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Thrombomodulin gene c.1418C>T polymorphism and risk of recurrent venous thromboembolism

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Running title: THBD polymorphism in recurrent VTE patients

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Abstract

Background: Thrombomodulin gene (THBD) is a critical cofactor in protein C anticoagulant system. THBD c.1418C>T polymorphism is reported to be associated with higher risk of primary venous thromboembolism (VTE) but its role in VTE recurrence is unknown. The aim of this study was to investigate the role of THBD polymorphism in VTE recurrence.

Material and methods: THBD c.1418C>T polymorphism was genotyped by using Taqman polymerase chain reaction in a prospective population based study of 1465 consecutive objectively verified VTE patients. Uni- and multivariate Cox regression were performed for the risk assessment of VTE recurrence.

Results: Patients who had VTE before inclusion or had recurrence or died during anticoagulant treatment were excluded. Among the remaining (N=1046) patients, 126 (12.05%) had VTE recurrence during the follow up period (from 1998-2008). THBD polymorphism was not significantly associated with risk of VTE recurrence in the univariate [Hazard ratio (HR) =1.11, 95% confidence interval (CI) = 0.78-1.59, p = 0.55] as well as the multivariate analysis adjusted for age, sex and thrombophilia (HR=1.11, 95% CI= 0.78-1.59, p =0.54). Similarly, in unprovoked first VTE (n=614), no association was observed between THBD polymorphism and risk of VTE recurrence (HR=1.22 and 95% CI = 0.78-1.89, p =0.38).

Conclusions

In this prospective study, our results do not suggest a predictive role for THBD c.1418C>T polymorphism in VTE recurrence.

Key words: Thrombomodulin, venous thromboembolism, recurrence, genotypes

Introduction:

Lower extremity deep vein thrombosis (DVT) and pulmonary embolism (PE) are two main clinical constituents of venous thromboembolism (VTE) that is the third most common vascular disease after coronary artery and cerebrovascular diseases [1]. The yearly incidence of VTE is approximately 1-2 per 1000 individuals [2, 3].

Venous thromboembolism is a chronic disease, which often recurs with a risk of 20-25% after 5 years and 30% after 8 years of first diagnosis for VTE [4-7]. It is also reported that rate of recurrence is higher in unprovoked VTE (patients without identified acquired risk factors for VTE e.g. older age, malignancy, trauma, major surgery, immobilization, female hormone therapy, pregnancy) as compared to provoked ones [6]. Case-fatality rate of VTE recurrence was reported as 5-12% [8]. Patients affected by VTE recurrence are predisposed to chronic pulmonary hypertension and recurrent PE that is reported to be fatal in 4-9% cases [8-10].

Standard treatment regimen of acute VTE is use of anticoagulant drugs e.g. heparin followed by vitamin K antagonist for several months. It is a "double-edged sword" which prevents from VTE recurrence at the cost of severe bleeding. Case-fatality rate of severe bleeding due to anticoagulation therapy was reported as 11.3% [11, 12]. Therefore, the duration of anticoagulation therapy after first VTE should be tailored according to the estimated risk for recurrence to minimize the side effects of anticoagulation treatment.

The thrombomodulin (THBD) gene is located at chromosome 20 [13]. It is expressed at endothelial cell membrane and has at least three major anticoagulant characteristics: 1) it catalyzes thrombin activation of protein C; 2) alters thrombin substrate specificity, which leads to inhibition of thrombin mediated clotting, platelet activation and procoagulant factors (V, VIII, XI, XIII); and 3) it has a significant role in the inhibition of thrombin by antithrombin [14, 15].

THBD's role in coagulation is well defined in pre-clinical models. A study on animal reveals the fact that transgenic mice with THBD mutations (targeted point mutation that substitute the glutamic acid 404 with proline) have a prothrombotic disorder [16]. In another study, it was found that mice with ablated THBD died soon after birth due to the consumptive coagulopathy [17].

Clinical studies have shown several genetic alterations in THBD and their role in arterial and venous thrombosis [15, 18-21]. A THBD c.1418C>T polymorphism (rs1042579), which encodes for the replacement of Ala455 by Val455 in THBD has been well described in previous studies [20, 23]. This polymorphism is located in the coding region of THBD that is responsible for the thrombin binding and protein C activation, signifying its potential role in regulating the thrombomodulin functions [20].

THBD c.1418C>T polymorphism has been shown to be associated with risk of arterial thrombosis [19, 22-24]. In a recent functional study of THBD c.1418C>T polymorphism, Navarro S *et al.*, reported a significant association of this polymorphism with the increased risk of primary VTE [18] while other studies suggest that this polymorphism is not associated with risk of primary VTE [25-28]. However, its role in VTE recurrence is not determined.

The aim of this study was to test the hypothesis whether the THBD c.1418C>T polymorphism has any role in risk assessment of VTE recurrence. To our knowledge, this is the first study in which the role of THBD c.1418C>T polymorphism in VTE recurrence is investigated.

Material and Methods

Study subjects

Malmö thrombophilia study (MATS), a prospective population based study of 1465 consecutive unselected VTE patients was performed at Skåne University Hospital from March 1998 to December 2008 [29]. For all MATS patients, the location of VTE, VTE events prior to study inclusion, and VTE recurrence during the follow up were recorded. Diagnosis of DVT and PE or recurrence was objectively confirmed by phlebography, duplex ultrasonography, computed tomography (CT), lung scintigraphy or magnetic resonance imaging (MRI). Included patients were required to leave blood samples and answer a questionnaire. They were also evaluated concerning risk factors for VTE. Acquired risk factors such as malignancy, hospitalization, surgical intervention, immobilization and cast therapy were assessed. In women, data on use of contraceptive pills, hormonal therapy, pregnancy and the postpartum period (defined as first six weeks after delivery) were also assessed.

Among 1465 patients, the participants who had one or more thrombotic events before inclusion (n=154) were excluded. The patients who had recurrence or died during anticoagulant treatment were also excluded (n=265). Among the remaining 1046 patients, 126 (12.05%) suffered from VTE recurrence during the follow-up. Primary end point was diagnosis for VTE (DVT or PE) during the follow up period. The follow up period (Mean ± SD, 3.9 ± 2.5) was counted in years and it started after stopping the anticoagulant treatment for the VTE diagnosed at inclusion until recurrence of VTE or the end of the study (December, 2008).

All patients were initially treated with unfractionated heparin (UFH) or low molecular weight heparin (LMH) during the initiation of oral anticoagulants (until INR (international normalized ratio) value is ≥ 2.0 for at least 5 days). The hospital treatment protocol recommends warfarin therapy for 3-6 months for first-time VTE with consideration of extended treatment in case of VTE recurrence. Thrombophilia was defined as presence of the factor V Leiden (FVL) mutation

(rs6025) or factor II G20210A mutation (rs1799963), or a level below the laboratory reference range of free protein S (women <0.5 kilo international unit (kIU)kIE/L, men <0.65 kIUE/L), protein C (<0.7 kIUE/L) or antithrombin (<0.82 kIEU/L) in patients without warfarin treatment. This study was approved by the ethical committee of Lund University and all the participants gave written permission for it.

Laboratory methods

DNA was isolated from whole blood using the QiAmp 96 DNA Blood Kit (Qiagen, Hilden, Germany). TaqMan® SNP Genotyping Assay was used for genotyping of THBD c.1418C>T polymorphism according to the manufacturer's instructions (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA). Briefly a PCR master mix was prepared for each sample as, Taqman gene specific assay (VIC and FAM probes for c.1418C>T polymorphism) 0.25µl, Taqman master mix 2.5µl and Deionized water 0.25µl. 3µl of this master mix was added to each well in 384 PCR plate followed by addition of 2µl DNA (5ng/µl). Plates were vortexed and centrifuged at 1000 rpm (revolutions per minute) for 30 seconds. Polymorphism analysis was performed by BioRad CFX384 real-time PCR (1000 Alfred Nobel Drive Hercules, California 94547 USA) according to manufacturer's instructions with following temperature conditions, 95°C for 10 minutes followed by 40x (92°C for 15 sec, 60°C for 1min). Different alleles of polymorphism were determined by

BioRad CFX manager software. TaqMan allele discrimination assays (Applied Biosystems) was used for DNA mutations analysis in FVL mutation and factor II G20210A as described previously [30]. Protein C levels were analyzed by a chromogenic method using the Berichrom® Protein C reagent (Siemens Healthcare Diagnostics, Upplands Väsby, Sweden) [31]. Analysis of free Protein S was done by latex immunoassay with Coamatic® Protein S-Free (Chromogenix, Haemochrom Diagnostica AB, Gothenburg, Sweden) [32]. Thrombin-based method using Berichrom Antithrombin (Siemens Healthcare Diagnostics) was used for

antithrombin analysis [33]. All the analyses were performed by using a BCS-XP coagulation analyzer (Siemens Healthcare Diagnostics).

Statistical analysis

SPSS version 21 (IBM, Armonk, NY, USA) was used for statistical measurements. Continuous variable were compared by Kruskal-Wallis H or Mann-Whitney *U* test. Dichotomous variables were compared by Chi-square test. Survival curves for time to recurrent VTE by THBD genotypes are presented and the log-rank test was used to compare recurrence-free survival between genotypes. Univariate and multivariate analyses, adjusting for age, sex, and thrombophilia were performed using Cox proportional hazards models and hazard ratios with 95% confidence intervals (CI) were calculated for each group of patients**.** Multivariate analyses were performed as sensitivity analyses by including all VTE patients with exception for those who had had one or more thrombotic event before inclusion. The follow-up period was calculated from time of inclusion and was adjusted for the duration of anticoagulation treatment.

Results:

Of the total 1465 patients, those who had had one or more thrombotic events before inclusion (n=154) were excluded. Baseline characteristics of the remaining patients (n=1311) are summarized in Table 1. THBD c.1418C>T polymorphism has 3 different genotypic forms, CC is homozygous wild type, CT is heterozygous and TT is homozygous mutated form. The genotypes containing T allele (CT and TT) were combined in the statistical analysis to compare with the homozygous CC genotype, which was used as reference. No significant difference was found in allelic frequencies of C and T between non-recurrent and recurrent VTE patients. 41% patients with FVL mutation had VTE recurrence compared to 29% without recurrence. There was no significant difference observed among recurrent and non-recurrent VTE patients in age, sex, body mass index (BMI), deep vein thrombosis (DVT), pulmonary embolism (PE), protein S, protein C and antithrombin deficiency ($p > 0.05$). To examine the distribution of THBD genotypes, DNA samples for genotyping were available for 1300 samples and THBD c.1418C>T polymorphism (CC, CT and TT) was thus analyzed in these patients. No significant difference was found between the distribution of THBD genotypes in age, sex, BMI, malignancy and the other variables tested as shown in Table 2.

Thrombomodulin polymorphism and risk of VTE recurrence

For the recurrence analysis, the patients who had recurrence or died during anticoagulant treatment were excluded (n=265) and analyses were performed on the remaining 1046 patients in which 126 (12.05%) had VTE recurrence. The aim of our study was to investigate the risk of VTE recurrence and patients were only followed after the anticoagulant treatment that was initiated at inclusion in the prospective population based study (MATS, see above) was stopped. Therefore, only those patients who had completed the scheduled period of anticoagulation treatment without experiencing VTE recurrence during treatment were included in this study.

A survival analysis by Kaplan-Meier curve was performed to determine whether THBD polymorphism influences recurrence-free survival. Patients having C and T allele were compared and no significant difference in recurrence-free survival (Figure 1, Log-rank test, p =0.55) was found.

Univariate Cox regression analysis showed no significant association between THBD polymorphism and risk of VTE recurrence (HR =1.15 and 95% CI = 0.80-1.66, p =0.439 and HR =0.79, 95% CI = 0.29-2.15, p =0.638 for CT and TT respectively where the CC genotype was used as reference). Multivariate Cox regression analysis including age, sex, THBD polymorphism and thrombophilia showed that only thrombophilia (HR = 1.67 and 95% CI = 1.17-2.37, p =0.005) was significantly associated with higher risk of VTE recurrence. Similar results were found when we combined the T containing alleles (CT and TT) and examined their association with risk of VTE recurrence in univariate [Hazard ratio (HR) =1.11, 95% confidence interval (CI) = 0.78-1.59, p = 0.55] as well as in multivariate analysis adjusted for age, sex and thrombophilia (HR=1.11, 95% CI= 0.78-1.59, p =0.54) (Table 3). Furthermore, a sensitivity analysis was performed for all patients except among those who had had VTE before inclusion. The follow up time was calculated from time of inclusion for this study and was adjusted for duration of anticoagulant treatment divided into 4 categories (3, 6, 12 and >12 months). Multivariate analyses were performed and THBD c.1418C>T polymorphism was not significantly associated with the risk of VTE recurrence (Table 1 in the Supplementary Appendix).

We also performed a sub-analysis on patients with unprovoked first VTE (n=614) in order to investigate the role of THBD in these high-risk patients. Our results showed no significant association between THBD polymorphism and risk of VTE recurrence in unprovoked first VTE either (HR=1.22 and 95% CI = 0.78-1.89, p =0.38).

Discussion:

THBD c.1418C>T polymorphism has previously been shown to be involved in amino acid change from Alanine to Valine (abbreviated as Ala455Val) [25]. In previous studies, this polymorphism has been investigated in coronary heart diseases and primary VTE [18, 19, 22, 25]. However, its role in VTE recurrence remained to be elucidated. Therefore, in this study we investigated the role of THBD c.1418C>T polymorphism in VTE recurrence and found that THBD c.1418C>T polymorphism is not significantly associated with risk of VTE recurrence. Multivariate models including thrombophilia and THBD genotypes showed that only thrombophilia was significantly associated with a higher risk of VTE recurrence.

In contrast to our findings, Navarro S *et al.*, reported a protective role of THBD c.1418C>T polymorphism in primary VTE. Navarro S *et al.*, also reported a significant association of THBD c.1418C>T polymorphism with lower levels of soluble THBD in plasma in primary VTE [18]. Similarly, Sugiyama S *et al.*, showed a significant association of the T allele of THBD c.1418C>T polymorphism with DVT in the Japanese population [34] while others have reported a protective role for the T allele in primary DVT [18].

However, beside these findings, a prospective study on primary VTE did not find any association between THBD c.1418C>T polymorphism and primary VTE [27]. Similar results were also shown in case control studies in which THBD c.1418C>T polymorphism was not found to be associated with risk of primary VTE [25, 26, 28, 34].

These results show that the role of THBD c.1418C>T polymorphism in primary VTE is controversial. However, in recurrent VTE, we did not find any evidence of an association between THBD c.1418C>T polymorphism and risk of VTE recurrence but further studies are needed on this under-investigated topic.

THBD c.1418C>T polymorphism has also been reported to be associated with risk of primary VTE in patients who had no acquired risk factors (Unprovoked VTE) [18]. We also analysed

the role of THBD polymorphism in VTE recurrence in patients with unprovoked VTE. However, our results showed that THBD c.1418C>T polymorphism has no association with risk of recurrence in unprovoked VTE either. A possible explanation for these results could be due to the fact that risk factors associated with primary VTE may not predict the risk of VTE recurrence [35-37].

A possible limitation of our study is that it was limited by a relatively small number of patients with mutant homozygous TT genotype. TT genotype was found to be protective for VTE recurrence but did not reach to statistical significance, which may be due to a lower frequency. Thus it cannot be ruled out that this polymorphism could be associated with risk of recurrence.

In conclusion, the present study is, to the best of our knowledge, the first study in which the association between THBD c.1418C>T polymorphism and VTE recurrence is analyzed. Our results indicate that THBD c.1418C>T polymorphism is not associated with VTE recurrence and therefore further studies are warranted.

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Conflict of Interest

Authors declare no conflict of interest.

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Legend to figure

Figure 1: Survival curve representing the different genotypes in THBD c.1418C>T polymorphism and their association with risk of VTE recurrence. p =log-rank test.

Table 1. Characteristics of studied population including the distribution of THBD c.1418C>T genotypes in recurrent and non-recurrent VTE patients.

	Mean (±SD) or %			
Parameters	**Non recurrent VTE n (%)**	**Recurrent VTE n (%)**	**Total n (%)**	**¶p-value**
Genotype				
CC	698 (60.5)	87 (59.2)	785 (60.4)	0.788
CT & TT	455 (39.5)	60 (40.8)	515 (39.6)	
Sex				
Male	565 (48.6)	78 (52.7)	643 (49.0)	0.383
Female	598 (51.4)	70 (47.3)	668 (51.0)	
Age at inclusion				
Years (Mean±SD)	62.9±17.5	61.3±15.3	62.7±17.3	0.087*
BMI				
Mean±SD	26.6±4.7	27.4±5.1	26.6±4.8	0.110*
FVL mutations				
Yes	330 (28.5)	60 (40.8)	390 (29.9)	0.002
No	829 (71.5)	87 (59.2)	916 (69.9)	
Factor II mutations				
Yes	39 (3.9)	9 (7.0)	48 (4.2)	0.104
No	969 (96.1)	120 (93.0)	1089 (95.8)	
DVT+PE				
DVT	736(68.2)	98 (68.5)	834 (68.2)	0.323
PE	277 (25.7)	32 (22.4)	309 (25.3)	
DVT+PE	66 (6.1)	13 (9.1)	79 (6.5)	
Malignancy				
Yes	140 (12.1)	13 (8.8)	153 (11.7)	0.278
No	1020 (87.9)	135 (91.2)	1155 (88.3)	
Protein C deficiency				
Yes	16 (1.6)	0 (0.0)	16 (1.4)	0.242
No	1009 (98.4)	1136 (100.0)	1145 (98.6)	
Protein S deficiency				
Yes	20 (2.0)	1 (0.7)	21 (1.8)	0.499
No	998 (98.0)	135 (99.3)	1133 (98.2)	
Antithrombin deficiency				
Yes	12 (1.2)	1 (0.7)	13 (1.1)	0.726
No	1013 (98.8)	135 (99.3)	1148 (98.9)	

DNA was not enough for genotyping in 11 samples, DVT, deep vein thrombosis; PE, pulmonary embolism; BMI, body mass index. P-value, Chi square test until unless indicated, *Mann-Whitney *U* test, ¶comparing non-recurrent with recurrent VTE.

Table 2. Distribution of different genotypes of THBD c.1418C>T polymorphism in studied population.

	THBD Genotypes [Mean (±SD) or n (%)]				
Parameters	**CC n (%)**	**CT n (%)**	**TT n (%)**	**Total n (%)**	**¶p-value**
Sex					
Male	386 (49.2)	232 (50.2)	20 (37.7)	638 (49.1)	0.228
Female	399 (50.8)	230 (49.8)	33 (62.3)	662 (50.9)	
Age at inclusion					
Mean±SD	62.8±17.2	62.3±17.5	64.9±16.6	62.7±17.3	0.587*
BMI					
Mean±SD	26.7±4.8	26.7±4.8	25.7±4.0	26.6±4.8	0.335*
FVL mutations					
Yes	236 (30.1)	139 (30.1)	14 (26.4)	389 (29.9)	0.862
No	549 (69.9)	323 (69.9)	39 (73.6)	911 (70.1)	
Factor II mutations					
Yes	26 (3.8)	19 (4.8)	3 (6.3)	48 (4.3)	0.477
No	661 (96.2)	375 (95.2)	45 (93.8)	1081 (95.7)	
DVT+PE					
DVT	495 (67.9)	299 (69.2)	33 (66.0)	827 (68.3)	0.147
PE	189 (25.9)	101 (23.4)	17 (34.0)	307 (25.4)	
DVT+PE	45 (6.2)	32 (7.4)	0 (0.0)	77 (6.4)	
Malignancy					
Yes	84 (10.7)	62 (13.4)	6 (11.5)	152 (11.7)	0.352
No	700 (89.3)	399 (86.6)	46 (88.5)	1145 (88.3)	
Protein C deficiency					
Yes	10 (1.4)	6 (1.5)	0 (0.0)	16 (1.4)	1.0
No	692 (98.6)	395 (98.5)	50 (100.0)	1137 (98.6)	
Protein S deficiency					
Yes	15 (2.2)	3 (0.8)	2 (4.0)	20 (1.7)	0.06
No	682 (97.8)	396 (99.2)	48 (96.0)	1126 (98.3)	
Antithrombin deficiency					
Yes	6 (0.9)	6 (1.5)	0 (0.0)	12 (1.0)	0.631
No	696 (99.1)	395 (98.5)	50 (100.0)	1141 (99.0)	
Recurrent VTE					
Yes	87 (11.1)	56 (12.1)	4 (7.5)	147 (11.3)	0.642
No	698 (88.9)	406 (87.9)	49 (92.5)	1153 (88.7)	

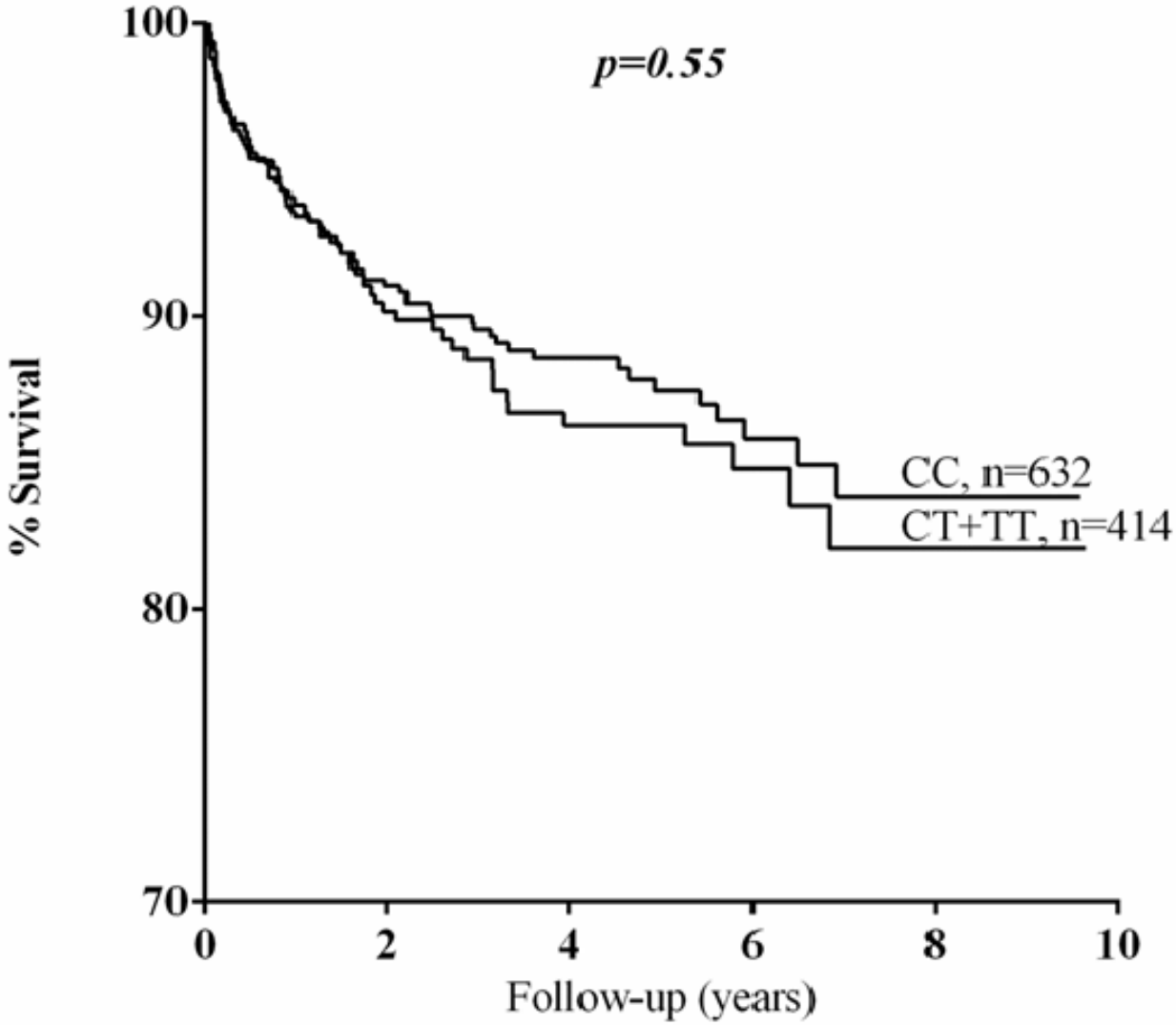
DVT, deep vein thrombosis; PE, pulmonary embolism; BMI, body mass index. P-value, Chi square test until unless indicated, * Kruskal-Wallis H Test, ¶comparing different genotypes of THBD c.1418C>T polymorphism.

Table 3. Uni- and multivariate analysis of THBD c.1418C>T polymorphism in recurrent VTE patients

	Univariate	p	Multivariate	p†
THBD c.1418C>T genotypes	HR (95% CI)		HR (95% CI)	
CC	Reference		Reference	
CT	1.15 (0.80-1.66)	0.439	1.15 (0.80-1.66)	0.438
TT	0.79 (0.29-2.15)	0.638	0.81 (0.29-2.21)	0.677
CT and TT	1.11 (0.78-1.59)	0.55	1.11 (0.78-1.59)	0.54
Thrombophilia* (yes/no)	1.65 (1.17-2.34)	0.005	1.67 (1.17-2.37)	0.005

*Thrombophilia: FVL and Factor II mutations, protein S, protein C and antithrombin deficiency. †Adjusted for age, sex and thrombophilia.

Figure 1


100
90
80
70
% Survival
p=0.55
CC, n=632
CT+TT, n=414
0
2
4
6
8
10
Follow-up (years)

Supplementary Table 1. Multivariate analyses of THBD c.1418C>T polymorphism in recurrent VTE patients with follow up from time of inclusion for this study and adjusting for duration of warfarin treatment.

THBD c.1418C>T genotypes	Model 1: adjusted HR (95% CI)	p	Model 2: adjusted HR (95% CI)	p
CC	Reference		Reference	
CT	1.17 (0.82-1.68)	0.391	1.17 (0.81-1.68)	0.404
TT	0.81 (0.30-2.22)	0.683	0.82 (0.30-2.26)	0.707
CT and TT	1.13 (0.80-1.61)	0.488	1.13 (0.79-1.61)	0.495
Thrombophilia* (yes/no)	1.65 (1.16-2.34)	0.005	1.66 (1.17-2.37)	0.005

*Thrombophilia: FVL and Factor II mutations, protein S, protein C and antithrombin deficiency, Model 1 adjusted= adjusted for duration of warfarin treatment, Model 2 adjusted= Adjusted for duration of warfarin treatment, age, sex and thrombophilia.