Int J Clin Exp Med 2019;12(10):12531-12537
www.ijcem.com /ISSN:1940-5901/IJCEM0099364

Original Article

Association between single nucleotide polymorphisms of the *MTHFR* gene and thromboangiitis obliterans

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Received July 8, 2019; Accepted September 3, 2019; Epub October 15, 2019; Published October 30, 2019

Abstract: *Background:* Hyperhomocysteinemia (HHcy) may cause endothelial dysfunction, playing a crucial role in vascular pathology. Previous studies have demonstrated that endothelial dysfunction is involved in occurrence and development of thromboangiitis obliterans (TAO). Methylenetetrahydrofolate reductase (MTHFR) is indispensable in the conversion of homocysteine (Hcy) to methionine. Single nucleotide polymorphisms (SNP) of the *MTHFR* gene have been demonstrated to be correlated with decreased enzyme activity, eventually causing elevated homocysteine (Hcy) levels. However, association levels between SNPs of the *MTHFR* gene have not been investigated. Objectives: The aim of the current study was to investigate association levels between SNPs of the *MTHFR* gene and TAO, exploring the underlying mechanisms. *Methods:* A total of 87 Uygur patients with TAO were recruited as the case group (TAO group). During the same period, 87 Uygur healthy people, matched according to gender and age (±2 years), were randomly chosen as the control group (non-TAO group). For all participants, demographic data, laboratory indexes, and cardiovascular risk factors were collected. SNPs of *MTHFR* rs1801133, rs2274976, rs3737964, and rs4846049 were genotyped and plasma Hcy levels were measured. Multivariate analysis was conducted, aiming to determine SNPs correlated with susceptibility to TAO. Plasma Hcy levels between different genotypes were compared with ANOVA. *Results:* The polymorphism rs1801133 was independently associated with susceptibility to TAO, adjusting for smoking, platelets, creatinine, CRP, and T-Chol. However, rs4846049 was not associated with susceptibility to TAO. TT and CT genotypes of *MTHFR* rs1801133 increased susceptibility to TAO. Moreover, the *OR* was higher in the TT genotype than in CT (2.216 vs 1.398). Plasma Hcy levels were highest in the TT genotype (20.16±7.61 pg/mL), intermediate in the CT genotype (17.23±5.54 pg/mL), and lowest in the CC genotype (15.06±4.72 pg/mL). *Conclusion:* The SNP of *MTHFR* rs1801133 was associated with susceptibility to TAO, affecting Hcy levels. Compared with the CC genotype, TT and CT genotypes showed increased susceptibility to TAO.

Keywords: Thromboangiitis obliterans, methylenetetrahydrofolate reductase, homocysteine, single nucleotide polymorphism, susceptibility

Introduction

Thromboangiitis obliterans (TAO) or Buerger disease is a rare non-atherosclerotic inflammatory arteritis, mainly affecting small- and medium-sized vessels and nerves of the upper and lower distal limbs [1]. It has shown a strong association with smoking [2, 3] and can induce the necessity for limb amputations in young and middle-aged people, especially smokers [4, 5]. The pathogenesis of TAO has not yet been clarified, though it has been named for over a century. Hyperhomocysteinemia (HHcy) can cause endothelial dysfunction, playing a crucial role in vascular pathology [6]. Previous studies have demonstrated that endothelial dysfunction is involved in occurrence and development of TAO [7-9]. Methylenetetrahydrofolate reductase (MTHFR) is an important regulatory enzyme in the transformation of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. Thus, it is indispensable in the conversion of homocysteine (Hcy) to methionine [10, 11]. The single nucleotide polymorphism (SNP) of the *MTHFR* gene (rs1801133, C667T) has been demonstrated to be correlated with decreased enzyme activity, eventually causing elevated homocysteine (Hcy) levels [12, 13].

However, association levels between SNPs of the *MTHFR* gene have not been investigated. In the current study, association levels between SNPs of the *MTHFR* gene (rs1801133, rs2274976, rs3737964, and rs4846049) and TAO were investigated, adjusting for confounders. Additionally, the underlying mechanisms were explored, measuring Hcy levels of different genotypes.

Materials and methods

Participants

A total of 87 Uygur patients with TAO were recruited as the case group (TAO group). They were selected from the People's Hospital of Xinjiang Uygur Autonomous Region, between July 2016 and July 2018. During the same period, 87 Uygur healthy people, matched according to gender and age (±2 years), were randomly chosen as the control group (non-TAO group). The current study was approved by the Ethics Committee of the People's Hospital of Xinjiang Uygur Autonomous Region (20160171038). All participants provided written informed consent.

TAO patients were diagnosed according to Shionoya's clinical criteria [14]. Criteria included: ① Onset age less than 50 years old; ② History of smoking; ③ Absence of other atherosclerotic risk factors, except for smoking; ④ Either phlebitis migrans or upper limb involvement; and ⑤ Presence of infra-popliteal arterial occlusions. Further confirming the diagnoses of TAO, angiographies were performed to assess arterial lesions. The patients demonstrated typical angiography findings, including abrupt interruption, absence of calcification, and a corkscrew appearance.

Exclusion criteria: Evidence of atherosclerotic disease or proximal emboli, connective-tissue (systemic lupus erythematosus, scleroderma, mixed connective-tissue disease, and rheumatoid arthritis), diabetes mellitus, thrombophilia, coronary heart disease, hypertension, other peripheral vascular occlusive diseases, and malignant diseases.

Variables investigated

Demographic data, laboratory indexes, and cardiovascular risk factors were collected for all participants. Demographic data included gender, age, weight, height, annual family income, occupation, and education level. Laboratory indexes included white blood cells (WBC), hemoglobin, platelets, fibrinogen, creatinine, total cholesterol (T-Chol), triglycerides, hemoglobin A1c, and C-reactive protein (CRP). Cardiovascular risk factors included hyperlipemia, smoking, drinking, and family histories.

SNP genotyping

The salting out method was employed to extract DNA from peripheral blood, as suggested by Hashemi et al. [15]. TaqMan Pre-Designed SNP Genotyping Assays (Applied Biosystems, Carlsbad, USA) were used to conduct SNP genotyping for *MTHFR* rs1801133, rs2274976, rs3737964, and rs4846049. An ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Carlsbad, USA) was used to conduct PCR amplification and allelic discrimination.

Measurement of plasma Hcy

Blood samples were collected from the antecubital veins after overnight fasting for all participants. They were separated through centrifugation at 3,000 g for 5 minutes. Levels of plasma Hcy were measured with the human Hcy ELISA kit (Beijing Donggeboye Biological Technology, Co., LTD, Beijing, China).

Statistical analysis

Calculation of allele frequencies and genotype frequencies, as well as Hardy-Weinberg equilibrium (HWE) testing, were performed using SNPstats software, a web tool for analysis of association studies: http://bioinfo.iconcologia.net/SNPstats [22]. Kolmogorov-Smirnov testing was used to determine the normality of quantitative data. Normal data are described as mean ± standard deviation (SD), while non-normal data are described as medians and interquartile ranges (IQR). Qualitative data are described as percentages or ratios (%). For univariate analysis, quantitative data were compared with Student's *t*-tests or Mann-Whitney U-tests. Qualitative data were compared with Chi-square tests. Variables with a *P*-value less than 0.10 were included in multivariate analysis. Multivariate analysis was conducted to determine SNPs correlated with susceptibility to TAO. Plasma Hcy levels between different genotypes were compared with ANOVA. Statistical analysis was performed using SPSS ver-

Table 1. Univariate analysis for demographic data, laboratory indexes, and cardiovascular risk factors

		TAO group (n=87)	Non-TAO group (n=87)	$\chi^2/t/Z$	*P*
Age (Years old)		39 (32-45)	39 (33-46)	0.357	0.849
BMI (Kg/m^2)		23.09±4.71	23.28±4.93	-0.260	0.874
Annual family income (RMB)	< 10 000	23 (26.44%)	21 (24.14%)	0.249	0.883
	10 000-20 000	30 (34.48%)	33 (37.93%)		
	> 20 000	34 (39.08%)	33 (37.93%)		
Occupation	Farmer	46 (52.87%)	43 (49.43%)	0.414	0.813
	Worker	27 (31.03%)	31 (35.63%)		
	Teacher/doctor/Civil servant	14 (16.10%)	13 (14.94%)		
Educational level	Primary school and below	49 (56.32%)	45 (51.72%)	0.37	0.831
	Junior high school	29 (33.33%)	32 (36.78%)		
	Senior high school and above	9 (10.35%)	10 (11.50%)		
Smoking	Yes	77 (88.51%)	59 (67.82%)	10.909	0.001
	No	10 (11.49%)	28 (32.18%)		
Drinking	Yes	60 (68.97%)	64 (73.56%)	0.449	0.503
	No	27 (31.03%)	23 (26.44%)		
Hyperlipidaemia	Yes	16 (18.39%)	12 (13.79%)	0.681	0.409
	No	71 (81.61%)	75 (86.21%)		
Family history	Yes	10 (11.49%)	7 (8.05%)	0.587	0.444
	No	77 (88.51%)	80 (91.95%)		
WBC (/μL)		5318 (4736-7342)	5297 (4695-7308)	1.074	0.285
Hemoglobin (g/dL)		13.91 (12.84-14.72)	14.27 (13.05-14.98)	0.938	0.346
Platelet (×10^4/μL)		24.52 (20.65-30.46)	15.86 (12.54-21.67)	6.452	0.028
Fibrinogen (mg/dL)		297 (269-325)	291 (259-316)	0.746	0.418
Creatinine (mg/dL)		0.83 (0.69-0.94)	0.41 (0.32-0.50)	8.706	0.009
T-Chol (mg/dL)		210 (181-223)	183 (152-197)	5.189	0.071
Triglycerides (mg/dL)		134 (81-149)	125 (74-140)	1.162	0.217
Hemoglobin A1c (%)		5.69 (5.48-6.32)	5.81 (5.57-6.43)	0.579	0.535
CRP (mg/dL)		0.15 (0.09-0.22)	0.08 (0.03-0.13)	7.643	0.014

TAO: Thromboangiitis obliterans, BMI: Body mass index, WBC: White blood cell, T-Chol: Total cholesterol, CRP: C-reactive protein.

sion 20.0 for Windows (SPSS Inc., USA). Significance is set at $P < 0.05$.

Results

Univariate analysis

The TAO group included 71 males and 16 females, with an average age of 39 (32-45) years old. Univariate analysis showed that smoking, platelets, creatinine, and CRP were statistically different between the TAO group and non-TAO group ($P < 0.05$), while other variables were not statistically different ($P < 0.05$, **Table 1**).

SNP analysis

The four SNPs, rs1801133, rs2274976, rs3737964, and rs4846049, were successfully genotyped in the TAO group and non-TAO group. As demonstrated in **Table 2**, genotype frequencies of the four SNPs in the TAO group and non-TAO group were not statistically different from those predicted through Hardy-Weinberg equilibrium. Chi-square testing showed that the genotype frequencies of rs1801133 (χ^2= 7.242, P=0.027) and rs4846049 (χ^2=6.451, P=0.040) were statistically different between the TAO group and non-TAO group, while rs2274976 and rs3737964 were not statistically different (all $P > 0.05$).

Multivariate analysis

To determine independent association levels between different genotypes of *MTHFR* rs1801133 and rs4846049, along with susceptibility to TAO, multivariate analysis was performed using a backward stepwise logistic regression model. Results showed that the poly-

Table 2. Univariate analysis results of allele and genotype frequencies

	Allele frequency		Genotype frequency			HWE *P*
rs1801133	C	T	CC	CT	TT	
TAO group*	69 (39.66%)	105 (60.34%)	13 (14.94%)	36 (41.38%)	38 (43.68%)	> 0.05
Non-TAO group	86 (49.43%)	88 (50.57%)	28 (32.18%)	30 (34.48%)	29 (33.33%)	> 0.05
rs2274976	A	G	AA	AG	GG	
TAO group	26 (14.94%)	148 (85.06%)	4 (4.60%)	18 (20.69%)	65 (74.71%)	> 0.05
Non-TAO group	30 (17.24%)	144 (82.76%)	5 (5.75%)	20 (22.99%)	62 (71.26%)	> 0.05
rs4846049	G	T	GG	GT	TT	
TAO group*	136 (78.16%)	38 (21.84%)	55 (63.22%)	26 (29.89%)	6 (6.90%)	> 0.05
Non-TAO group	129 (74.14%)	45 (25.86%)	42 (48.28%)	29 (33.33%)	16 (18.39%)	> 0.05
rs3737964	A	G	AA	AG	GG	
TAO group	33 (18.97%)	141 (81.03%)	8 (9.20%)	17 (19.54%)	62 (71.26%)	> 0.05
Non-TAO group	36 (20.69%)	138 (79.31%)	7 (8.05%)	22 (25.29%)	58 (66.67%)	> 0.05

*: $P < 0.05$, *vs* allele frequency and genotype frequency of non-TAO group. HWE: Hardy-Weinberg equilibrium.

Table 3. Independent correlation between different genotypes of *MTHFR* rs1801133 and rs4846049 and susceptibility to TAO

	Regression coefficient	Standard error	Wald	*OR*	95% *CI*	*P*
rs1801133			7.417			0.005
CC				---	---	Ref=1
CT	0.312	0.164	5.637	1.398	1.127-2.260	0.031
TT	0.409	0.211	9.285	2.216	1.759-4.063	< 0.001
Rs4846049			0.886			0.361
AA				---	---	Ref=1
AC	0.172	0.113	0.659	0.821	0.511-1.509	0.452
CC	0.225	0.134	1.038	0.726	0.517-1.368	0.269

MTHFR: Methylenetetrahydrofolate reductase, TAO: Thromboangiitis obliterans, *OR*: odds ratio, *CI*: confidence interval.

Table 4. Plasma Hcy levels between different genotypes of *MTHFR* rs1801133

Genotype	n	Plasma Hcy levels (pg/mL)
CC	44	15.06±4.72
CT	67	17.23±5.54*
TT	63	20.16±7.61*,◇
F		9.287
P		< 0.001

*: $P < 0.05$, *vs* CC genotype; ◇: $P < 0.05$, *vs* CT genotype.

morphism of rs1801133 was independently associated with susceptibility to TAO, adjusting for smoking, platelets, creatinine, CRP and T-Chol. However, rs4846049 was not associated with susceptibility to TAO (**Table 3**). TT and CT genotypes of *MTHFR* rs1801133 showed increased susceptibility to TAO. Moreover, the *OR* was higher in the TT genotype than in CT (2.216 vs 1.398).

Plasma Hcy levels

Plasma Hcy levels were compared between *MTHFR* rs1801133 TT, CT, and CC genotypes, employing ANOVA. Results demonstrated that plasma Hcy levels were highest in the TT genotype, intermediate in the CT genotype, and lowest in the CC genotype (**Table 4**).

Discussion

A known risk factor in the early onset of leg-vein thrombosis and arteriosclerotic occlusive disease [16], HHcy happens frequently in TAO patients. It may play a critical role in the pathogenesis of TAO [17, 18]. Endoplasmic reticulum (ER) stress and cellular oxidative stress may result in endothelial dysfunction, atherosclerosis, and inflammation [19-21]. HHcy can induce endothelial dysfunction via ER stress and cellular oxidative stress, which have been reported to

be associated with vascular pathology [6]. Previous studies have demonstrated that endothelial dysfunction is involved in occurrence and development of TAO [7-9].

Mechanisms associated with endothelial dysfunction induced by HHcy have been explored. High reactivity of the sulfhydryl group of Hcy has been associated with multiple physiological effects of Hcy. Reactive oxygen species (ROS), produced by thiol (-SH) auto-oxidation of Hcy, are of crucial importance in Hcy-induced susceptibility to inflammatory progression of atherosclerotic lesions and B lymphocyte proliferation [22]. Hcy has also been demonstrated to induce the production of ROS through endothelial NO synthase uncoupling and NADPH oxidases in endothelial cells [23-26]. Elevated ROS levels can lead to damage of DNA and proteins. This can further induce inflammation and cell death [27]. Additionally, elevated Hcy levels can activate unfolded protein response (UPR) and elicit ER stress. Moreover, prolonged activity of UPR can result in ER stress-associated cell death [28].

HHcy is correlated with modulation of ER redox homeostasis through regulating expression of endoplasmic reticulum oxidoreductin 1-α (Ero1α) and glutathione peroxidase 7 (GPx7) [6]. It can induce expression of Ero1α and inflammation in the arteries of HHcy mice and in human umbilical vein endothelial cells. It upregulates expression of Ero1α through promoting the binding of hypoxia-inducible factor 1α to the ERO1A promoter. At the same time, HHcy can inhibit expression of GPx7. Inhibition of GPx7 further leads to an ER redox imbalance. Possible mechanisms associated with inhibition of GPx7 are that Hcy can be metabolized to methionine. This can rapidly form S-adenosyl methionine, a methyl donor for methylation of RNA and DNA [29, 30]. GPx7 can be inhibited by hypermethylation of promoter DNA [31]. Thus, HHcy can further exaggerate an ER redox imbalance through upregulating expression of Ero1α and downregulating expression of GPx7 in endothelial tissues. This induces HHcy-associated vasculopathy.

A key controlling enzyme in the metabolism of Hcy, MTHFR is indispensable in the conversion of homocysteine to methionine [10, 11]. It is encoded by the *MTHFR* gene found on chromosome 1p36.3 [32]. The most common SNP of this gene is the cytosine (C) to thymine (T) substitution at position 677 (rs1801133) in the encoding region. This results in the conversion from alanine to valine at amino acid 222 [33]. This SNP is associated with a decrease in enzyme activity. This decrease in enzyme activity eventually results in the elevation of Hcy levels [34, 35]. It was speculated that the SNP of the *MTHFR* gene (rs1801133, C667T) was associated with susceptibility to TAO through affecting Hcy levels.

According to present results, the SNP of *MTHFR* rs1801133 was independently associated with susceptibility to TAO, adjusting for confounders. CT and TT genotypes showed increased susceptibility to TAO, compared to the CC genotype. Moreover, the *OR* was higher in the TT genotype than in the CT genotype. Additionally, plasma Hcy levels of different genotypes showed the tendency of TT > CT > CC. Therefore, the SNP of *MTHFR* rs1801133 was associated with susceptibility to TAO through affecting Hcy levels.

Disclosure of conflict of interest

None.

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