Original Article
Association between thrombomodulin (THBD) gene polymorphism and pulmonary thromboembolism in a Chinese population

Peng Zhang¹, Cen-Feng Xia², Min Jiang³, Shao-Jin Wang¹, Jin Zhang¹

¹Department of Respiratory Critical Care Medicine, General Hospital of Ningxia Medical University, Yinchuan 750004, China; ²Department of Respiratory Critical Care Medicine, Cardio-Cerebrovascular Disease Hospital affiliated to General Hospital of Ningxia Medical University, Yinchuan 750004, China; ³Beijing National Biochip Center Sub-center in NingXia, Yinchuan 750004, China

Received January 23, 2017; Accepted February 27, 2017; Epub April 15, 2017; Published April 30, 2017

Abstract: Objective: Thrombomodulin (TM) is an essential component of the anticoagulant protein C system. Polymorphism 1418C>T of the thrombomodulin gene (THBD) that encodes TM is associated with venous thrombosis in both American and Japanese populations; however, the effect of this polymorphism in Chinese populations is unknown. Therefore this study aimed to investigate the relationship between the THBD polymorphisms 1418C>T and/or -151G>T, and pulmonary thromboembolism (PTE) in a Chinese population. Patients and methods: The current study assessed 111 PTE patients (PTE group) and 100 healthy patients (control group). These patients were screened for 1418C>T and -151G>T polymorphisms using high-resolution melt (HRM) analysis, and their plasma TM concentration was examined by ELISA. Results were statistically analysed to evaluate a possible relationship between THBD 1418C>T and/or -151G>T polymorphism, plasma TM concentration, and PTE incidence. Results: Patients were identified as having one of the three genotypes at THBD position 1418: C/C, C/T, or T/T. The frequency of the T allele was higher in the control group than in PTE group; however, this difference was not statistically significant (P>0.05). Patients were shown to have either a G/G or a G/T genotype at THBD position -151. The frequency of the T allele at this locus was significantly higher in PTE patients than in the control group (P<0.05). The results of the patient screening at the THBD 1418 and -151 loci demonstrate that 1418C>T polymorphism is associated with a decreased risk of PTE (OR=0.373, P<0.05). Similarly, -151G>T polymorphism appears to significantly reduce the plasma concentration of soluble TM (sTM) compared to the standard G/G genotype at this locus (P<0.05). Conclusion: THBD 1418C/T genotype is a protective factor against the development of PTE, and the THBD -151G/T genotype reduces expression of plasma sTM.

Keywords: Pulmonary thromboembolism, thrombomodulin, gene polymorphism

Introduction
Pulmonary thromboembolism (PTE) is a disease characterized by the blockage of a pulmonary artery by a blood clot that originates at another site in the body. This blockage disrupts pulmonary circulation and thereby prevents normal respiratory function. Ranking third amongst the most common cardiovascular diseases internationally, PTE causes chronic disease, disability, and even death [1]. Venous thromboembolism (VTE) is another common disease similar to PTE, and is induced by a combination of genetic and environmental factors that vary with ethnicity. For example, both factor V (FV) Leiden and prothrombin gene G20210A mutation are thrombophilic risk factors that occur at a rate of 3%-7%, amongst Caucasian or European populations, but are rarely observed within Asian populations [2-5]. In China, common genetic risk factors for PTE affect the physiological anticoagulant proteins, such as Antithrombin III (AT-III), protein C (PC), and protein S (PS). Thrombomodulin (TM) is a vital element of the PC anticoagulant system. If the formation and/or function of TM are abnormal, the coagulation and anticoagulation systems in the body lose homeostatic balance, resulting in PTE.

TM is a transmembrane glycoprotein expressed at the surface of vascular endothelial cells, and
Thrombomodulin gene polymorphism and PTE plays an essential role in both anticoagulation and anti-inflammation. It is encoded by the 6.1 kb-long thrombomodulin (THBD) gene, located on chromosome 20 and consisting of a promoter region and a coding sequence composed of 18 exons (with no intronic regions). Animal models have previously demonstrated that THBD genetic defects are associated with thrombogenesis. For example, homozygous TM-knockout rats accumulate large amounts of fibrous protein that spontaneously form a thrombus [6]. Similarly, human thrombotic diseases such as coronary heart disease, cerebral infarction, and venous thrombosis are associated with THBD point mutations. Importantly, the relationship between THBD polymorphism and PTE is not yet established, perhaps due to a failure of the current literature to adequately investigate genetic variation amongst PTE patients of varying ethnicities. For example, very few studies have investigated the incidence or effect of the 1418C>T (rs1042579) THBD polymorphism in Chinese populations. Thus, the current study aimed to explore the relationship between PTE and the 1418C>T (rs1042579) and -151G>T (rs16984852) THBD polymorphisms in this ethnic background.

Materials and methods

Patient recruitment

One hundred and eleven PTE patients from the General Hospital of Ningxia Medical University were selected for inclusion in this study between January 2014 and January 2015. These included 51 men and 60 women, with an overall average age of (52.64±12.12) years. The inclusion criteria first stipulated that patients be diagnosed as having PTE by spiral CT pulmonary angiography (CTPA) and radionuclide pulmonary perfusion imaging, according to ACCP-9. Second, patients had to be more than 18 years of age, and lastly, they must have signed an informed consent form. The exclusion criteria for this group stipulated that patients should not be younger than 18 years of age; should not have refused to sign an informed consent form; nor have any of the following: a medical history of PTE; a PTE diagnosis determined by imagiological examination; chronic thromboembolic pulmonary hypertension; suspected gas embolism, amniotic fluid embolism, or fat embolism; coronary heart disease; diabetes; autoimmune disease; a malignant tumour; inflammatory disease; and chronic disease, such as hepatopathy, renal failure. One hundred healthy patients were selected during the same period to form a control group. These comprised 54 men and 46 women, with an average age of 49.63±10.02 years. The inclusion criteria for these control patients first stipulated that they be deemed healthy and show normal liver and kidney function, during an outpatient examination. Second, patients had to be more than 18 years of age. Lastly, they must have signed an informed consent form. The exclusion criteria for the control group was identical to that stipulated for PTE group patients.

Genomic DNA extraction

Genomic DNA was extracted from peripheral blood, using an OMEGA blood genomic DNA isolation kit according to the manufacturer’s instructions.

Genotype identification

The genotype of extracted genomic DNA samples was identified via high-resolution melt (HRM) analysis. After PCR amplification, 20 samples, (including appropriate wild type and mutant polymorphic controls), were sent to Sangon Biotech (Shanghai) Co. Ltd., to be sequenced using the following primers: -151G>T (rs16984852) sense primer 5’AGAGAACCCAGCAATCCGAG3’; -151G>T (rs16984852) reverse primer 5’CCAGACACTTCTTGCCGCT3’; 1418C>T (rs1042579) sense primer 5’AGAGAACCCAGCAATCCGAG3’; 1418C>T (rs1042579) reverse primer 5’CCAGACACTTCTTGCCGCT3’.

ELISA for plasma TM

After thawing plasma samples at room temperature, TM concentration was determined using the ‘sandwich’ ELISA method, using an American RD kit according to the manufacturer’s instructions.

Statistical analysis

A database was constructed using SPSS17.0 software, and the collected data were analysed. For general conditions, we used a chi-squared test to evaluate potential relationships between genetic (e.g. THBD genotype) and non-genetic risk factors, with PTE incidence, such that a P value of <0.05 was deemed to indicate
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Table 1. Comparison of non-genetic risk factors between PTE patients and control group

<table>
<thead>
<tr>
<th>Patient history</th>
<th>PTE group</th>
<th>Control group</th>
<th>$\chi^2$</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male, n (%)</td>
<td>51 (45.9%)</td>
<td>54 (54%)</td>
<td>1.365</td>
<td>0.243</td>
</tr>
<tr>
<td>Age (years)</td>
<td>52.6±12.13</td>
<td>48.76±10.12</td>
<td>1.782</td>
<td>0.760</td>
</tr>
<tr>
<td>Smoking history, n (%)</td>
<td>49 (44.1%)</td>
<td>18 (18%)</td>
<td>16.593</td>
<td>0.000*</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>54 (48.6%)</td>
<td>27 (27%)</td>
<td>10.424</td>
<td>0.001*</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>25.23±2.18</td>
<td>23.08±2.91</td>
<td>6.139</td>
<td>0.000*</td>
</tr>
<tr>
<td>sTM (pg/ml)</td>
<td>445.18±168.00</td>
<td>374.5±125.06</td>
<td>3.431</td>
<td>0.001*</td>
</tr>
</tbody>
</table>

*P<0.05.

Table 2. Comparison of allelic and genotypic frequencies at THBD gene loci -151 (rs16984852) and 1418 (rs1042579) in PTE cases versus control group

<table>
<thead>
<tr>
<th>THBD locus</th>
<th>Genotype/allele</th>
<th>PTE group (n/%)</th>
<th>Control group (n/%)</th>
<th>t</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>-151 (rs16984852)</td>
<td>GG</td>
<td>106 (95.5)</td>
<td>100 (100)</td>
<td>4.559</td>
<td>0.032*</td>
</tr>
<tr>
<td></td>
<td>GT</td>
<td>5 (4.5)</td>
<td>0 (0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>217 (97.7)</td>
<td>200 (100)</td>
<td>4.614</td>
<td>0.033*</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>5 (2.3)</td>
<td>0 (0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1418 (rs1042579)</td>
<td>CC</td>
<td>69 (62.2)</td>
<td>56 (56.0)</td>
<td>0.83</td>
<td>0.661</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>40 (36.0)</td>
<td>42 (42.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>2 (0.18)</td>
<td>2 (2.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CT+TT</td>
<td>42 (37.8)</td>
<td>44 (44.0)</td>
<td>0.827</td>
<td>0.363</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>178 (80.2)</td>
<td>154 (77.0)</td>
<td>0.63</td>
<td>0.426</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>44 (19.8)</td>
<td>46 (23.0)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P<0.05.

Results

Non-genetic risk factors for PTE

There was no observed difference in either gender or age between the case and control-group patients (P>0.05). In contrast, smoking status, hypertension, body mass index (BMI), and plasma soluble TM (sTM) concentration, were all significantly higher in the case group than in the control group (P<0.05) (Table 1).

Distribution of THBD genotypic and allelic frequencies amongst case versus control PTE patients

The distribution of the G/G and G/T genotypes at position -151 (rs16984852) of THBD, and similarly the frequencies of G and T alleles at this locus, were found to be significantly different between case and control patients (P<0.05). This is reflective of the fact that no control patients were observed to carry the T allele at this genetic locus (Table 2). The two groups meet Hardy-Weinberg Equilibrium (HWE), $P$ value (0.808, 1.00).

The frequency with which the C/C genotype was shown to occur at position 1418 (rs1042579) of THBD in the control group was higher than in the case group, while in contrast, the frequencies of the C/T and T/T genotypes at this locus were higher in the case than in the control group. Notably however, the distribution of each of these genotypes was not statistically significant (P>0.05). Similarly, there was no significant difference in the allelic frequencies of C or T at this genetic locus, between the case and control group patients (P>0.05). A comparison between a dominance model for the C/C versus the C/T + T/T genotypes was shown to have no statistical significance (Table 2). Again, the case and control groups meet HWE, $P$ value (0.159, 0.163).

Evaluating potential relationships between THBD gene polymorphism, non-genetic risk factors (BMI, age) and plasma sTM concentration

Patient screening revealed that the presence of a G/G genotype at THBD position -151 (rs16984852) was significantly associated with an increased plasma sTM concentration, as compared to the presence of a G/T genotype at this locus (P<0.05). The sTM of patients with
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Table 3. Comparison of plasma sTM concentration in PTE group versus control group, with respect to \textit{THBD} genotype, age and BMI

<table>
<thead>
<tr>
<th>\textit{THBD} locus</th>
<th>Genotype</th>
<th>sTM (pg/ml)</th>
<th>Age (years)</th>
<th>BMI (kg/m$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-151 (rs16984852)</td>
<td>GG</td>
<td>456.17±163.23</td>
<td>56.88±12.79</td>
<td>25.17±2.19</td>
</tr>
<tr>
<td></td>
<td>GT</td>
<td>212.24±75.82</td>
<td>39.60±9.07</td>
<td>26.55±1.49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>\textit{t}=3.313, \textit{P}=0.001*</td>
<td>\textit{t}=0.942, \textit{P}=0.341</td>
<td>\textit{t}=1.395, \textit{P}=0.166</td>
</tr>
<tr>
<td>1418 (rs1042579)</td>
<td>CC</td>
<td>429.85±164.00</td>
<td>51.93±11.70</td>
<td>25.07±2.38</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>469.80±175.66</td>
<td>62.73±10.25</td>
<td>25.55±1.80</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>481.66±170.65</td>
<td>67.50±7.78</td>
<td>24.49±1.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>\textit{F}=0.761, \textit{P}=0.470</td>
<td>\textit{F}=1.003, \textit{P}=0.370</td>
<td>\textit{F}=0.743, \textit{P}=0.478</td>
</tr>
</tbody>
</table>

\* \textit{P}<0.05.

a polymorphism at this locus was not affected by either age or BMI. At \textit{THBD} position 1418 (rs1042579), there was no observed change to plasma sTM concentration induced by any of the studied genotypes (C/C, C/T or T/T) (\textit{P}>0.05), or by either patient age or BMI (Table 3).

\textbf{Evaluating the relationship between \textit{THBD} gene polymorphism and PTE risk}

Using a logistic regression analysis to evaluate the various PTE risk factors, we found that a \textit{THBD} C/T genotype at position 1418 (rs1042579) is a protective factor against PTE (OR=0.373, \textit{P}=0.013).

\textbf{Discussion}

TM has been demonstrated to assume two forms \textit{in vivo}: membrane bound (Mb-TM) and soluble (sTM) [7]. Some reports suggest that sTM is a marker for blood vessel endothelium injury [8]. In PTE, injury to the blood vessel endothelium induces the abnormal secretion of TM into the blood, causing an increase in the level of sTM [9]. This is supported by the results of the current study, which show the concentration of sTM to be much higher in the PTE case than in the control group patients. Some studies report that sTM is also increased in patients with recurrent pulmonary embolism [10]. Similarly, in acute respiratory distress syndrome (ARDS), an increased level of sTM is closely related to an increased risk of patient mortality [11]. Notably, however, increased levels of sTM in these cases are induced by blood vessel endothelium injury caused by inflammatory disease, rather than by genetic mutation or polymorphism of \textit{THBD} [11]. In the current study, we observed the level of sTM to decrease in patients with a -151G>T \textit{THBD} polymorphism. However, the sTM level in the PTE group overall was much higher than that exhibited by the control group, suggesting that the increased levels of sTM observed in PTE patients is indicative of blood vessel endothelium injury, rather than of genetic mutation.

Our screening results identify three genotypes to occur at \textit{THBD} locus 1418 in the Chinese population studied. The frequencies of the C/C, C/T and T/T genotypes at this locus are 62.2%, 36% and 1.8% respectively in the case group, whereas they were 56%, 42% and 4% respectively in the control group patients. The frequencies of the C and T alleles at this locus are 80.2% and 19.8% respectively in the case group, and 77% and 23% respectively in the control group. To place these results in context, we surveyed previous research reporting the incidence of \textit{THBD} 1418 polymorphisms in various populations internationally. For example, a recent study on coronary heart disease compared the frequencies of the C/C, C/T and T/T genotypes at this locus, and reported them as occurring at a frequency of 62.2%, 36% and 1.8% respectively amongst 159 Swedish patients, 66.5%, 30.1% and 3.4% in 356 American patients, and 93.3%, 5.7% and 3.4% in 105 African American patients [12]. In Japanese populations, the allelic frequencies of C and T at the 1418 \textit{THBD} locus are 65.1% and 34.9% respectively [13]. We conclude that the genotype frequency at the \textit{THBD} 1418 locus in the studied Chinese population is most similar to that reported in Swedish and American populations, whilst the allelic frequency at this locus is similar to that occurring in Japanese populations.

At the \textit{THBD}-151 locus, the Chinese population studied here showed two genotypes: G/G and
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G/T. Amongst PTE patients, these genotypes occurred at a frequency of 95.5% and 4.5% respectively, whereas in contrast, only the G/G genotype was observed in control group patients. The G and T allelic frequencies at this locus were 97.7% and 2.3% in the case group, and 100% and 0% in the control group. These results correlate closely with a previous study on 1269 VTE patients from Wuhan China, which reported the THBD G/G and G/T genotypes at position -151 to occur at a rate of 97.32% and 2.68% respectively amongst case patients, and 99.03% and 0.97% respectively amongst control patients [14].

The results of the current study show no difference in plasma sTM concentration amongst patients with varying genotypes at the THBD 1418 locus. In contrast, the sTM concentration was found to be much lower in patients with a C/T genotype at THBD position-151 than in those with a C/C genotype at this locus. This observation is strongly supported, again by the recent study of Chinese VTE patients which reported a reduction in sTM levels associated with a C/T genotype at THBD position-151 [14], and also by various other studies which suggest that genetic mutation of THBD can result in abnormal sTM expression. For example, Arg385Ser, Pro477Ser, and Pro483Leu mutation of THBD can each reduce the expression level of TM, and similarly a THBD del791-801 polymorphism results in abrogation of TM expression at the endothelial cell surface [15]. In contrast, a TM Asp468Tyr polymorphism has been shown not to affect either the plasma sTM concentration or the observed anticoagulant activity [15]. Similarly, previous studies investigating the effect of THBD 1418 polymorphism on plasma sTM concentration report mixed results, with some showing no difference in sTM expression amongst patients with C/C, C/T or T/T genotypes at this locus [16], while others report a reduction in sTM expression associated 1418C>T polymorphism [17]. Thus while it seems probable that -151G>T polymorphism can reduce sTM expression, further study is required to confirm the effect of polymorphism at THBD locus 1418 on plasma sTM concentration.

Using a logistic retrospective analysis, we demonstrate in the current study that THBD 1418C>T polymorphism can reduce patient risk of developing PTE (OR=0.373, P=0.013). This is supported by a previous study conducted in the Netherlands, in which the same mutation increased TM expression in human umbilical vein endothelial cells, thereby increasing the level of activated protein C and reducing the risk of PTE [17]. In contrast, the combined effects of THBD 2729C>T and 1418C>T polymorphisms were reported to be associated with VTE in a Japanese population [13], and 1418C>T polymorphism is thought to have no effect on VTE risk in American populations. The effect of THBD-151G>T polymorphism is shown here to have no effect on patient risk of developing PTE, in contrast to previous research linking this polymorphism with an increased risk of VTE, as compared to patients with a G/G genotype at this locus [14]. Thus the relationships between both the THBD 1418C>T and -151G>T polymorphisms, and PTE, requires clarification through further study.

Conclusions

In conclusion, the current study identifies two genotypes (G/G and G/T) at THBD position-151 (rs16984852), and three genotypes (C/C, C/T and T/T) at THBD position 1418 (rs1042579), in the studied Chinese population. Analysis of patient screening results reveals that the THBD 1418C/T genotype acts as a protective factor against PTE in this population, and THBD-151G>T genotype reduces the expression of plasma sTM.

Disclosure of conflict of interest

None.

Address correspondence to: Jin Zhang, Department of Respiratory Critical Care Medicine, General Hospital of Ningxia Medical University, 804 Shengli Road, Xingqing District, Yinchuan 750004, China. Tel: +86-951-6743066; E-mail: jinzhangnew@hotmail.com

References


