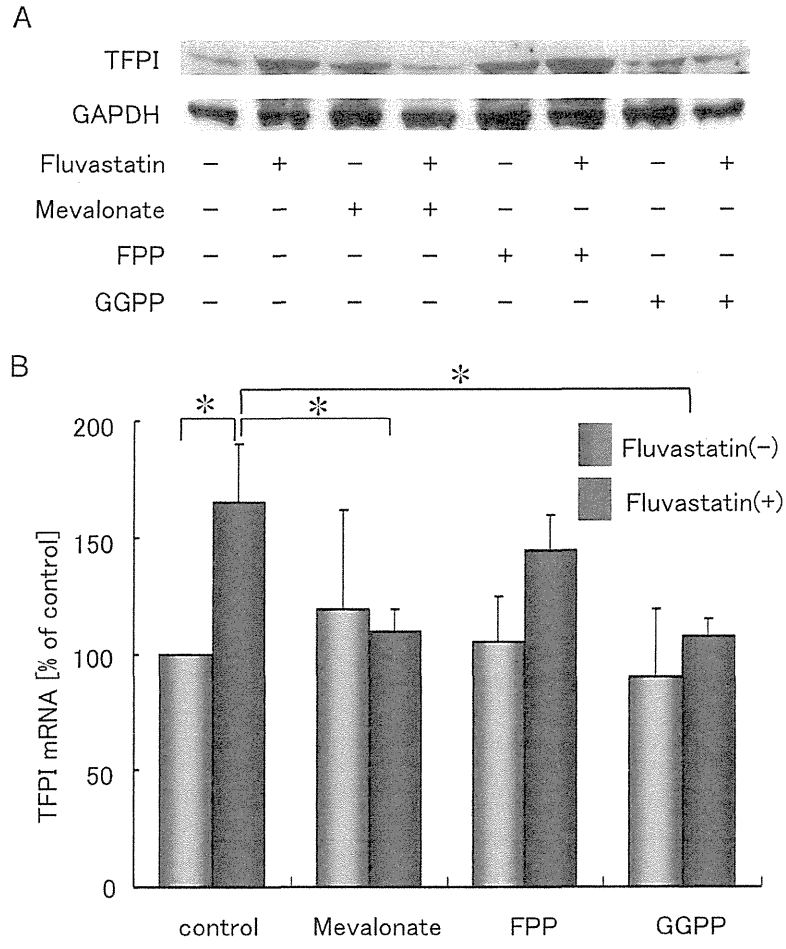

Fig. 2. Fluvastatin-induced upregulation is reversed by mevalonate and GGPP, but not FPP.

HUVECs were incubated with mevalonate (200 μM), FPP (10 μM), or GGPP (10 μM) in the presence or absence of fluvastatin (1 μM) for 24 h. mRNA and protein levels were determined by RT-PCR (B) and western blotting (A), respectively. The data represent the mean ± SD of 3 separate experiments. $^*p<0.05$ vs. control.

Results

Fluvastatin Upregulates TFPI Antigen and mRNA Expression in HUVECs

Treatment of HUVECs with fluvastatin (0.1–10 μM) for 24 h significantly increased TFPI antigen levels in a concentration-dependent manner (**Fig. 1A**). The increase of TFPI antigen levels was accompanied by the increased expression of TFPI mRNA, which showed a 2-fold increase compared to untreated cells after treatment with 1 μM fluvastatin for 24 h ($p<0.01$, **Fig. 1B**). Treatment of HUVECs with 10 μM fluvastatin for 24 h did not affect their viability (data not shown).

Fluvastatin Upregulates TFPI Expression Through Inhibition of the Mevalonate Pathway

To clarify further whether the induction of TFPI expression by fluvastatin was mediated by inhibition of the mevalonate pathway, we incubated HUVECs with mevalonate (200 μM), FPP (10 μM), or GGPP (10 μM) in the presence or absence of fluvastatin (1 μM) for 24 h. As shown in **Fig. 2**, treatment of HUVECs with mevalonate and GGPP reversed the induction of TFPI antigen (**Fig. 2A**) and mRNA (165 ± 25 vs. 110 ± 10%, $p<0.05$ and 165 ± 25 vs. 108 ± 7%, $p<0.05$, respectively, **Fig. 2B**). In contrast, FPP did not significantly alter the effect of fluvastatin on TFPI induction (165 ± 25 vs. 145 ± 15%, **Fig. 2B**).

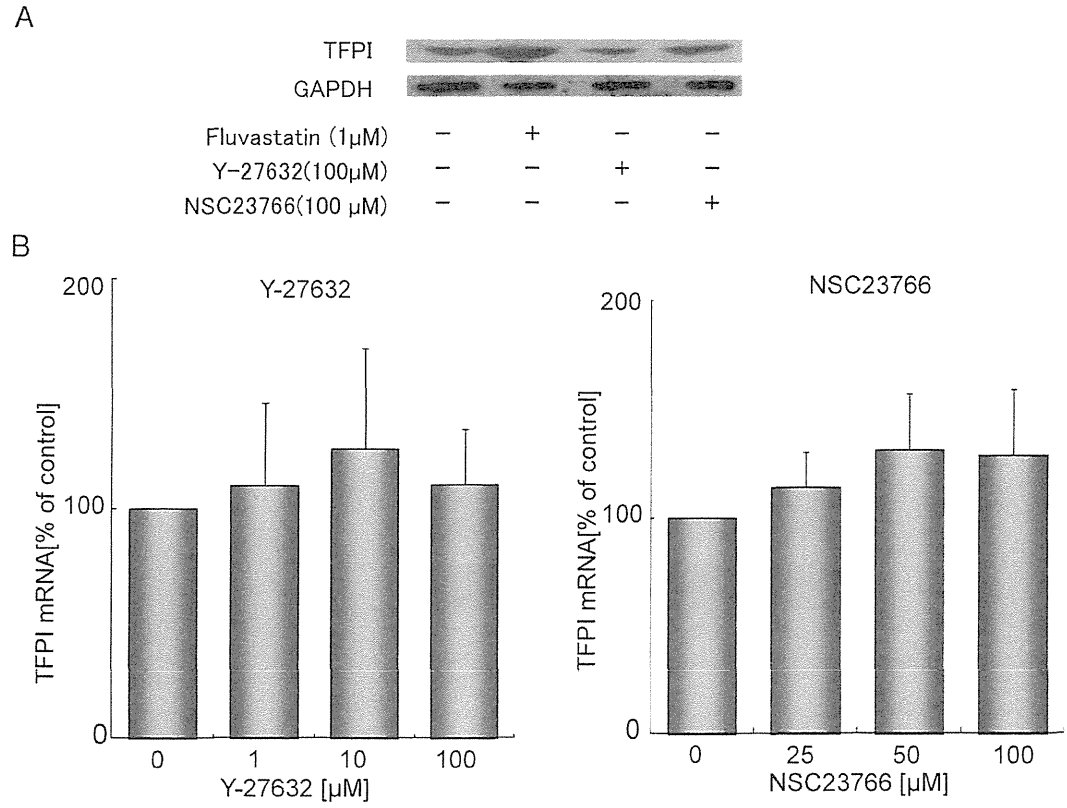

Fig. 3. Y-27632 and NSC23766 have no effect on the fluvastatin-induced upregulation of the TFPI expression in HUVECs.

HUVECs were incubated with Y-27632 (1–100 μM) or NSC23766 (25–100 μM) for 24 h. After incubation, mRNA and protein levels were determined by RT-PCR (B) and western blotting (A), respectively. The data represent the mean ± SD of 3 separate experiments.

Rho and Rac Inhibition are not Involved in Fluvastatin-Induced TFPI Expression

Since isoprenylation of the small GTP-binding proteins Rho and Rac is mediated by GGPP, we tested whether inhibition of these small GTP-binding proteins is involved in TFPI induction. Treatment of HUVECs with NSC23766 (25–100 μM) and Y-27632 (1–100 μM), specific inhibitors of Rac- and Rho-dependent kinases, respectively, showed no significant effect on TFPI protein (**Fig. 3A**) and mRNA (**Fig. 3B**) expression.

p38 MAPK, PKC, and PI3K Pathways are Involved in Fluvastatin-Induced TFPI Expression

We then examined the involvement of signal transduction pathways in the upregulation of TFPI expression by fluvastatin. Treatment of HUVECs with SB203580 (p38 MAPK inhibitor, 20 μM), LY294002 (PI3K inhibitor, 10 and 20 μM), and GF109203 (PKC inhibitor, 0.1 and 1 μM) reduced fluvastatin-induced TFPI expression (**Fig. 4A** and **B**). In contrast, U0126 (ERK1/2 inhibitor, 20 μM) and SP600125 (JNK inhibitor, 20 μM) did not have a significant effect (**Fig. 4A**). In addition, increased phosphorylation of p38 MAPK was detected after fluvastatin treatment of HUVECs (**Fig. 4C**).

Fluvastatin Upregulates TFPI Expression without Enhancing its Promoter Activity Through mRNA Stabilization

To confirm the effects of fluvastatin on TFPI gene transcription, we transfected EA.hy926 cells with the TFPI promoter construct. Luciferase assays did not show a significant increase of TFPI promoter activity after treatment of the cells with fluvastatin (0–10 μM) for 24 or 48 h (**Fig. 5A**). We also confirmed that treatment with fluvastatin for 48 h induced TFPI mRNA expression in EA.hy926 cells (1 μM: 1.5-fold, 10 μM: 2.0-fold, data not shown).

We next investigated whether fluvastatin-induced

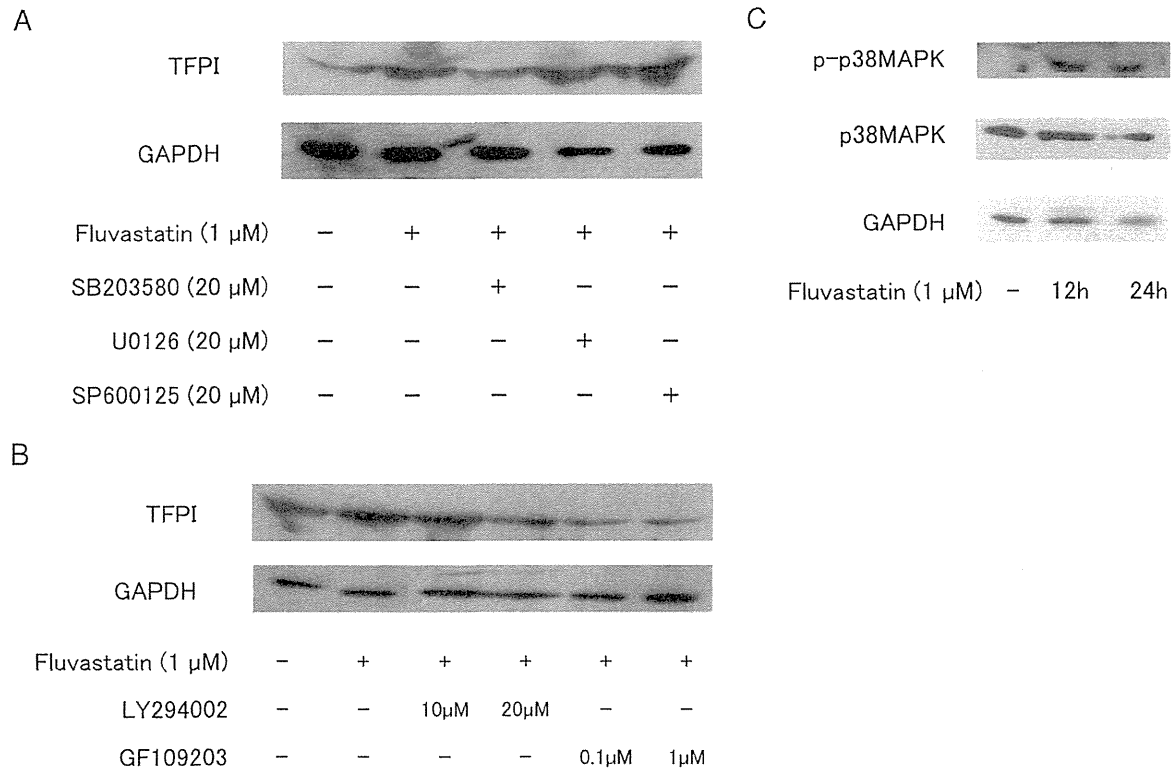

Fig. 4. Fluvastatin-induced TFPI expression is mediated via the activation of p38 MAPK, PKC, and PI3K in HUVECs.
A, HUVECs were incubated with fluvastatin (1 μM) in the presence or absence of SB203580 (p38 MAPK inhibitor, 20 μM), U0126 (ERK1/2 inhibitor, 20 μM), or SP600125 (JNK inhibitor, 20 μM) for 24 h; and B, GF109203 (PKC inhibitor, 0.1, 1 μM) or LY294002 (PI3K inhibitor, 10, 20 μM) for 24 h. After incubation, protein levels were determined by western blotting. C, HUVECs were incubated with fluvastatin (1 μM) for 12 or 24 h. Total or phosphorylated p38 MAPK was determined by western blotting.

TFPI expression was regulated via post-transcriptional mechanisms. The stability of TFPI mRNA was estimated by quantitating TFPI mRNA at various time points after the administration of a transcriptional inhibitor, actinomycin D (AD; 1 μg/mL), in the presence or absence of fluvastatin (1 μM). The amount of TFPI mRNA decreased gradually from 4 h after AD administration. In contrast, the amount of TFPI mRNA in the presence of fluvastatin remained steady (Fluvastatin + AD: 93 ± 9%, AD alone: 70 ± 6%, $p < 0.05$, **Fig. 5B**).

Discussion

Fluvastatin is reported to penetrate into vascular walls more effectively than other statins[13]. Previous reports have described that among all statins currently available, only fluvastatin interferes with proliferation of arterial smooth muscle cells at therapeutic levels[13, 14]. Therefore, we selected fluvastatin for use in this study, in combination with cultured human endothelial cells. We adjusted the experimental concentration of fluvastatin to 0.1–10 μM in consideration of the maximum blood concentration currently used (0.5–1 μM). It was demonstrated that fluvastatin upregulates TFPI expression in HUVECs. Moreover, some of underlying mechanisms of TFPI induction were identified. This is the first evidence to show the direct contribution of statins to TFPI expression. Together with the previously described anticoagulant effect of statins, their effect on TFPI induction seems to be a prospective therapeutic application for thrombotic diseases.

Our data showed that the fluvastatin-induced upregulation of TFPI was mediated by inhibition of the mevalonate and GGPP pathways. Nevertheless, it was shown that downstream small GTP-binding proteins, Rho and Rac, were not involved in the regulation of TFPI expression. Thus, inhibiting the remaining small GTP-binding protein, Cdc42, may contribute to TFPI induction by statins. Furthermore, it was

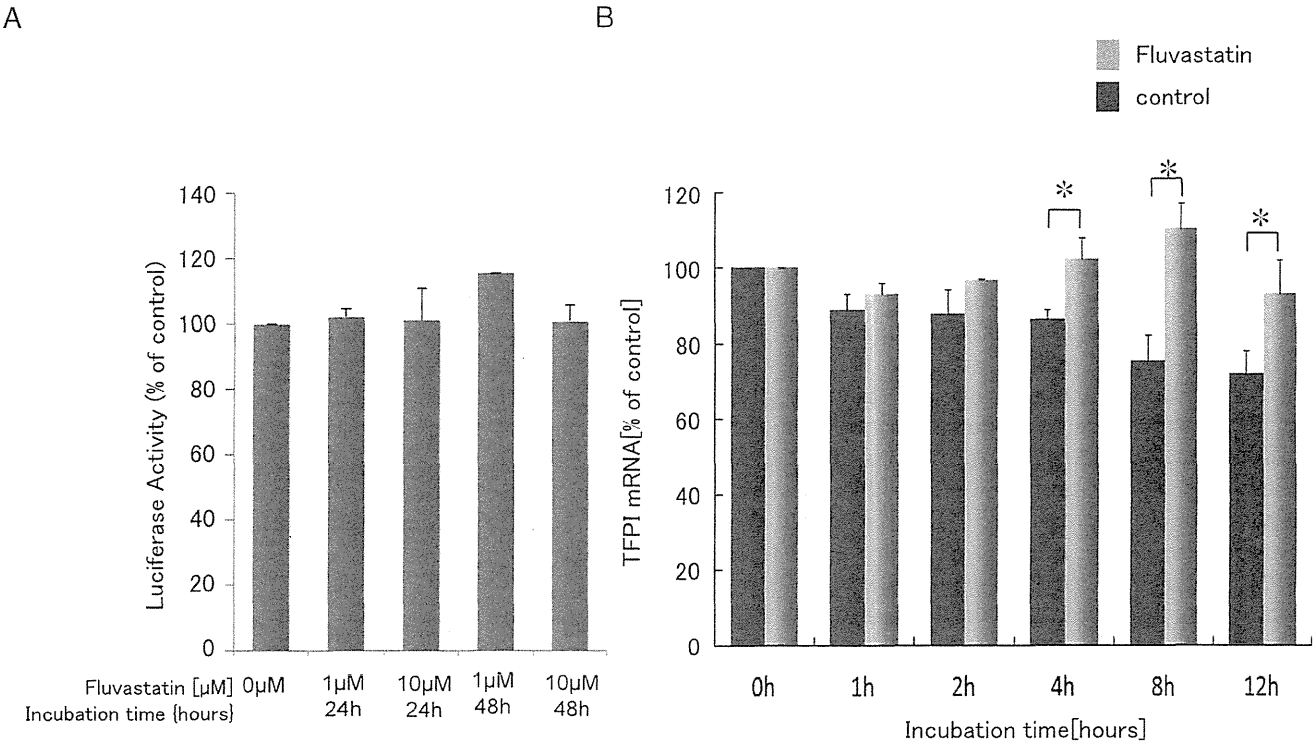

Fig. 5. Fluvastatin has no effect on the TFPI promoter activity in transiently transfected EA.hy926 cells and stabilizes TFPI mRNA in HUVECs.

A, EA.hy926 cells were transfected with a luciferase reporter gene construct containing the 5′-flanking region of the TFPI gene and were incubated with fluvastatin (0–10 μM) for 24 or 48 h. The data represent the mean ± SD of 5 separate experiments. B, HUVECs were incubated with fluvastatin (1 μM) for 24 h, and then actinomycin D (AD; 1 μg/mL) was added alone or with fluvastatin. The cells were harvested at the indicated time points and TFPI mRNA levels were determined by RT-PCR. The data represent the mean ± SD of 5 separate experiments. $^{*}p < 0.05$ vs. control.

suggested that activation of p38 MAPK, but not ERK or JNK, was involved in the upregulation of TFPI by fluvastatin. The statin-induced phosphorylation of p38 MAPK was demonstrated in some reports[15, 16]. Conversely, other reports described that statins showed their pleiotropic effects by attenuating the increased phosphorylation of p38 MAPK[17, 18]. The effect of statins on p38 MAPK phosphorylation may differ according to cell type and pretreatment condition of the cells. In our experiments, treatment of HUVECs with fluvastatin increased p38 phosphorylation; in addition, inhibition of p38 MAPK by SB203580 reversed the induction of TFPI by fluvastatin, suggesting that p38 MAPK activation by fluvastatin was involved in TFPI upregulation. The induction of TFPI was also reversed by the presence of LY294002 and GF109203, indicating that fluvastatin increased TFPI expression via the activation of PI3K and PKC. It is well documented that statins upregulate endothelial nitric oxide (NO) production via the PI3K/Akt signaling pathway[19]. The PI3K/Akt signaling pathway has various roles including cell metabolism, apoptosis, and proliferation. Of those, some are mediated by the induction of NO production, while others are independent of NO[20, 21]. We confirmed that fluvastatin-mediated TFPI upregulation was not reversed by L-NAME, indicating that TFPI upregulation through PI3K/Akt activation is independent of NO production (data not shown).

The transcriptional activity of the TFPI promoter cloned in front of the luciferase gene was not altered by fluvastatin treatment. As explained in the Materials and Methods, our construct consisted of exon 1 of the TFPI gene and its 5′-flanking region, which contains three GATA-2, one SP-1, and two c-Mic binding sites[22]. Our results showed that the upregulation of TFPI by fluvastatin is not mediated by those transcription factors. Instead, fluvastatin treatment of HUVECs delayed the degradation of TFPI mRNA in the presence of actinomycin D, indi-

cating that fluvastatin increased TFPI synthesis by improving its mRNA stability. Indeed, mRNA stabilization by statins has been reported for various genes other than TFPI. Habara *et al.* reported that pitavastatin increases iNOS gene expression through stabilization of its mRNA in rat hepatocytes. The presence of its 3′-untranslated region (UTR) containing AU-rich elements (ARE, AUUU[U]A), which are associated with ARE-binding proteins such as HuR, played a key role in this stabilization[23]. According to the ARE database (http://brp.kfshrc.edu.sa/ARED/), the full-length TFPIα mRNA contains 17 AREs within its 3′-UTR, which may contribute to the regulation of TFPI expression. In contrast to TFPIα, TFPIβ mRNA expression seems to be less dependent on AREs since it has only 2 AREs within its 3′-UTR. Besides, the expression of TFPIβ is reportedly affected by the alternative splicing of exon 2 rather than AREs[24]. Since all three signaling pathways that contributed to TFPI induction in our study, p38 MAPK, PI3K/Akt, and PKC, reportedly have a role in ARE-mediated mRNA stabilization[25], the contribution of each signaling pathway to TFPI induction is unclear and still needs to be investigated.

Our luciferase reporter assay does not reflect the consequence of alternative splicing of exon 2 nor mRNA stabilization through the 3′-UTR. It might be worth investigating if fluvastatin regulates TFPI expression by affecting alternative splicing of the 5′- or 3′-regions of the TFPI gene. As described, TFPI has two major isoforms with different tissue distribution. Since the primers that we used to quantitate TFPI mRNA can bind to both TFPIα and -β mRNA, it is uncertain which of these isoforms was upregulated, and this still needs to be investigated.

The favorable point of the use of TFPI as an anticoagulant, as compared with other anticoagulant drugs currently in use, is that it targets TF-FVIIa and FXa, thereby contributing to the inhibition of the very early phase of clot formation. In this way, TFPI has been expected to become a prospective therapeutic agent for anticoagulation and endothelial protection. Nevertheless, several clinical trials of recombinant TFPI administration did not achieve favorable results[26, 27]. It is tempting to speculate that inducing endogenous TFPI in the endothelium and platelets by statins may inhibit clot formation more effectively than administering exogenous TFPI, as endothelial- and platelet-derived TFPI may have a role as an on-demand and locus-specific clotting inhibitor during the rupture of an atherosclerotic plaque. Previously reported downregulation of TF by statins may contribute synergistically with the induction of TFPI to prevent intravascular coagulation. Further studies including animal experiments are needed.

Conclusion

We demonstrated that fluvastatin increases TFPI expression in HUVECs by inhibiting the GGPP- and Cdc42-dependent signaling pathways and the activation of the p38 MAPK, PI3K, and PKC pathways. This study revealed unknown mechanisms underlying the anticoagulant effects of statins and gave a new insight to its therapeutic potential for the prevention of thrombotic diseases.

Conflicts of Interest

There are no conflicts of interest associated with this manuscript.

Acknowledgements

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Letter to the Editor-in-Chief

Late onset thrombosis in two Japanese patients with compound heterozygote protein S deficiency

Dear Editor,

Protein S (PS; gene symbol *PROS1*; MIM #176880) is a vitamin K-dependent plasma glycoprotein that acts as a nonenzymic cofactor for activated protein C (APC) in the degradation of activated coagulation factors V and VIII [1]. PS is a single-chain protein of 676 amino acids that consists of a γ-carboxyglutamic acid (Gla) domain, a thrombin-sensitive region (TSR), four epidermal growth factor (EGF)-like domains and a carboxy-terminal region homologous to sex hormone binding globulins (SHBG-like domain). Hereditary PS deficiency is an autosomal dominant disorder associated with venous thromboembolism (VTE) [2]. The prevalence of PS deficiency is 0.03-0.13% in the general Caucasian population [3], whereas it is estimated to be 1.12% in the Japanese population [4]. In particular, the Lys196Glu classified qualitative PS deficiency has been reported to be a polymorphism in the Japanese population, known as PS Tokushima [5]. A homozygous or a compound heterozygote mutation for *PROS1* gene has been reported in several families, but it is rare [6]. Generally, patients with a homozygote or compound heterozygote mutation for *PROS1* developed life-threatening thrombosis, for example, neonatal purpura fulminans. Two patients with late-onset thrombosis who had compound heterozygote PS deficiency of non PS Tokushima type, which is reported to have a high frequency in Japanese, are reported.

Proband 1 was a 14 year-old Japanese girl who suffered from deep vein thrombosis (DVT) from the right calf to the right common iliac vein and pulmonary embolism (PE) involving bilateral lungs (Fig. 1). She had been thrombosis-free for 14 years. Although the trigger of her thrombosis was unclear, dehydration due to exercise might cause development of thrombosis because she belonged to an ice hockey team. Plasma levels of antithrombin activity, protein C activity, PS activity (Staclot Protein S; Clot, Roche, Basel, Switzerland), total PS antigen (Asserachrom Total Protein S;Roche) and free PS antigen (STA(R)Liatest(R)Free Protein S; Roche) were 128%, 78%, <10%, 30%, and 8%, respectively. Tests were done while the patient was on warfarin therapy. Concentration of antiphospholipid antibodies (including anti-cardiolipin and anti-beta-2-glycoprotein I antibodies) were below the respective cut off values, and screening tests for lupus anticoagulant (LA) were negative. Her parents and her two brothers showed low PS activities (Fig. 2A). However, her family history was negative for thrombotic episodes, but her mother had three miscarriages and her younger brother has epilepsy.

Proband 2 was an 18-year-old Japanese man who suffered from cerebral venous sinus thrombosis. The trigger of thrombosis was unclear. Plasma levels of antithrombin activity, protein C activity, PS activity, total PS antigen and free antigen were 115%, 110%, <10%, 47% and 9% respectively. Concentration of antiphospholipid antibodies (including anti-cardiolipin and anti-beta-2-glycoprotein I antibodies) were below the respective cut off values, and screening tests for LA were not done. His father and younger brothers also had low PS activities (Fig. 2B). However, his family history was negative for thrombotic episodes.

After obtaining informed consent to identify mutations in the *PROS1* gene, genomic DNA was isolated from peripheral blood leukocytes and sequenced for all exon and exon/intron boundaries of *PROS1* gene. The DNA sequence analysis revealed that proband 1 was a compound heterozygote for missense and frameshift mutations. The c.757C > T substitution in exon 5 resulted in p.Ala139Val (Fig. 3A), previously reported in a Japanese patient who developed DVT [7]. Her mother and younger brother had this mutation. A novel c.2135delA in exon 14 is suggested to result in Asp599ThrfsX12 (Fig. 3B). Her father and older brother had this frameshift mutation. On the other hand, a heterozygous c.757C > T substitution that resulted in p.Ala139Val (Fig. 3C) was identified in proband 2, his mother, and younger brother. Since his father's PS activity was low, and both the proband and his brother had quite low PS activities, it was suspected that the proband and his brother had another paternal mutation in *PROS1* gene. However, DNA sequencing and multiplex ligation-dependent probe amplification (MLPA) analysis found no paternal mutation.

Two Japanese patients with hereditary PS deficiency were analyzed. In proband 1, compound heterozygote *PROS1* mutations were detected. On the other hand, although proband 2 was suspected to have compound heterozygote PS deficiency, only a maternal missense mutation was detected. Since the PS levels of proband 2 and his brother were lower than expected for a heterozygous mutation, and since their father had low PS activity, and low total and free PS antigen, it is possible that they have another mutation of *PROS1* which causes a quantitative PS deficiency. Further analysis to detect a mutation which is undetectable by direct sequencing and MLPA analysis (i.e. intron mutation resulting in aberrant splicing or a chromosomal inversion) remains to be investigated.

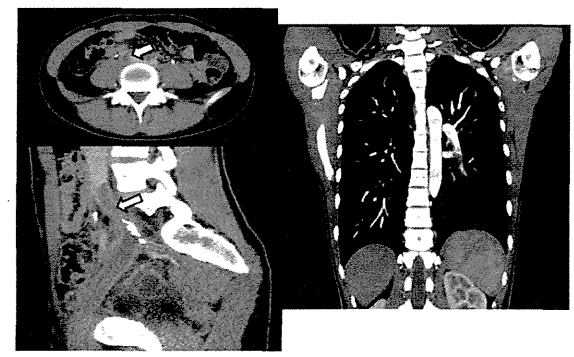

Fig. 1. Enhanced whole-body CT shows DVT from the right calf to the right common iliac vein with phleboliths in the right external iliac vein and PTE of bilateral lungs. Thrombi are marked by arrows.

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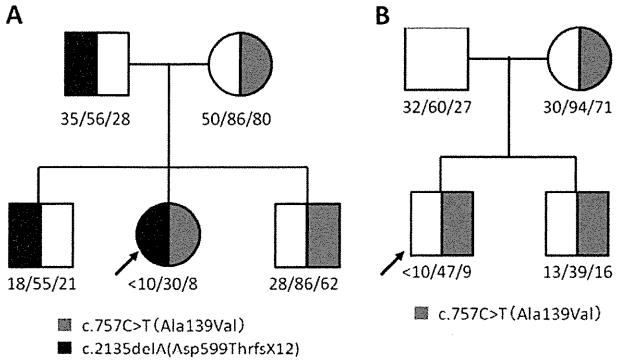

Fig. 2. Family pedigrees of probands 1 (A) and 2 (B). The arrows indicate the probands. The numbers are expressed in the order of PS activity (%), total PS antigen (%) and free PS antigen (%). Ala139Val is indicated in the pedigree as dark gray and Asp599ThrfsX12 as black.

In the present patients, one recurrent and one novel mutation in the *PROS1* gene causing hereditary PS deficiency were detected. The residue Ala139 is highly conserved among PS of different mammalian species such as chimpanzee, dog, cow, and mouse, which suggests that this residue is important for the conformation or function of PS. Both Ala and Val are nonpolar and hydrophobic amino acids, so that Ala139Val mutation might cause slight structural changes. The Ala139 residue is located in the EGF1 domain, which interacts with APC directly and has an important role of APC cofactor activity [8]. Moreover, Bruno et al. showed that Lys138 is in contact with the Gla module via Gla36, which may contact the membrane surface [9]. We hypothesized that Ala139Val mutation may influence the interaction of PS-APC or affect PS membrane binding by interruption of the Gla-EGF interaction. Proband 1's mother and younger brother and proband 2's mother had Ala139Val mutation heterozygously. The plasma levels of their PS activities were low (proband 2's mother's activities were within normal limits), and the plasma levels of their total PS antigen were normal.

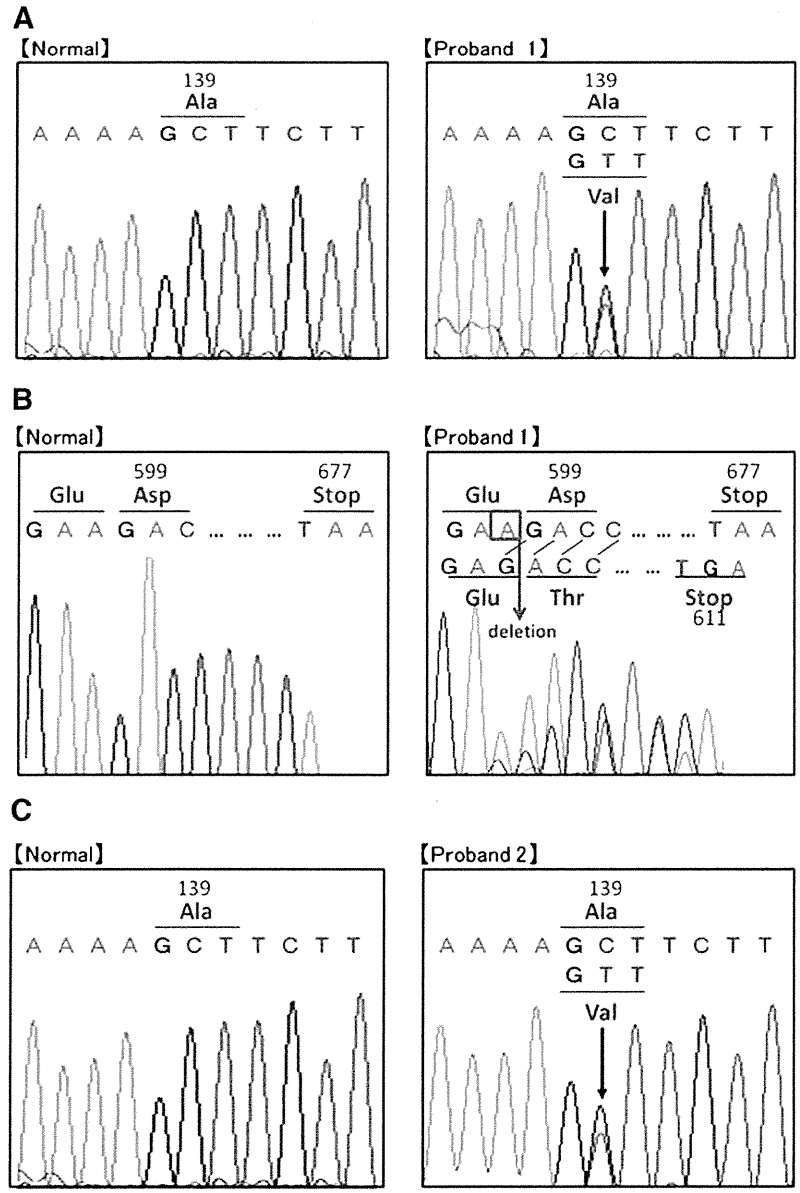

Fig. 3. DNA sequence analysis of *PROS1* of the probands. (A) *PROS1* exon 5 of proband 1. The c.757C>T resulted in Ala139 being replaced by Val. (B) *PROS1* exon 14 of proband 1. The upper is the normal allele, and the lower is the mutant allele. The c.2135delA disrupts the normal reading frame and results in a premature stop codon at +611. (C) *PROS1* exon 5 of proband 2. The c.757C>T resulted in Ala139 being replaced by Val.

Given these findings, it appeared that there was almost normal secretion of PS Ala139Val, but there was loss of APC cofactor activity, suggesting that missense mutation may induce qualitative PS deficiency.

The novel c.2135delA mutation in exon 14 caused a frameshift of 67 amino acids upstream from the termination codon, which generated an aberrant amino acid sequence +599 to +610 and, thus, finally led to a stop codon at +611, which led to Asp599ThrfsX12 (Fig. 3B). The deletion changes the amino acid sequence EDLQRQLAVLDKMK to ETFKDNLPSWTKQstop, and it is predicted to result in a truncated PS lacking the C-terminal 66 residues. This mutation probably resulted in the transcription of abnormal mRNA that contains a premature stop codon and the synthesis of a short aberrant polypeptide. If the translation product of the abnormal mRNA is synthesized, the short aberrant polypeptide might be unstable and degraded by the cellular proteolysis system. Since this mechanism would cause low PS antigen and activity, c.2135delA mutation may result in a quantitative PS deficiency.

This case report described two patients with late onset thrombosis who were suspected to have compound heterozygote PS deficiency. In general, compound heterozygote PS deficiency results in neonatal purpura fulminans or severe thrombosis in early childhood. However, both probands remained free of thrombosis until they became teenagers. An Ala139Val mutation was identified in a Japanese DVT patient with a decrease in PS activity to 28% [7]. We also previously encountered a man with PS activity of 30% who developed DVT at age 41 years. Including present cases, this mutation has been reported in at least four cases with DVT in Japanese, although there are no reports in other countries. Thus, this mutation might be common in Japan. Despite proband 1's and proband 2's mother being heterozygotes for this mutation with PS activities of 50% and 30%, respectively, they were all asymptomatic. Therefore this mutation might have a mild phenotype, although Ala139Val mutation causes molecular abnormalities of PS. The present patients had compound heterozygote mutations for *PROS1*, but thrombosis did not develop until their teenage years. The reason why they presented with late-onset thrombosis is probably because one of which was the Ala139Val mutation with a mild phenotype.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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ORIGINAL ARTICLE

A donor thrombomodulin gene variation predicts graft-versus-host disease development and mortality after bone marrow transplantation

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Abstract Thrombomodulin, encoded by the *THBD* gene, is a critical regulator of coagulation and innate immunity. Its gene variant (rs3176123, 2729A>C) in the 3′ untranslated region has been reported to be associated with vasculopathies. The present study analyzed the impact of *THBD* variation on transplant outcomes in a cohort of 317 patients who underwent unrelated HLA-matched bone marrow transplantation (BMT) for hematologic malignancies through the Japan Marrow Donor Program. The donor A/C or C/C genotype vs. the donor A/A genotype resulted in a lower incidence of grades II–IV acute graft-versus-host disease [GVHD; hazard ratio (HR) 0.66; 95 % confidence interval (CI) 0.44–0.99; $P = 0.05$] according to a multivariate analysis. In patients with grades II–IV acute GVHD, the donor A/C or C/C genotype vs. the donor A/A genotype was associated with significantly better overall survival rates (HR 0.45; 95 % CI 0.21–0.99, $P = 0.05$), while this effect was absent in other patients. A functional analysis using lymphocytes obtained from healthy individuals revealed that the 2729C allele has a higher level of *THBD* mRNA than the 2729A allele. These findings suggest the functional relevance of the rs3176123 variation and indicate that higher thrombomodulin expression by individuals with the 2729C allele likely accounts for their decreased risk for acute GVHD development and subsequent mortality.

For the Japan Marrow Donor Program.

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Keywords *THBD* · Unrelated donor · Bone marrow transplantation · Single nucleotide variation · Graft-versus-host disease

Introduction

The *THBD* gene encodes thrombomodulin (TM), also referred to as CD141 or blood dendritic cell antigen (BDCA) 3, which is expressed on endothelial cells, keratinocytes, osteoblasts, monocytes, neutrophils, and dendritic cells (DCs) [1–4]. TM functions as a cofactor to activate protein C, which leads to endothelial thromboresistance, and also plays pivotal roles in cytoprotection by regulating inflammation. TM binds to deactivate high-mobility group protein box 1 protein (HMGB1), a key mediator of inflammation that is mainly secreted by macrophages, monocytes and DC, and stimulates the production of inflammatory cytokines, such as interleukin (IL) 1β, IL-6 and tumor necrosis factor alpha (TNF-α) [4, 5]. Two types of TM have been found: namely, membrane-bound TM and soluble TM circulating in the blood. A form of soluble TM that lacks protein C cofactor activity may be present, even in a form in which the anti-inflammatory property is maintained [6].

Graft-versus-host disease (GVHD) is a main cause of mortality and morbidity after allogeneic hematopoietic stem cell transplantation (HSCT). Several studies have showed the association among the increasing levels of soluble TM, the endothelial damage, and the disease activity of GVHD [7–14]. Treatment with recombinant soluble TM, which was approved for the treatment of disseminated intravascular coagulation in Japan in 2008 [15], resulted in the disappearance of GVHD manifestations along with decreasing plasma levels of HMGB1 in a patient with steroid-refractory acute GVHD [16], thus suggesting that soluble TM may ameliorate GVHD through the suppression of inflammatory mediators.

A single nucleotide variation of the *THBD* gene, rs3176123 (2729A>C), is associated with susceptibility to coronary arterial diseases, deep vein thrombosis, placental abruption, and acute lung injury in Caucasian and Asian populations [17–23]. A recent study using samples from the recipients [21] showed that rs1042579, which was in complete linkage disequilibrium with rs3176123 of the *THBD* gene, predicted the mortality in the patients who developed acute GVHD. We herein investigate the impact of the donor and recipient 2729A>C variations in the *THBD* gene on the clinical outcomes of the patients who underwent allogeneic bone marrow transplantation (BMT) using HLA allele-matched unrelated donors through the Japan Marrow Donor Program (JMDP). We find that the donor 2729A allele is associated with a significantly higher incidence of grades II–IV acute GVHD, as well as inferior survival rates for the patients who developed grades II–IV acute GVHD. The present study also shows that the 2729A allele correlates with a lower expression of the *THBD* mRNA.

Patients and methods

Patients

THBD genotyping was performed on 317 transplantation recipients with hematological malignancies and their unrelated donors who underwent BMT through the JMDP with T cell-replete marrow from HLA-A, -B, -C, -DRB1, -DQB1 and -DPB1 allele-matched donors between January 2006 and December 2009. All available data and samples for eligible patients and their donors were analyzed. The study cohort included Asian patients only and excluded patients who had a history of any prior transplantation. The final clinical survey of these patients was completed by 1 September 2014. The diagnoses included acute myeloid leukemia (AML) in 153 patients (48 %), acute lymphoblastic leukemia (ALL) in 67 patients (21 %), chronic myeloid leukemia (CML) in 12 patients (4 %), myelodysplastic syndrome (MDS) in 53 patients (17 %) and malignant lymphoma (ML) in 32 patients (10 %) (Table 1). The median follow-up duration in the cohort was 1034 days in the survivors (range 49–1918 days), and 58 recipients

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Table 1 Donor and recipient characteristics

Variable	No.	Ratio (%)
No. of cases	317	
Recipient age, years		
Median	48	
Range	1–67	
Donor age, years		
Median	34	
Range	25–66	
Year of transplant		
Median	2008	
Range	2006–2009	
Recipient *THBD* genotype		
A/A	172	54
A/C	114	36
C/C	31	10
Donor *THBD* genotype		
A/A	162	51
A/C	128	40
C/C	27	9
Recipient sex		
Male	181	57
Female	136	43
Donor sex		
Male	181	63
Female	136	37
Donor/recipient sex		
Sex matched	176	56
Female/male	54	17
Male/female	87	27
Disease		
Acute myeloid leukemia	153	48
Acute lymphoblastic leukemia	67	21
Myelodysplastic syndrome	53	17
Malignant lymphoma	32	10
Chronic myeloid leukemia	12	4
Disease stage		
Standard risk	173	59
High risk	122	41
Performance status		
0–1	295	93
2–4	22	7
ABO matching		
Major or/and minor mismatch	125	39
Major mismatch	68	22
Minor mismatch	73	23
Bidirectional	16	5
Conditioning regimen		
Myeloablative	169	53
Reduced intensity	148	47
With total body irradiation (TBI)	236	74
Pre-transplant CMV serostatus		
CMV positive recipient	237	79
Missing	17	5
GVHD prophylaxis		
With cyclosporine	82	26
With tacrolimus	235	74
With short course methotrexate	306	97
TNC, $\times 10^8$ per kg		
Median	2.7	
Range	0.5–8.8	

TNC total nucleated cell count harvested

(18 %) relapsed or progressed and 120 (38 %) died. Thirteen patients (4 %) died before engraftment. The recipients were defined as having standard-risk disease if they had AML or ALL in the first complete remission, CML in any chronic phase, ML in any complete remission or MDS. All others were designated as having high-risk disease. The observed myeloid malignancies included AML, CML and MDS and the observed lymphoid malignancies included ALL and ML. Cyclosporine- or tacrolimus-based regimens were used in all the patients undergoing GVHD prophylaxis, while anti-T cell therapies, such as anti-thymocyte globulin or ex vivo T cell depletion, were not used in any of the patients. All the patients and donors gave their written informed consent to participate in the molecular studies of this nature at the time of transplantation according to the Declaration of Helsinki. This project was approved by the Institutional Review Board of Kanazawa University Graduate School of Medicine and the JMDP.

THBD genotyping

Genotyping of *THBD* was performed using the TaqMan-Allelic discrimination method as previously described [24]. The genotyping assay was conducted in 96-well PCR plates using specific TaqMan probes for the *THBD* gene single nucleotide variations rs3176123 (C__32272154_10) and rs1042579 (C___2531431_10) in a StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA).

Allele-specific transcript quantification

Allelic expression analyses were performed using the TaqMan assay using single nucleotide variation genotyping probes as previously described [25, 26]. A standard curve was generated by measuring the fluorescent intensity data for mixtures of the genomic DNAs from the two

homozygote controls (the rs3176123 genotype of A/A and C/C) at 5 different ratios (4:1, 3:2, 1:1, 2:3, and 1:4). The ratio of the fluorescent intensity for the A and C alleles was then measured using cDNA and genomic DNA from 6 heterozygote controls, and the allelic ratio on the gene expression was calculated based on the standard curve.

Data management and statistical analysis

The data were collected by the JMDP using a standardized report form. Follow-up reports were submitted at 100 days, 1 year and thereafter annually after transplantation. The pre-transplant cytomegalovirus (CMV) serostatus was routinely tested in the patients only and not in the donors. Engraftment was confirmed by an absolute neutrophil count of more than 0.5×10^9/L for at least 3 consecutive days. The outcome classification, including GVHD, did not change over time in the present study. After collecting the data, acute and chronic GVHD were diagnosed and graded based on classically defined criteria [27, 28], namely, acute GVHD was defined as GVHD that developed within the first 100 days post-transplant, while the manifestations of GVHD occurring after day 100 were classified as chronic GVHD. The data using the updated criteria for the assessment of GVHD [29, 30] were not available in our cohort. The overall survival (OS) rate was defined as the number of days from transplantation to death from any cause. Disease relapse was defined as the number of days from transplantation to disease relapse. Non-relapse mortality (NRM) was defined as death without relapse. Any patients who were alive at the last follow-up date were censored. The data regarding the causative microbes of infections, postmortem changes in the causes of death and supportive care, including prophylaxis for infections and therapy for GVHD given on an institutional basis, were not available for this cohort.

All statistical analyses were performed with the EZR software package (Saitama Medical Center, Jichi Medical University), a graphical user interface for R (The R Foundation for Statistical Computing, version 2.13.0) [31], as described in a previous report [32, 33]. The probability of OS was calculated using the Kaplan–Meier method and compared using the log-rank test. The probabilities of TRM, disease relapse, acute GVHD, chronic GVHD and engraftment were compared using the Gray's test [34] and analyzed using a cumulative incidence analysis [35] while considering relapse, death without disease relapse, death without acute GVHD, death without chronic GVHD and death without engraftment as respective competing risks. The variables included the recipient's age at the time of transplantation, sex, pre-transplant CMV serostatus, disease characteristics (disease type, disease lineage and disease risk at transplantation), donor characteristics (age, sex, sex compatibility and ABO compatibility), transplant characteristics [conventional or reduced-intensity conditioning [36], tacrolimus versus cyclosporine and total nucleated cell counts harvested per recipient weight (TNC)] and year of transplantation. The median was used as the cutoff point for the continuous variables. The Chi-square test and the Mann–Whitney U test were used to compare the data between two groups. The Hardy–Weinberg equilibrium for the TM gene variant was determined using the Haploview software program [37].

Multivariate Cox models were used to evaluate the hazard ratio associated with the *THBD* variation. All of the potential confounding factors were included in the multivariate analyses and then were removed in a stepwise fashion from the model to exclude factors with a P value of 0.1 or higher. Finally, the *THBD* genotype was added to the model. For both the univariate and multivariate analyses, P values were two sided and statistical significance was considered to exist at values of $P \leq 0.05$.

Results

Frequencies of the *THBD* genotypes

The rs3176123 single nucleotide variation (2729A>C) in the *THBD* gene was genotyped in 317 unrelated bone marrow donor–transplant recipient pairs (Table 1). The genotype frequencies of A/A, A/C and C/C were 54, 36 and 10 % in recipients and 51, 40 and 9 % in donors, respectively. These results were in accord with the Hardy–Weinberg equilibrium ($P = 0.87$) and were similar to the HapMap data in the Japanese population. A linkage disequilibrium analysis using the genotype data from the patient population and donor population showed that the rs3176123 variation was in linkage disequilibrium with the rs1042579 single nucleotide variation as previously reported [38]. The donor and recipient *THBD* genotype did not significantly influence the cumulative incidence of engraftment (data not shown).

Transplant outcomes according to the *THBD* genotype

The transplant outcomes according to the *THBD* genotype are summarized in Table 2. The donor A/C or C/C genotype was significantly associated with a lower incidence of grades II–IV acute GVHD (26 %) compared to that observed in the donor A/A genotype (40 %, $P = 0.02$) (Fig. 1a), thus suggesting the homozygous dominant effects of the A allele. The incidence of grades III–IV acute GVHD did not significantly differ according to the genotype (Table 2) as well as that of grades I–IV (data not shown). After adjusting for the clinical factors by a stepwise deletion method in the multivariate model, the donor

A/C or C/C genotype remained statistically significant compared to the donor A/A genotype for the development of grades II–IV acute GVHD [hazard ratio (HR) 0.66; 95 % confidence interval (CI) 0.44–0.99; $P = 0.05$; Table 3 and Supplementary Table 1]. Although this association of the donor *THBD* genotype with grades II–IV acute GVHD did not significantly influence the OS or NRM in the univariate analysis, the donor A/C or C/C genotype showed a tendency toward a better OS in the multivariate analysis (HR 0.71; 95 % CI 0.48–1.04; $P = 0.08$; Table 3). It should be noted that in the patients who developed grades II–IV acute GVHD, the donor A/C or C/C genotype predicted a better OS (HR 0.45; 95 % CI 0.21–0.99; $P = 0.05$; Fig. 1b; Table 4 and Supplementary Table 1), which was not observed in the remaining patients (Fig. 1c). The recipient *THBD* genotype revealed no significant effects on the transplant outcomes. The main causes of death according to the donor *THBD* genotype are illustrated in Fig. 3. Transplants from A/C or C/C donors tended to result in a reduced incidence of death compared to those from A/A donors. It may be noted that death due to acute GVHD or thrombotic microangiopathy (TMA) was absent in patients receiving transplants from A/C or C/C donors.

The impact of the rs3176123 single nucleotide variation on the gene expression

The rs3176123 single nucleotide variation is located in the 3′ untranslated region (3′UTR), which contains regulatory regions that influence the post-transcriptional gene expression as well as the localization and stability of mRNA [39]. To address whether the rs3176123 single nucleotide variation correlates with the transcription of mRNA, an allele-specific quantitative PCR assay was performed using lymphocytes obtained from healthy individuals possessing the rs3176123 2729A/C heterozygous genotypes according to previous reports [25, 26]. The results revealed that the mean proportion of the allele C in the amplicons of cDNA was significantly higher than that of genomic DNA (47 vs. 43 %; $P = 0.006$; Fig. 2), thus suggesting that the 2729C allele of the rs3176123 single nucleotide variation has higher transcriptional activity of *THBD* mRNA than the 2729A allele.

Discussion

The present study showed that the donor 2729A/C or C/C genotype of the *THBD* gene variation (rs3176123) was associated with a lower incidence of grades II–IV acute GVHD after unrelated HLA-matched BMT through JMDP. In addition, the donor 2729A/C or C/C genotype predicted better OS rates in the patients who developed grades II–IV

Table 2 The results of a univariate analysis regarding the association between the *THBD* variations and clinical outcomes after transplantation

Variable	No.	3-Year OS (%)	*P*	3-Year NRM (%)	*P*	3-Year relapse (%)	*P*	II–IV Acute GVHD (%)	*P*	III–IV Acute GVHD (%)	*P*	Chronic GVHD (%)	*P*
Recipient *THBD* genotype													
A/A	172	59		20		20		33		6		29	
A/C	114	64	1.00	16	1.00	19	0.97	31	0.76	9	0.69	39	0.36
C/C	31	54	1.00	18	1.00	31	0.62	41	0.76	14	0.42	37	0.79
A/C or C/C	145	61	0.36	16	0.30	22	0.67	33	0.93	10	0.20	38	0.11
Donor *THBD* genotype													
A/A	162	56		21		22		40		9		32	
A/C	128	64	0.73	18	0.57	19	1.00	**25**	**0.04**	6	0.87	35	0.42
C/C	27	64	0.52	9	0.15	22	1.00	30	0.92	11	0.87	33	0.24
A/C or C/C	155	64	0.22	16	0.25	20	0.43	**26**	**0.02**	7	0.44	35	0.65

Bold results represent $P \leq 0.05$

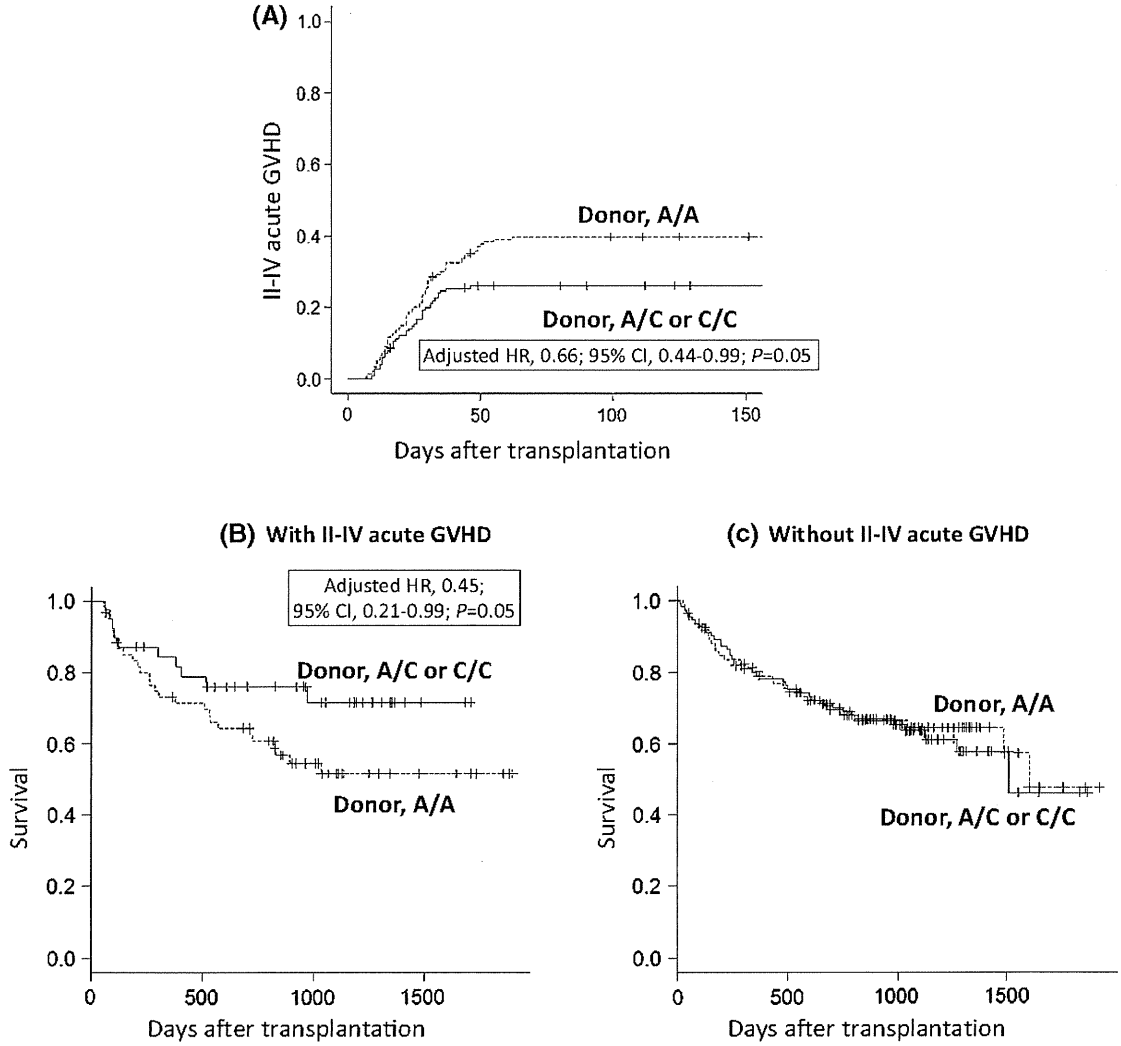

Fig. 1 The estimated cumulative incidence curves of grades II–IV acute GVHD according to the donor *THBD* genotype (**a**), and the Kaplan–Meier analysis of the overall survival rates after transplantation according to the donor *THBD* genotype in the patients who developed grades II–IV acute GVHD (**b**) or who did not develop grades II–IV acute GVHD (**c**). The *solid lines* represent the donor A/A genotype, the *dashed lines* represent the donor A/A genotype and the *dotted lines* represent the donor A/C or C/C genotype

acute GVHD. Of note, the functional analysis of the *THBD* variation revealed that the 2729C allele was associated with a higher transcriptional activity in the lymphocytes compared to the 2729A allele, thus suggesting that the expression of TM in the donor blood cells transplanted likely accounts for their decreased risk for acute GVHD development and the subsequent mortality.

The mechanisms through which the 2729A>C variation of the *THBD* gene affects the mRNA expression remain unclear. One plausible explanation is that the 2729A>C variation in the 3′UTR might affect the mRNA stability because the 3′UTR of *THBD* mRNA was found to play pivotal roles in the post-transcriptional decay of mRNA [40]. It may be speculated that microRNAs, negative regulators of gene expression [41], could differently interact with the 3′UTR of mRNA according to the 2729A>C variation, leading to a change in its stability. These hypotheses may be supported by our previous observations [33, 42] that NK cells possessing the lower affinity allele with the microRNA in the 3′UTR NKG2D variation exhibited higher NKG2D levels and higher NK cytotoxicity.

Previous studies have identified three variations (rs1042579, rs1042580 and rs3176123) in the *THBD* gene which were associated with susceptibility to vascular diseases [17–23, 43]. The coding region of the 1418C>T variation (rs1042579) was reported to be in complete disequilibrium with the 3′UTR 2729A>C variation (rs3176123) in Caucasian and Asian populations [17, 19] as observed in the present study. The rs3176123 was considered to be suitable for the functional analysis based on the hypothesis

Table 3 The results of a multivariate analysis regarding the association between the *THBD* variations and clinical outcomes after transplantation

Variable (no.)	OS			NRM			Relapse		
	Adjusted HR	95 % CI	*P*	Adjusted HR	95 % CI	*P*	Adjusted HR	95 % CI	*P*
Recipient *THBD* genotype, A/C (114) vs. A/A (172)	0.93	0.61–1.43	0.75	1.22	0.64–2.32	0.55	0.82	0.44–1.53	0.54
Recipient *THBD* genotype, C/C (31) vs. A/A (172)	0.89	0.46–1.70	0.72	0.72	0.27–1.93	0.52	1.37	0.58–3.22	0.48
Recipient *THBD* genotype, A/C or C/C (145) vs. A/A (172)	0.92	0.62–1.36	0.68	1.11	0.61–2.02	0.72	0.94	0.54–1.63	0.82
Donor *THBD* genotype, A/C (128) vs. A/A (162)	0.73	0.48–1.10	0.13	0.79	0.42–1.49	0.47	0.73	0.40–1.34	0.31
Donor *THBD* genotype, C/C (27) vs. A/A (162)	0.62	0.28–1.36	0.23	0.20	0.03–1.31	0.09	0.82	0.33–2.01	0.66
Donor *THBD* genotype, A/C or C/C (155) vs. A/A (162)	0.71	0.48–1.04	0.08	0.70	0.38–1.27	0.24	0.74	0.42–1.28	0.28
Variable (no.)	II–IV acute GVHD			III–IV acute GVHD			Chronic GVHD		
	Adjusted HR	95 % CI	*P*	Adjusted HR	95 % CI	*P*	Adjusted HR	95 % CI	*P*
Recipient *THBD* genotype, A/C (114) vs. A/A (172)	0.94	0.62–1.44	0.79	1.70	0.71–4.10	0.24	1.46	0.93–2.28	0.10
Recipient *THBD* genotype, C/C (31) vs. A/A (172)	1.40	0.74–2.63	0.30	3.08	0.90–10.47	0.07	1.38	0.62–3.09	0.43
Recipient *THBD* genotype, A/C or C/C (145) vs. A/A (172)	1.03	0.70–1.52	0.88	1.94	0.84–4.50	0.12	1.44	0.94–2.21	0.10
Donor *THBD* genotype, A/C (128) vs. A/A (162)	**0.64**	**0.41–0.98**	**0.04**	0.74	0.30–1.83	0.52	1.20	0.77–1.88	0.43
Donor *THBD* genotype, C/C (27) vs. A/A (162)	0.79	0.36–1.76	0.57	1.45	0.43–4.94	0.55	1.05	0.49–2.25	0.90
Donor *THBD* genotype, A/C or C/C (155) vs. A/A (162)	**0.66**	**0.44–0.99**	**0.05**	0.86	0.38–1.91	0.70	1.17	0.76–1.79	0.47

Bold results represent $P \leq 0.05$

Table 4 The results of the multivariate analysis of the association between the *THBD* variations and clinical outcomes after transplantation in the patients who developed grades II–IV acute GVHD

Variable (no.)	OS			NRM			Relapse		
	Adjusted HR	95 % CI	*P*	Adjusted HR	95 % CI	*P*	Adjusted HR	95 % CI	*P*
Recipient *THBD* genotype, A/C (34) vs. A/A (54)	0.77	0.38–1.58	0.48	0.95	0.50–1.81	0.88	0.82	0.44–1.53	0.54
Recipient *THBD* genotype, C/C (12) vs. A/A (54)	1.03	0.50–2.16	0.93	1.13	0.57–2.22	0.72	1.37	0.58–3.22	0.48
Recipient *THBD* genotype, A/C or C/C (46) vs. A/A (54)	0.31	0.07–1.36	0.12	0.47	0.17–1.25	0.13	0.94	0.54–1.63	0.82
Donor *THBD* genotype, A/C (31) vs. A/A (61)	0.51	0.23–1.16	0.11	0.99	0.50–1.96	0.98	0.73	0.40–1.34	0.31
Donor *THBD* genotype, C/C (8) vs. A/A (61)	0.24	0.03–1.79	0.16	0.27	0.04–1.98	0.20	0.82	0.33–2.01	0.66
Donor *THBD* genotype, A/C or C/C (39) vs. A/A (61)	**0.45**	**0.21–0.99**	**0.05**	0.70	0.38–1.27	0.24	0.74	0.42–1.28	0.28

Bold results represent $P \leq 0.05$

that the 3′UTR variation in the *THBD* gene could influence the expression of TM according to the microRNA–mRNA interaction as mentioned above. The 2521A>G variation (rs1042580) in the 3′UTR was found to be associated with vascular disease in the Caucasian population but not in the Asian population [17–23, 43]; instead the 2729A>C

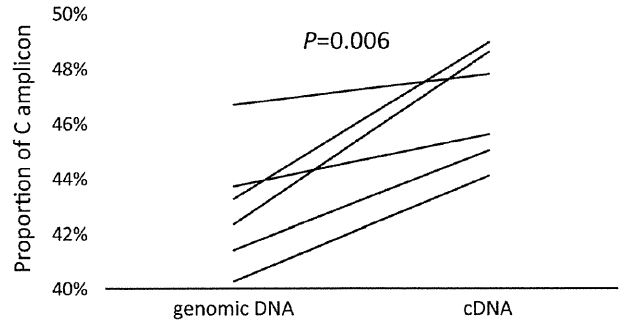

Fig. 2 Allele-specific gene expression of *THBD* rs3176123 single nucleotide variation using real-time PCR. The allelic ratio of the A and C alleles in genomic DNA and cDNA from 6 heterozygote controls are shown

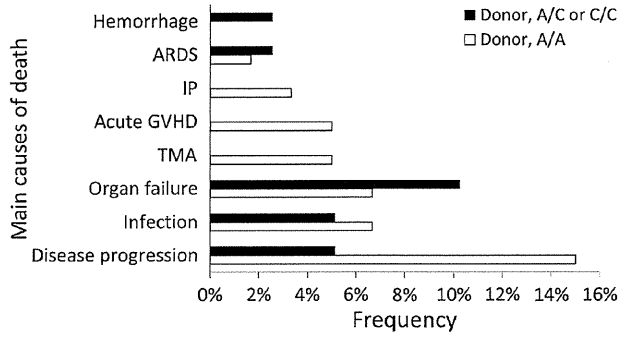

Fig. 3 Main causes of death after transplantation according to the donor *THBD* genotype. *ARDS* acute respiratory distress syndrome, *TMA* thrombotic microangiopathy, *IP* interstitial pneumonia

variation (rs3176123) was associated with deep vein thrombosis. Accordingly, we have focused on the analysis of the rs3176123 variation in the 3′UTR of the *THBD* gene.

A recent study [21] investigated the impact of the recipient *THBD* variations, including rs3176123 (2729A>C), on the transplant outcomes in a cohort of European patients who underwent hematopoietic stem cell transplantation for hematological malignancies. The authors found that the recipient *THBD* 2729C allele was associated with a higher NRM in the patients who developed acute GVHD, contrasting with the present findings in which the recipient *THBD* 2729A>C variation did not show significant effects on the transplant outcomes, and instead the donor *THBD* 2729C allele predicted a lower incidence of acute GVHD and the subsequent better survival. Although the reasons for the disparity between the two studies are unclear, ethnic differences between the two cohorts (Japanese vs. European) and in the frequency and severity of acute GVHD (grades I–IV, 45 vs. 60 %; grades III–IV, 9 vs. 22 %) may have influenced the results. This concern should be clarified by further investigations in the patients at higher risk for acute GVHD, including those receiving peripheral blood stem cell or HLA-mismatched transplants.

The role of TM in the pathogenesis of acute GVHD remains unclear. When recombinant soluble TM was used in the patients who underwent allogeneic HSCT, decreasing plasma levels of IL-6, IFN-γ, TNF-α, and HMGB1 and the amelioration of steroid-refractory acute GVHD were found [16, 44]. In the mouse acute GVHD models, treatment with recombinant soluble TM improved GVHD and survival along with decreasing the plasma levels of IL-6, IFN-γ, and HMGB1 as well as increasing the number of regulatory T cells (Tregs), in which the graft-versus-leukemia effect was not hampered [45]. Inflammatory mediators, such as IL-6, IFN-γ, TNF-α, and HMGB1, are critical in the initiation and progression of acute GVHD [46, 47], in which Tregs conversely operate [48, 49]. In the present study, the donor 2729C allele in the *THBD* rs3176123 variation, which was associated with a reduced risk of acute GVHD and the subsequent mortality, exhibited a higher transcriptional activity of *THBD* mRNA, thus leading to a hypothesis that the *THBD* rs3176123 variation plays functional roles by influencing the production of soluble TM from the donor blood cells, such as monocytes, neutrophils, and DCs, thereby affecting the production of inflammatory mediators related to acute GVHD and the generation of Tregs after HSCT.

The second hypothesis is that the expression of TM, videlicet BDCA3, on donor DCs influences their functions to regulate acute GVHD, since BDCA3-positive DCs were found to polarize T cells into T helper (Th) 2 cells more efficiently than DCs lacking BDCA3 [50], and Th2 cells are considered to suppress acute GVHD [51–53]. This possibility may be supported by the observation that BDCA3-positive DCs displayed an inhibitory activity on GVHD via producing IL-10 and inducing the generation of Tregs in a humanized mouse model [54]. Because obtaining blood samples from the patients and their donors and measuring the plasma levels of TM and inflammatory mediators were not within the scope of the present study, further studies to test these hypotheses are warranted.

It is unclear in the present study why the donor genotype would significantly influence the development of GVHD and its outcome without any influence from the recipient's genotype; however, one reason may be due to the higher expression of TM although the expression of TM is quite complex and is upregulated and downregulated by many factors [4]. One plausible explanation is the influence of endothelial chimerism on the outcome. It has been previously reported that endothelial cells derived from donor bone marrow stem cells can replace damaged host endothelium in organs and tissues affected by GVHD, such as the skin, gut and lung in the form of physiological cell replacement, even during the effector phase of acute GVHD [55–60]. The reported numbers of donor-type bone

marrow-derived endothelial cells after SCT varied from 2 to 40 %. In the present study, the donor A/C or C/C genotype predicted a lower incidence of grades II–IV acute GVHD compared to the donor A/A genotype (26 vs. 40 %), and the subsequent higher probability of survival (72 vs. 52 %); however, the donor A/C or C/C genotype did not influence the development of grades I–IV acute GVHD (59 vs. 65 %), suggesting that the donor A/C or C/C genotype may have decreased the risk of the progression of acute GVHD from grade I to grade II or higher, as well as subsequent mortality. The replacement of endothelial cells derived from the favorable A/C or C/C genotype donor could function to improve organ and tissue impairment caused by acute GVHD and microangiopathy through its higher expression of thrombomodulin. The present findings, namely that patients who received transplants from donors with the A/C or C/C genotype and developed grades II–IV acute GVHD did not die due to acute GVHD or TMA (Fig. 2) could thus support this hypothesis. However, these hypotheses are highly speculative due to the lack of in vivo data. Elucidating endothelial chimerism and the functional roles of the *THBD* variation after SCT will thus provide useful information on this issue (Fig. 3).

One major limitation of this study is that the results of the transcription activity test are insufficient to definitively conclude the functional roles of the *THBD* variation in the development and mortality of acute GVHD. The association between the serum concentrations of soluble TM and *THBD* variations may offer useful information on this issue. Unfortunately, no serum samples were available in the present study.

In conclusion, the present data suggest that the donor *THBD* variation predicts the development of acute GVHD and the subsequent survival after allogeneic HSCT from unrelated donors and plays a functional role in the regulation of the *THBD* mRNA expression. Therefore, *THBD* genotyping in transplant donors may be a useful tool for optimizing donor selection and evaluating pretransplantation risks that, combined with other currently known risk factors, can form the basis for appropriate tailoring of transplantation strategies. Furthermore, the current findings may positively contribute to the development of novel prophylactic and therapeutic strategies for acute GVHD using recombinant soluble TM. However, care should be made before drawing any conclusions because further experimental evidence is required to substantiate the effects of the *THBD* gene according to the allelic variation. Finally, transplant outcomes, including acute GVHD, are multifactorial and single variations in one gene are unlikely to determine the majority of the outcomes. Further studies are warranted to ascertain whether the findings of this study can be extended to other stem cell sources or to HLA-mismatched transplantation and to validate the present data in other ethnic groups.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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