aSAH in Han nationality from Henan province of China.

The current research indicated that eNOS polymorphism might be one of the factors that affected the incidence and prognosis of ruptured intracranial aneurysms in Caucasian [1]. Khurana VG et al. [9, 10] verified that eNOS T786C genotype may be a factor influencing the size at which an aneurysm ruptures and predict susceptibility to post-subarachnoid hemorrhage vasospasm, which was similar to that found by Ko NU et al. [12]. Philipp et al. [6] demonstrated that the C allele of the eNOS SNP T786C was independently associated with an increased risk for cerebral ischemia following aSAH while Starke RM et al. [19] found that the patients with the T allele of the eNOS gene T768C SNP are more likely to have severe vasospasm. Ozüm et al. [17] found that the polymorphism of eNOS G894T seems to be a possible risk

Table 4 Logistic regression analysis on the occurrence of aSAH

Risk factors		*B*	OR	95% CI		*P*
				Lower limit	Upper limit	
T786C	TT					0.004
	CT	0.844	2.326	1.346	4.020	0.002
	CC	0.831	2.295	1.052	5.004	0.037
G894T	GG					0.001
	TG	0.654	1.922	1.071	3.449	0.028
	TT	1.368	3.928	1.798	8.582	0.001
Hypertension		0.859	2.361	1.427	3.905	0.001

Table 5 Logistic regression analysis on the prognosis of aSAH

Risk factors		*B*	OR	95% CI		*P*
				Lower limit	Upper limit	
G894T	GG					0.001
	TG	1.131	3.100	1.331	7.220	0.009
	TT	1.860	6.423	2.365	17.446	0.000
H-H grade		1.019	2.770	1.312	5.850	0.008
Hypertension		1.377	3.962	1.783	8.800	0.001

factor for intracranial aneurysm rupture. So, there was correlation between the eNOS polymorphism, particularly T786C SNP and the development and prognosis of aSAH. But there were different conclusions in Asians. In a Korean population, Song MK et al. [18] studied that the eNOS T786C mutation was not found to be associated with either a susceptibility to SAH or vasospasm after SAH, which was consistent with the research of Kim TG [11]. And the eNOS polymorphisms were not found to be a risk factor for aSAH among south Indian patients [13] and Japanese patients [14]. Chinese scholar Xu Liang et al. [21] found that polymorphism in exon 7 of eNOS G894T was a possible risk factor for the onset of aneurismal subarachnoid hemorrhage and eNOS GT+TT genotype was independently associated with an unfavorable outcome of aSAH in Suzhou of China. Therefore, there was no consistent conclusion about the correlation between the eNOS polymorphism and the development and prognosis of aSAH among Asians. The possible reasons might be ① The distribution of the genotype and allele frequency were influenced by the factors of ethnicity, environment, and geography. The participants in this study were all Han nationality from Henan province of China and may influence the results; ② Sample size was insufficient or the inclusion criteria was not consistent; ③ There was linkage disequilibrium in polymorphism locus; ④ The occurrence of aSAH was affected by the interaction among multiple genes or between genes and environment, which meant that a single gene analysis was seemed unconvincing [16].

In this study, the results showed that the T786C and G894T polymorphisms of eNOS were correlated with the occurrence and prognosis of aSAH, and the logistic regression analysis further indicated that the two SNPs of eNOS were independent influencing factors for the occurrence of aSAH, while G894T SNP of eNOS was independent risk factor for the prognosis of aSAH. And the potential mechanism was speculated that the G894T or T786C polymorphism of eNOS might affect the expression of eNOS, decreasing the concentration of NO and induced the occurrence and development of the intracranial aneurysm. And G894T located in the coding region of eNOS and the 894G → T mutation, which causes the 289th amino acid Glu → Asp missense mutation, might influence the activity of eNOS and the binding of substrates to eNOS and reduce the generation of NO, resulting in adverse prognosis [4, 15]. However, there were many defects, which might be the reasons why the results were not the same as the existing research: ① The participants in this study were all Han nationality from Henan province of China, do not represent the Han nationality from other regions; ② Sample size was small, which limited by the study time and region; ③ The interaction effects of the two SNPs of eNOS have not been analyzed, and other SNPs of eNOS gene such as 4-VNTR27, which might result in T786C SNP not the independent factor for the prognosis of aSAH; and ④ In the methodology, not all variables were included in the multi-factor logistic regression analysis, and the variables included in the regression analysis are different, resulting in different results. So the results need further verification and additional studies will be necessary for better, comprehensive understanding of the association between the eNOS polymorphisms and the occurrence and prognosis of aSAH.

Conclusion

In conclusion, T786C and G894T polymorphisms of eNOS were correlated with the occurrence and prognosis of aSAH, and the G894T might be an independent influencing factor. However, further studies on the interaction among different SNPs of eNOS and a large sample might verify the correlation between the eNOS polymorphism and the occurrence and prognosis of aSAH to provide clinical basis for the prevention and prognosis evaluation of aSAH.

Compliance with ethical standards

The study was reviewed and approved by the Ethical Inspection Committee of Zhengzhou University and all participants conformed and signed the informed content.

Conflict of interest The authors declare that they have no conflicts of interest.

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