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Combined Effect of MTHFR C677T and PAI-1 4G/5G Polymorphisms on the Risk of Venous Thromboembolism in Chinese Lung Cancer Patients

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Abstract

Venous thromboembolism (VTE) is a common and potentially fatal complication in cancer patients. Although several genetic risk factors related to thrombophilia have been identified, their contributions for the occurrence of VTE in cancer patients have conflicting results. The aim of this study was to evaluated the gene polymorphisms of methylenetetrahydrofolate reductase (MTHFR) C677T and plasminogen activator inhibitor-1 (PAI-1) 4G/5G in lung cancer patients, with and without VTE, and the combined effect on the risk of VTE. 92 lung cancer patients diagnosed with VTE (VTE group) and 122 lung cancer patients without VTE (non-VTE group) were enrolled in the study. The gene polymorphisms were analyzed by the method of polymerase chain reaction-restriction fragment length polymorphism. Gene mutation of factor V Leiden was not detected both in non-VTE group and VTE group. The frequency of MTHFR C677T homozygous mutation in VTE group was 25.00%, higher than that in the non-VTE group without statistical difference. It was found that the PAI-1 4G4G genotype is associated with a higher risk of VTE (OR: 2.62, 95%CI: 1.19-5.75). Interestingly, the interaction between MTHFR C677T and PAI-1 4G/5G polymorphisms showed that the coexistence of the 2 homozygous mutation could further increase the risk of VTE. In conclusion, PAI-1 4G/5G polymorphism may be an increased risk factor for VTE among lung cancer patients in Chinese population. The homozygous MTHFR C677T mutation may be not a risk factor for VTE but increases the risk, accompanied with PAI-1 4G5G genotype.

Keywords

venous thromboembolism, MTHFR C677T, PAI-1 4G/5G, lung cancer, Chinese population

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Introduction

Cancer produces a hypercoagulable state which increases the risk of thrombosis.[1] The incidence of venous thromboembolism (VTE) was as many as 20% of patients diagnosed with cancer.[2] A previous study has demonstrated that patients with hematological malignancy had the highest risk of VTE, followed by lung and gastrointestinal cancers.[3] In China, lung cancer has the highest incidence and currently is the leading cause of cancer mortality.

VTE manifests itself as a multifactorial disease and the pathogenesis for thrombosis generation are endothelial damage, hypercoagulable states and stasis, known as Virchow's Triade.[4] Besides the well-established acquired risk factors, the influence of inherited determinants has made considerable head-way and several genetic factors for thrombophilia have been identified, including deficiencies of protein C, protein S and antithrombin III, mostly related to the clotting system and inherited hypercoagulable states.[5,6] In the past few decades, the genetic mutations in coding for coagulation factor V (Factor V Leiden) and factor II (prothrombin G20210A) are 2 of the more common variants in association to an increased

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risk of VTE.[7,8] However, Factor V Leiden (FVL) and prothrombin G20210A mutations are extremely scarce in the Chinese population.[9]

In addition to genetic mutations related to the clotting factors, there are other genetic mutations that increase the risk of thrombosis. Methylenetetrahydrofolate reductase (MTHFR) is a key regulatory enzyme involved in folate metabolism. The genetic polymorphism, caused by a cytosine to thymidine transition at nucleotide 677 of the gene (MTHFR C677T), leads to the reduction of enzyme activity and increase of enzyme thermolability. The MTHFR gene mutation could aggravate hyperhomocysteinemia, which is identified as a significant risk factor for VTE.[10] The presence of allele 4G of −675 4G/5G sequence in plasminogen activator inhibitor-1 (PAI-1), one of the main regulators of endogenous fibrinolytic system, leads to the over expression of PAI-1 which may impair the function of fibrinolytic system and increase the incidence of thrombotic events.[11]

Although there are much data regarding the correlation between the well-characterized genetic defects and increased risk of thrombosis, most the epidemiological evidences have been restricted to Caucasian populations or single genetic mutation.[12-14] Whether there are obvious racial differences in the prevalence of these mutation with VTE remains controversial. Moreover, there is few published study that evaluates the combined effect of multiple gene polymorphisms in the occurrence of VTE in cancer patients. Some studies, investigating the role of genetic mutation on the thrombosis risk in cancer have yielded conflicting results. Eroglu et al investigated MTHFR C677T polymorphism in 187 cancer patients and concluded that mutation of the gene encoding this enzyme was not a major risk factor for developing VTE in cancer patients.[15] A meta-analysis evaluated the potential relationship between MTHFR C677T polymorphisms and VTE risk in Chinese population, and elucidated that MTHFR C677T mutation was associated with increased risk of VTE.[16] However, the patients with VTE who met the inclusion criteria of this study were not just cancer patients.

To date, the effect of hereditary factors in Chinese with VTE is still unclear, particularly in cancer patients. Thus the purpose of this study was to assess the frequency of FVL, MTHFR C677T and PAI-1 4G/5G polymorphisms in lung cancer patients, with or without VTE, and to investigate whether genetic predisposition plays an important role in the occurrence of VTE in lung cancer patients.

Materials and Methods

Study Design and Data Source

214 lung cancer patients at Nanjing Drum Tower Hospital were enrolled in the study with informed consent from January 2017 to September 2019. Included patients were accorded with the diagnostic criteria of lung cancer, including squamous cell carcinoma, adenocarcinoma, small cell carcinoma, large cell carcinoma. The diagnosis of VTE was confirmed by using venography, Doppler ultrasonography or CT pulmonary angiography in addition to clinical signs and symptoms. The general characteristics of patients involved in this study were analyzed, including gender, age, type of thrombosis, type of cancer and environmental risk factors. Written informed consent was obtained from all patients and the study was approved by the Institutional Ethics Committees.

Gene Polymorphisms Detection

Genomic DNA isolation was prepared from peripheral venous blood by standard phenol/chloroform extraction. The polymorphisms of FVL, MTHFR C677T and PAI-1 4G/5G were determined by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method.[17,18]

The polymorphisms of factor V gene were determined using the primer pairs 5′-TTTCAGGCAGGAACAACA-3′ and 5′-CTTCGGCAGTGATGGTAC-3′. PCR was performed for 35 cycles in a volume of 25 μl. Denaturation was at 94 °C, annealing at 55 °C, and a final extension at 72 °C, all for 30 s. The 424-bp PCR products were digested with a Mnl I restriction enzyme, then separated on 2% agarose gel and stained with ethidium bromide.

The polymorphisms of MTHFR gene were determined using the primers 5′-TGAAGGAGAAGGTGTCTGCGGGA-3′ and 5′-AGGACGGTGCGGTGAGAGTG-3′. PCR was performed for 35 cycles in a volume of 25 μl. The PCR reaction mixtures were heated to 94 °C for 5 min for initial denaturation and underwent denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. Finally, extension was conducted at 72 °C for 5 min. The amplified products were digested with Hinf I at 37°C for 1.5 h, then subjected to 2% agarose gel and stained with ethidium bromide.

The polymorphisms of PAI-1 gene were determined using an upstream control primer 5′-AAGCTTTTAC-CATGGTAACCCCTGGT-3′, allele-specific primer (5′-GTCTGGACACGTGGGGA-3′ for the 4G allele and 5′-GTCTGGACACGTGGGGG-3′ for the 5G allele), and a common downstream primer 5′-TGCAGCCAGCCACGT-GATTGTCTAG-3′. The PCR reaction was performed in a total volume of 25 μL with 35 cycles of denaturation at 94 °C for 60 s, annealing at 60°C for 60 s, and extension at 72°C for 60 s. For each PCR, 2.5 U of Taq polymerase was used. The PCR products were separated on 2% agarose gel containing ethidium bromide.

All these fragments were analyzed by electrophoresis.

Statistical Analysis

Statistical analysis was performed with SPSS software. Data were expressed as the mean or frequency. The *t*-test, chi-square test or Fisher's Exact test were used to evaluated the statistic difference. The odds ratios (OR) and 95% confidence intervals were also calculated. P-value < 0.05 was considered significant.

Table 1. General Characteristics of Lung Cancer Patients With and Without VTE.

Characteristics	non-VTE group	VTE group	*P* value
Number of subjects	122	92	
Mean age	61.38 ± 10.78	60.03 ± 10.97	$P > 0.05$
Male/Female	78/44	63/29	$P > 0.05$
Type of thrombosis			
Isolated DVT		81(88.04%)	
DVT and PE		11(11.96%)	
Type of cancer			
Squamous cell carcinoma	66(54.10%)	50(54.35%)	
Adenocarcinoma	40(32.78%)	29(31.52%)	
Large cell carcinoma	5(4.10%)	4(4.35%)	
Small cell carcinoma	11(9.02%)	9(9.78%)	$P > 0.05$
Environmental risk factors			
Recent surgery	11(9.02%)	8(8.70%)	
immobilization	6(4.92%)	4(4.35%)	
Trauma	3(2.46%)	1(1.09%)	$P > 0.05$
D-dimer level(mg/L)	1.54 ± 1.09	3.82 ± 2.24	$P < 0.05$

Results

Baseline Characteristics

General characteristics of 214 lung cancer patients are listed in Table 1. The mean ages of patients in non-VTE group and VTE group were 61.38 and 60.03 years, respectively. Of the 92 patients with VTE, 81 (88.04%) had isolated deep venous thrombosis (DVT) and 11 (11.96%) had DVT complicated by symptomatic PE. We compared the type of cancer between the 2 groups and no difference was observed. Moreover, in addition to the exposing factor of cancer, there was no difference in other environmental risk factors for VTE between the 2 groups. The D-dimer level in non-VTE group (1.54 ± 1.09 mg/L) was significantly lower than that in VTE group (3.82 ± 2.24 mg/L), but higher than normal level.

Gene polymorphisms of FVL, MTHFR C677T and PAI-1 4G/5G

Of all subjects recruited, mutation of FVL was not detected both in non-VTE group and VTE group (Table 2), indicating that FVL mutation is very rare in Chinese population, consistent with the published research.[9]

The Hardy-Weinberg equilibrium test showed that the distribution of MTHFR and PAI-1 genotypes conformed to the law of genetic equilibrium ($P > 0.05$, Table S3 and Table S4). The MTHFR genotype distribution in patients was also shown in Table 2. The frequencies of the CC, CT, and TT genotypes in non-VTE group were 32.79%, 50.00% and 17.21%, respectively, whereas the corresponding frequencies in VTE group were 32.61%, 42.39% and 25.00%, respectively. The frequency of TT genotype in VTE group appeared to be higher than that in the non-VTE group without statistical difference (OR: 1.46, 95%CI: 0.69-3.12).

Table 2. Gene Polymorphisms of FVL, MTHFR C677T and PAI-1 4G/5G in Cancer Patients With and Without VTE.

	non-VTE group (n = 122)	VTE group (n = 92)	*P* value
FVL			
GG	122	92	
MTHFR C677T			
CC	40 (32.79%)	30 (32.61%)	1.00*
CT	61 (50.00%)	39 (42.39%)	0.85 (0.49-1.59)
TT	21 (17.21%)	23 (25.00%)	1.46 (0.69-3.12)
PAI-1 4G/5G			
4G4G	29 (23.77%)	38 (41.31%)	2.62 (1.19-5.75)
4G5G	63 (51.64%)	39 (42.39%)	1.07 (0.52-2.23)
5G5G	30 (24.59%)	15 (16.30%)	1.00*

*Reference category.

Table 3. Distribution of Plasma Homocysteine Concentration (μmol/L) in Different MTHFR Genotypes Among Cancer Patients With and Without VTE.

	CC	CT	TT
non-VTE group	11.99 ± 2.88	12.64 ± 4.24	14.44 ± 4.16
VTE group	11.83 ± 5.11	13.42 ± 5.31	16.35 ± 5.94

In the non-VTE group, the frequencies of different PAI-1 4G/5G genotype were 23.77% for 4G4G, 51.64% for 4G5G, and 24.59% for 5G5G. In the VTE group, the distributions were 41.31% for 4G4G, 42.39% for 4G5G, and 16.30% for 5G5G (Table 2). There was significant difference in the frequency of 4G4G genotype (OR: 2.62, 95%CI: 1.19-5.75).

Plasma Homocysteine Level

The level of plasma homocysteine was also compared between the 2 groups (Table 3). The homozygous TT genotype were associated with higher levels of plasma homocysteine level than CT and CC genotypes. The plasma homocysteine of VTE group in TT genotype was 16.35 ± 5.94 compared to 14.44 ± 4.16 for non-VTE group with no statistical difference ($P > 0.05$).

Combined Effect of MTHFR C677T and PAI-1 4G/5G Polymorphisms

The OR value in patients with both homozygous mutations or either one was calculated, in contrast to patients with neither (Table 4). The OR value of the homozygous mutant PAI1 gene alone was 2.02. However, the OR value of the double homozygous mutations was 7.59, which was higher than the gene-gene interaction calculated by additive model and multiplicative model. Excluding the influence of environmental risk factors, the combined effect of TT and 4G4G genotypes further increased the risk of VTE (OR: 11.65, 95%CI: 1.54-37.49). Prevalence and OR (95%CI) for VTE associated with MTHFR

Table 4. Effect of the Association Between MTHFR C677T and PAI1 4G/5G.

MTHFR	PAI-1	OR (95% CI)	No risk factor, OR (95% CI)
− (CC/CT)	− (5G5G/5G4G)	1.00*	1.00*
− (CC/CT)	+ (4G4G)	2.02 (1.10-4.03)	2.50 (1.23-5.05)
+ (TT)	− (5G5G/5G4G)	1.50 (0.69-3.27)	1.64 (0.69-3.99)
+ (TT)	+ (4G4G)	7.59 (1.54-37.49)	11.65 (1.35-100.70)

*Reference category.

C677T and PAI1 4G/5G (with or without environmental risk factors) were showed in Supplementary file: Table S1 and Table S2.

Discussion

Cancer patients are at an increased risk for thrombosis. Until now, the morbidity and mortality of lung cancer has shown a continuously rising trend in China. In these patients, VTE complicates the clinical management of cancer and is associated with poor patient prognosis. To understand the causes of VTE in cancer patients is conducive to achieve better prevention and treatment of VTE. In addition to the recognized acquired risk factors, genetic risk factors have also been shown to have an important role in the occurrence of thrombosis in Caucasian populations. However, the risk varies evidently in different racial populations. Actually, previous studies that evaluated genetic risk factors for cancer-associated thrombosis are still rare and revealed conflicting results.

FVL has been demonstrated to be the most common genetic risk factor of VTE. The genetic defect leads to the resistance to activated protein C, resulting in higher level of thrombin in the blood and an increased risk of VTE.[7] Based on the results of previous studies, FVL mutation has a remarkable incidence in Caucasian populations but was almost scarce in Asian populations. In our study, we did not find any FVL mutation, suggesting that FVL mutation could not be considered as a genetic risk factor for VTE. This result further confirms the hypothesis that there are significant ethnic differences in the incidence of FVL mutation.[9]

Another gene polymorphism that has been widely studied is the C677T mutation in MTHFR gene, which could increase homocysteine levels. The elevated homocysteine level could damage the structure and function of the endothelial cells, associated with the occurrence of thrombosis.[19] However, some studies have failed to prove a direct relationship between MTHFR C677T mutation and venous thrombosis. A meta-analysis demonstrated that the MTHFR C677T gene polymorphism was weakly associated with an increased risk of VTE.[20] Moreover, this relevance was slightly more significant after excluding cases with malignancy or situational VTE or a thrombophilia factor. Another meta-analysis from Asian populations showed a significant association between gene polymorphism and the risk of VTE in a recessive model.[16] Nevertheless, these studies were not limited to cancer patients. In our study, there was no difference in the distribution of MTHFR C677T genotypes between the 2 groups. These conflicting results may be attributed to the varying frequency of MTHFR gene mutations among different races or the influence of external environmental factors.

The levels of plasma homocysteine were also measured in patients with or without VTE. There was an obvious gradient in homocysteine levels, with high value in TT genotype group. Increased plasma homocysteine concentration has consistently been identified as a risk factor for the development of thrombosis in many previous studies.[21,22] However, there is no statistic difference of homocysteine concentrations for TT genotype between the 2 groups in our study. This is probably because the plasma homocysteine concentration could be modulated by both environmental and genetic factors. It is well known that lower plasma folate levels, mostly due to the insufficient dietary intake, are associated with higher homocysteine plasma levels. Unfortunately, we did not perform plasma folate measurements in this study to evaluate this influence. In our study, perhaps the slight increase in homocysteine level accompanied by homozygous mutation is not sufficient to induce VTE.

PAI-1, the physiological inhibitor of tissue plasminogen, could reduce fibrinolytic capacity.[23] Since a guanosine deletion/insertion polymorphism (4G5G) at the −675 bp position of the PAI-1 gene promoter was confirmed to be closely related to the plasma PAI-1 level. In the recent decade, impaired fibrinolytic capacity might play a role important in the pathogenesis of thrombotic diseases possibly by affecting the prothrombotic state, including coronary artery disease and ischemic stroke. However, the effect of PAI-1 4G5G genotype on venous thrombosis in Chinese has been rarely reported, especially in cancer patients. In our study, in terms of the distribution of genotypes between the VTE and non-VTE groups, there was a significant increase in the frequency of 4G4G genotype in VTE group. A meta-analysis by Tsantes et al found that the presence of 4G allele appears to increase the risk of VTE in Caucasian population, particularly in combination with other genetic thrombophilic defects.[24] Another meta-analysis by Wang et al concluded that PAI-1 gene promoter 4G5G polymorphism was considered as a genetic risk factor for VTE.[25] Moreover, in the analysis of ethnic subgroups, significant results were achieved in both Asian and Caucasian population. The higher prevalence of 4G4G genotype in patients with VTE in our study was consistent with the hypothesis that PAI-1 4G5G polymorphism was a genetic risk factor for VTE. Moreover, the combined effects of MTHFR C677T and PAI-1 4G5G polymorphisms were evaluated. Our results indicated that lung cancer patients, due to the synergistic effect of homozygous mutants of the MTHFR and PAI-1 genes, were at higher risk of VTE than patients with either mutation alone.

There are several possible reasons to explain the conflicting results observed in previous studies. Firstly, the effect of genetic polymorphism is likely to be weak, and therefore the reliable association would be obtained through a large sample

study. Secondly, most of the current studies only investigated the effect of single gene polymorphisms, and ignored the association between the combined effects of thrombophilic gene polymorphisms and VTE. Thirdly, in addition to the genetic factors, other acquired factors also play a non-ignorable role in the occurrence of VTE, such as surgery, fracture or immobilization. Currently, some other novel risk factors associated with thrombosis risk have been identified, such as iron deficiency. Unfortunately, the serum ferritin level was not routinely determined in this study. Therefore, it was difficult to demonstrate its role on the occurrence of thrombosis. Finally, the MTHFR C677T polymorphism accounts for only part of the variation in plasma homocysteine levels at baseline, and several other important physiologic factors are also involved in the influence of the plasma levels, such as metabolic syndrome, inflammation or injury.

Conclusion

The results of this study suggest that PAI-1 4G/5G polymorphism may be an increased risk factor for development of VTE, especially in lung cancer patients in Chinese population. Moreover, patients with the double homozygous mutations of MTHFR and PAI1 gene were more prone to experiencing VTE. There are some limitations in the article. Further and larger studies are needed to draw definite conclusions. There are no adequate multiple comparisons or replication which may limit the extrapolation of experimental results. Therefore, it is necessary to make more depth analyses in the future studies.

Authors' Note

HX contributed to the study design. QS and SMY carried out blood sampling and data analysis. BYW and PJX performed the experiments, wrote the manuscript and contributed equally to this work as the first author. All authors read and approved the final manuscript. Ethical approval to report this case was obtained from the Ethics Committee of Nanjing Drum Tower Hospital (Approval Number: 2017-100-01). Written informed consent was obtained from the patients for their anonymized information to be published in this article.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

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