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ORIGINAL RESEARCH

Identification of the Novel Variants in Patients With Chronic Thromboembolic Pulmonary Hypertension

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BACKGROUND: Although chronic thromboembolic pulmonary hypertension (CTEPH) and acute pulmonary embolism (APE) share some clinical manifestations, a limited proportion of patients with CTEPH have a history of APE. Moreover, in histopathologic studies, it has been revealed that pulmonary vasculature lesions similar to pulmonary arterial hypertension existed in patients with CTEPH. Thus, it remains unknown whether these 3 disorders also share genetic backgrounds.

METHODS AND RESULTS: Whole exome screening was performed with DNA isolated from 51 unrelated patients with CTEPH of Japanese ancestry. The frequency of genetic variants associated with pulmonary arterial hypertension or APE in patients with CTEPH was compared with those in the integrative Japanese Genome Variation Database 3.5KJPN. Whole exome screening analysis showed 17 049 nonsynonymous variants in patients with CTEPH. Although we found 6 nonsynonymous variants that are associated with APE in patients with CTEPH, there was no nonsynonymous variant associated with pulmonary arterial hypertension. Patients with CTEPH with a history of APE had nonsynonymous variants of *F5*, which encodes factor V. In contrast, patients with CTEPH without a history of APE had a nonsynonymous variant of *THBD*, which encodes thrombomodulin. Moreover, thrombin-activatable fibrinolysis inhibitor, which is one of the pathogenic proteins in CTEPH, was significantly more activated in those who had the variants of *THBD* compared with those without it.

CONCLUSIONS: These results provide the first evidence that patients with CTEPH have some variants associated with APE, regardless of the presence or absence of a history of APE. Furthermore, the variants might be different between patients with CTEPH with and without a history of APE.

Key Words: chronic thromboembolic pulmonary hypertension ■ gene variants ■ pulmonary hypertension

Chronic thromboembolic pulmonary hypertension (CTEPH) is a fatal disease entity of pulmonary hypertension characterized by obstruction of the major pulmonary arteries by organized thrombus and pulmonary vascular remodeling.[1,2] CTEPH is believed to occur following acute pulmonary embolism (APE).[2] Although CTEPH and APE share some clinical manifestations, a limited proportion of patients with CTEPH have a history of APE.[3] Moreover, risk factors for the development of CTEPH are different from those of traditional risk factors for APE.[4] Thus, it is uncertain whether patients with CTEPH, especially who did not have a history of APE, have some variants associated with APE. It has been reported that endothelial dysfunction is involved in the pathogenesis of CTEPH.[5] Moreover, patients with CTEPH show distal pulmonary artery remodeling, which is similar to pulmonary arterial hypertension (PAH).[2] These reports implicate a potential overlap of CTEPH and PAH pathogenesis.

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CLINICAL PERSPECTIVE

What Is New?

- Although patients with chronic thromboembolic pulmonary hypertension (CTEPH) did not have any variants associated with pulmonary arterial hypertension, patients with CTEPH have some variants associated with acute pulmonary embolism.
- Patients with CTEPH without the history of acute pulmonary embolism had some variants associated with acute pulmonary embolism, especially thrombomodulin.
- Thrombin-activatable fibrinolysis inhibitor was activated in patients with CTEPH who had the variant of thrombomodulin.

What Are the Clinical Implications?

- As thrombin-activatable fibrinolysis inhibitor was activated especially in patients with CTEPH who had the variant of thrombomodulin, the inhibition of thrombin-activatable fibrinolysis inhibitor might be novel therapy in those patients.

Nonstandard Abbreviations and Acronyms

APC	activated protein C
APE	acute pulmonary embolism
CTEPH	chronic thromboembolic pulmonary hypertension
PAH	pulmonary arterial hypertension
TAFI	thrombin-activated fibrinolysis inhibitor
TAFIa	activated thrombin-activated fibrinolysis inhibitor

We and others have just begun to elucidate the cause of CTEPH. Some risk factors associated with CTEPH have been reported, including antiphospholipid antibodies,[6] increased von Willebrand factor,[7] increased factor VIII,[8] and activated platelets.[9] In addition, as we have recently demonstrated, activated thrombin-activatable fibrinolysis inhibitor (TAFI) promotes the development of CTEPH.[10,11] TAFI is activated by the thrombin-thrombomodulin complex on the surface of the endothelium, and the activated TAFI (TAFIa) removes the C-terminal lysines from fibrin and reduces the binding of tPA (tissue-type plasminogen activator) and plasmin to fibrin.[12] On the basis of this background, we examined the serum levels of TAFI and TAFIa in patients with CTEPH, demonstrating that the levels were significantly higher than in healthy controls.[10,11] However, the mechanism involved in the excessive activation of TAFI in patients with CTEPH remains to be elucidated.

It is widely known that some rare variants play an important role in the pathogenesis of PAH.[13–16] In 2000, mutations in the bone morphogenetic protein receptor-2 gene (BMPR2) were identified as a cause of familial PAH.[13] Subsequently, variants in genes involved in BMP-BMPR2 signaling (eg, *SMAD9* and *ACVRL1*) were identified.[14,15] Recently, through studies using whole exome screening, variants associated with cytoskeletal function and the Wnt signaling pathway have been identified in idiopathic PAH.[16] In contrast, a limited number of reports on the genetic background of CTEPH are available in the literature. However, there are a few reports about common variants of CTEPH in recent years.[17–20] Especially, a large cohort of variants of a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13, has been reported.[19] In this study, patients with CTEPH had decreased a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13, antigen levels, and there were 5 variants in the a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13, ±40 kb region significantly associated with a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13, antigen levels. Moreover, although variants involved in the BMP-BMPR2 signaling pathway have also been implicated in CTEPH,[20] opposite findings have also been reported elsewhere.[21] Although variants of factor V, known as factor V Leiden, have been identified in patients with CTEPH,[7] these variants have not been identified in eastern Asia.[22] Moreover, there was no report that patients with CTEPH have the genetic background associated with APE. In contrast, it has been reported that human leukocyte antigen-DPB1*0202 and B*5201 are associated with CTEPH.[22,23] More important, human leukocyte antigen is associated with autoimmune diseases.[24] Thus, it is considered that some rare and common variants are associated with pathophysiological characteristics of CTEPH, although CTEPH is a nonheritable disease.

Herein, we examined, using whole exome screening, whether patients with CTEPH have the rare or common variants associated with APE or PAH and whether the allele frequency of some variants in patients with CTEPH without a history of APE was different from those with a history of APE.

METHODS

The study protocol was approved by the Ethics Committees of Tohoku University, and all patients provided written informed consent (No. 2014-1-599).

The data that support the findings of this study are available from the corresponding author on reasonable request.

Patients and Samples

We enrolled patients who were aged >20 years and underwent right heart catheterization at our institute from April 2015 to January 2016. All patients were diagnosed as having CTEPH using the following methods. First, precapillary pulmonary hypertension was defined as mean pulmonary artery pressure >25 mm Hg and pulmonary capillary wedge pressure ≤15 mm Hg at rest.[25] CTEPH was diagnosed by ventilation-perfusion scintigraphy, computed tomography, and pulmonary angiography after treatment with anticoagulants for 6 months.[25] In the present study, we enrolled 51 consecutive patients with CTEPH. To determine whether they had a history of APE, we checked the information of their former hospitals and enhanced computed tomography. After patients provided written informed consent, we obtained 10 mL of whole blood, and DNA was purified from neutrophils by the SRL Laboratory Co (Tokyo, Japan).

Whole Exome Screening and Data Processing Pipeline for Illumina HiSeq Exome Sequencing

Library preparation and exome capture were performed using the SureSelectXT Target Enrichment System (Agilent Technologies, Santa Clara, CA) on a Bravo Automated Liquid Handling Platform (Agilent Technologies) as follows. Briefly, 1 μg of genomic DNA was sheared to 150- to 200-bp fragments using a Covaris S220 instrument (Covaris, Woburn, MA), followed by end repair, A-tailing, and adapter ligation. Precapture libraries were amplified by 6 cycles of polymerase chain reaction and analyzed using the Agilent 2200 TapeStation (Agilent Technologies) to evaluate quality and yield. Exome capture was performed with the SureSelectXT Human All Exon V5 Plus Regulatory kit (Agilent Technologies), followed by library amplification with 11 or 12 cycles of polymerase chain reaction. We assessed the quality of sequencing libraries by quantitative MiSeq methods.[26] Paired-end sequencing with 2×10^1 bp reads was performed on Illumina HiSeq 2500 (Illumina Inc, San Diego, CA). The mean output was 6.4 Gb per sample, and mean coverage depth was 38.8-fold. We used the data from the integrative Japanese Genome Variation Database 3.5KJPN (https://ijgvd.megabank.tohoku.ac.jp/) to determine the allele frequency in the general population. Samples from 51 patients with CTEPH and the general population as references were obtained in the same area, eastern Japan, specifically in the Tohoku district.

Data Processing Pipeline for Illumina HiSeq Exome Sequencing

Paired 2×101 bp reads were aligned to the reference human genome (hGRC37, hg19) using alignment software (version 0.7.5a-r405; BWA-MEM, http://bio-bwa.sourceforge.net). Reads that were potential duplicates based on polymerase chain reaction amplifications were flagged using the Picard program (http://picard.sourceforge.net). Single-nucleotide variants, insertions, and deletions were detected using the UnifiedGenotyper tool in Genome Analysis Toolkit (version 2.5-2; http://www.broadinstitute.org/gatk) with the default options after the base quality score recalibration,[27] and the variants were stored with the variant call format version 4.1.

Western Blotting Analysis for TAFI and TAFIa

We obtained 5 mL of whole blood with 0.313% citric acid, which was centrifuged at 1100*g* for 10 minutes to obtain plasma. Then, the plasma was diluted 50 times with PBS and samples were loaded on the SDS-PAGE and transferred to polyvinylidene difluoride membranes (GE Healthcare, Buckinghamshire, UK), following blocking for 1 hour at room temperature with 5% BSA in Tris-buffered saline with Tween 20. The anti-TAFI antibody (1000:1; Abcam, Cambridge, UK) was used as the primary antibody. The regions containing proteins were visualized by the enhanced chemiluminescence system (ECL Prime Western Blotting Detection Reagent; GE Healthcare). Densitometric analysis was performed using ImageJ Software (NIH, Bethesda, MD).

ELISA Assay for Soluble Thrombomodulin

We obtained plasma from whole blood with 0.313% citric acid. We excluded patients with CTEPH with chronic kidney disease (estimated glomerular filtration rate <45 mL/min) and collagen disease because soluble thrombomodulin is elevated in those diseases.[28,29] Finally, we measured plasma levels of soluble thrombomodulin in 41 patients with CTEPH with ELISA assay (R and D Systems Inc, Minneapolis, MN).

Statistical Analysis

All statistical analyses were performed using R, version 3.1.3 (R Foundation for Statistical Computing, Vienna, Austria; http://www.R-project.org/). We used the data from the integrative Japanese Genome Variation Database 3.5KJPN (https://ijgvd.megabank.tohoku.ac.jp/) and 1000G project (ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp/technical/working/20130723_phase3_wg/shapeit2) to

determine the allele frequency in the general population. Samples from 51 patients with CTEPH and the general population as references were obtained in the same area, eastern Japan, specifically in the Tohoku district. The comparison of allele frequency between the general population (3.5KJPN; n=3554) and patients with CTEPH was analyzed by the Fisher exact test. Moreover, multiple comparisons were analyzed with the Holm method. The comparison of TAFI and TAFIa plasma levels was analyzed by the Tukey honestly significant difference multiple comparison. To determine the cutoff point of the plasma levels of TAFIa, we performed receiver operating characteristic curve analysis and determined the Youden index. $P<0.05$ was considered to be statistically significant.

RESULTS

Patient Characteristics

Among the 51 unrelated patients with CTEPH, none had a family history of CTEPH (Table 1). Our patient population was composed predominantly of women (n=41; 80%) with a mean age of 65±15 years. All patients underwent right heart catheterization, which showed mean pulmonary artery pressure of 43.6±10.1 mm Hg, pulmonary capillary wedge pressure of 9.5±3.1 mm Hg, cardiac index of 2.47±0.53 L/min per m^2, and pulmonary vascular resistance of 9.7±4.6 Wood units. In this patient population, 45.1% of patients had a history of APE.

Comparison of Allele Frequencies of Common Variants Between Cases and Controls

The present study comprised a large prospective genome cohort in the Tohoku area of northeast Japan. Although all patients with CTEPH in the present study were enrolled from the same area, it was unknown whether the genetic background of the cases was similar to that of controls. At first, we compared the genetic background of patients with CTEPH with that of controls. We were unable to obtain individual variant data of controls. Thus, we were unable to perform principle component analysis. Next, we compared the frequency of the common variants in cases and controls to investigate whether the genetic background in cases and controls was same or not. We screened the synonymous and nonsynonymous variants of which allele frequency was >5% as common variants. A total of 8727 variants were identified. We compared the frequency of these variants in cases with 3.5KJPN (Figure 1A). It was revealed that the frequency of these variants in cases was significantly correlated with that in 3.5KJPN (R=0.99; y=0.9865x+0.0026). Next, we compared the frequency of these variants in cases with 1000GEAS project, which has sequenced 1008 healthy subjects from East Asia. It was revealed that they were significantly correlated with that in 1000GEAS (R=0.97; y=0.968x+0.0085) (Figure 1B). The regression line of Figure 1A approximates $y=x$ compared with Figure 1B. Thus, we concluded that the genetic background of the cases was the same as that of 3.5KJPN.

Table 1. Clinical Characteristics of Study Subjects

Variable	Subjects With CTEPH (n=51)
Clinical characteristics	
Age, y	65±15
Women, %	80
Body mass index, kg/m^2	24.6±4.5
Diabetes mellitus, %	9.8
History of smoking, %	27.5
History of acute pulmonary embolism, %	45.1
History of deep vein thrombosis, %	11.7
6-min Walk distance, m	473±123
NYHA class, %	
I	0
II	52.9
III	45.1
IV	2.0
Hemoglobin, g/dL	13.3±1.9
Platelets, ×10^3/μL	231±58
B-type natriuretic peptide, pg/mL	269±357
Epoprostenol, %	0
Oral PGI_2 analogue, %	25.5
Endothelin receptor antagonist, %	19.6
Phosphodiesterase-V inhibitor, %	25.5
Warfarin, %	100
Hemodynamic data	
RAP, mm Hg	6.7±3.4
Systolic PAP, mm Hg	77.2±20.4
Diastolic PAP, mm Hg	26.0±7.1
Mean PAP, mm Hg	43.6±10.1
PCWP, mm Hg	9.5±3.1
Systolic BP, mm Hg	121.6±18.3
Diastolic BP, mm Hg	73.5±12.3
PVR, Wood unit	9.7±4.6
CI, L/min per m^2	2.47±0.53

Results are expressed as mean±SD, unless otherwise indicated. BP indicates blood pressure; CI, cardiac index; CTEPH, chronic thromboembolic pulmonary hypertension; NYHA, New York Heart Association; PAP, pulmonary arterial pressure; PGI, prostaglandin I; PCWP, pulmonary capillary wedge pressure; PVR, pulmonary vascular resistance; and RAP, right arterial pressure.

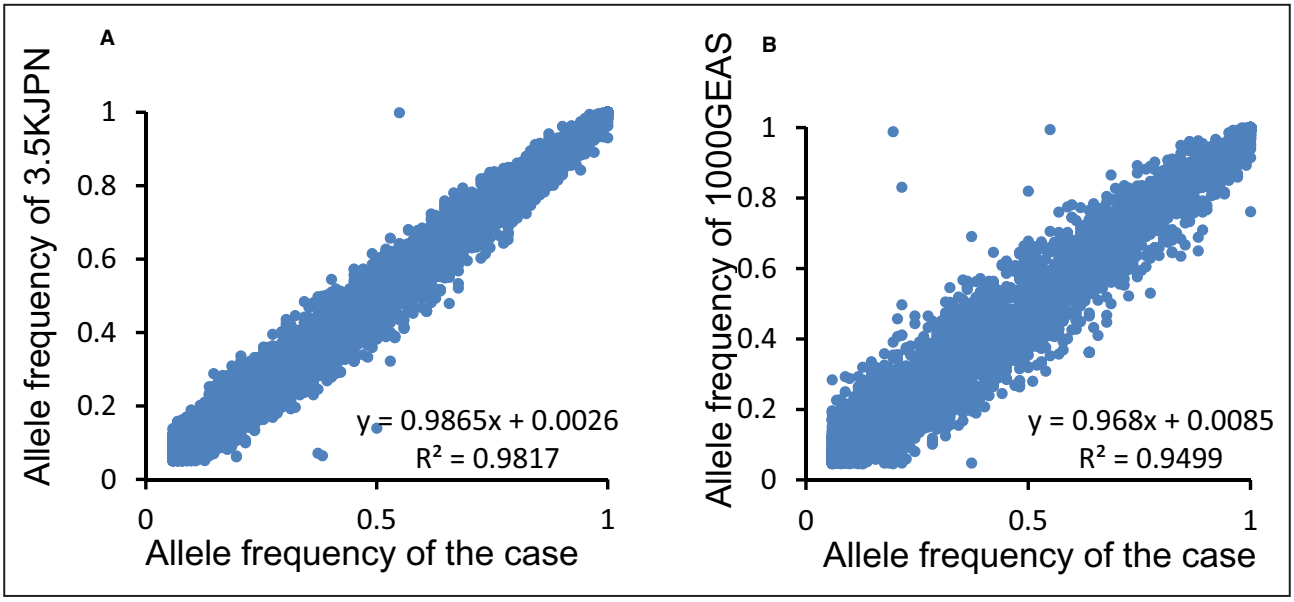

Figure 1. Comparisons of allele frequency of the case and controls.
Allele frequency of the case in this study was compared with that of 3.5KJPN (**A**) and 1000G project (**B**).

Screening of PAH-Associated Variants in Patients With CTEPH

Table 2 summarizes the variants identified in our population with CTEPH. A total of 82 825 variants were identified in 51 patients with CTEPH. In the past report,[30] the average coverage sequencing depth of 3.5KJPN was 32.4 times. Thus, we screened the variants in which read depth was >30 times. Among the variants, 32 005 variants were identified, which were considered as high genotype quality. Then, we removed synonymous variants and identified 17 049 variants. We filtered these variants with LJB-SIFT score >0.95 and LJB-Phylop score >0.85 as the prediction of deleteriousness. Finally, 3665 variants were identified. First, we screened the rare or common variants of *BMPR2*,[13] *ENG*,[31] *SMAD9*,[32] *ACVRL1*,[33] *CAV1*,[34] *KCNK3*,[35] *CBLN2*,[36] *TOPBP1*,[37] *SOX17*,[38] *TBX4*,[38] and *ABCC8*,[38] which have been reported to be associated with PAH. Although one nonsynonymous variant of *ENG* (NM_000118: c.1096G>C: p.366D>H) was identified in our study as in the previous study,[20] this allele frequency was also comparable between patients with CTEPH and the general population (4.9% versus 6.2%, respectively; *P*=0.83).

Any gene variants for *BMPR2*, *SMAD9*, *ACVRL1*, *CAV1*, *KCNK3*, *CBLN2*, *TOPBP1*, *SOX17*, *TBX4*, and *ABCC8* were not identified in the present study.

Thus, in the present study, any variants associated with PAH were not detected in patients with CTEPH.

Table 2. Genetic Variants Identified in Population With CTEPH Using WES

Variant Type	CTEPH
Patients, n	51
All variants, n	82 825
Variants (read depth >30), n	32 005
Synonymous variants, n	14 956
Nonsynonymous variants, n	17 049
Missense variants, n	16 793
Nonsense variants, n	235
Frameshift indels, n	21
Rare (<1% MAF) nonsynonymous, n	8078
Rare (<1% MAF) indels, n	21

CTEPH indicates chronic thromboembolic pulmonary hypertension; indel, insertion/deletion; MAF, minor allele frequency; and WES, whole exome screening.

Screening of APE-Associated Variants in Patients With CTEPH

Some patients develop CTEPH following APE.[7,8] Indeed, a limited percentage of patients with CTEPH have a history of APE.[3] Moreover, most risk factors of CTEPH differ from those of APE.[7,8] However, there is no report currently available suggesting that patients with CTEPH had some variants associated with APE. A total of 51 patients with CTEPH were screened for rare or common nonsynonymous variants of *SRPINC1*,[39] *THBD*,[40] *F5*,[41] *F2*,[42] *RGS7*,[43] *KNG1*,[44] *MTHFR*,[45] *VWF*,[7] and *PROCR*,[45] all of which have been reported as candidates for variants associated with APE. Interestingly, we identified 2 nonsynonymous variants of *F5*, which encodes factor V, in patients with CTEPH (Table 3). Moreover, the allele frequency of a nonsynonymous

Table 3. Nonsynonymous Variants Associated With APE in Patients With CTEPH

Gene	Variation, cDNA	LJB-SIFT Score	LJB-Phylop Score	Allele Frequency in 3.5KJPN (n=3554), %	Allele Frequency in CTEPH (n=51), %	*P* Value (vs 3.5KJPN)
THBD	c.1418C>T	0.85	0.96	27.6	35.2	0.38
F2	c.494C>T	0.92	0.97	62.0	67.6	0.52
F5	c.6665A>G	0.99	1.00	8.5	13.7	0.36
*F5**	c.3980A>G*	0.94*	0.99*	6.2*	13.7*	0.04*
MTHFR	c.665C>T	0.99	0.99	38.1	36.3	0.76
VWF	c.4585G>C	0.99	0.98	0.1	1.0	0.42

APE indicates acute pulmonary embolism; and CTEPH, chronic thromboembolic pulmonary hypertension.
*The variants of which frequency was significantly higher in patients with CTEPH compared with those of general population.

variant (NM_000130: c.3980A>G: p.1327H>R) of *F5* was significantly higher in patients with CTEPH than in the general population (Table 3). The predicted damage of this variant was 0.99 in silico (LJBSIFT). The frequency of another variant of *F5* was comparable between the patients with CTEPH and the general population. Although nonsynonymous variants of *F2*, *VWF*, and *MTHFR* were identified in patients with CTEPH, these allele frequencies were comparable between the patients with CTEPH and the general population. Furthermore, we identified specific variants in patients with CTEPH with or without deep vein thrombosis. In this study, deep vein thrombosis was confirmed in 6 patients with CTEPH with lower limb venous ultrasound and enhanced computed tomography. We screened for nonsynonymous variants associated with APE (Table 4). In patients with CTEPH without deep vein thrombosis, allele frequencies of c.3980A>G of *F5* were significantly higher compared with general population. Although the group of patients with CTEPH with deep vein thrombosis was a small one, the allele frequency of c.3980A>G of *F5* was significantly higher compared with general population.

Variants Associated With APE in Patients With CTEPH With or Without a History of APE

Next, we aimed to identify specific variants in patients with CTEPH with or without a history of APE. The baseline characteristics of patients with CTEPH with or without a history of APE are shown in Table 5. The allele frequencies of NM_000130: c.6665A>G: p.2222D>G and NM_000130: c.2450A>C: p.817N>T in *F5* were significantly higher in patients with CTEPH with a history of APE than in the general population. In contrast, these allele frequencies were comparable between patients with CTEPH without a history of APE and the general population (Table 6). The predicted functional damage of these variants in silico was 1.00 in LJBSIFT and 0.985 in Polyphen2. Thus, these variants may affect the function of factor V.

The allele frequency of NM_000361: c.1418C>T: p.473T>M in *THBD*, which encodes thrombomodulin, was significantly higher in patients with CTEPH without a history of APE than in the general population (Table 6). The predicted damage of this variant was 0.96 in LJBSIFT, suggesting that this variant may damage the function of thrombomodulin. In contrast, some allele frequencies were comparable between patients with CTEPH with a history of APE and the general population (Table 6). Although variants of factor V were identified in patients with CTEPH with a history of APE, the thrombomodulin variants were detected in patients with CTEPH without a history of APE. These differences in genetic variants might explain differences in pathogenesis and any partially overlapping mechanisms of CTEPH and APE.

Table 4. Nonsynonymous Variants Associated With APE in Patients With CTEPH With or Without DVT

Gene	Variation, cDNA	Allele Frequency in 3.5KJPN (n=3554), %	Allele Frequency in CTEPH With DVT, %	*P* Value (vs 3.5KJPN)	Allele Frequency in CTEPH Without DVT, %	*P* Value (vs 3.5KJPN)
THBD	c.1418C>T	27.6	41.7	0.33	34.4	0.15
F2	c.494C>T	62.0	83.3	0.15	65.6	0.51
F5	c.6665A>G	8.5	25.0	0.07	12.2	0.25
*F5**	c.3980A>G*	6.2*	25.0*	0.03*	12.2*	0.03*
MTHFR	c.665C>T	38.1	25.0	0.55	37.8	1.00
VWF	c.4585G>C	0.1	0	1.00	1.1	0.12

APE indicates acute pulmonary embolism; CTEPH, chronic thromboembolic pulmonary hypertension; and DVT, deep vein thrombosis.
*The variants with significantly higher frequencies in patients with CTEPH with or without a history of DVT compared with those of general population.

Table 5. Clinical Characteristics of Patients With CTEPH With or Without a History of APE

Variable	CTEPH With a History of APE (n=23)	CTEPH Without a History of APE (n=28)	*P* Value
Clinical characteristics			
Age, y	63±14	66±15	0.45
Women, %	82.6	78.6	0.50
Body mass index, kg/m^2	24.5±4.0	24.7±4.9	0.93
History of smoking, %	34.7	25.0	0.45
History of deep vein thrombosis, %	26.0	0	<0.01
6-min Walk distance, m	495±118	455±123	0.37
NYHA class, %			
I–II	56.5	53.5	
III–IV	47.8	46.5	0.53
Hemoglobin, g/dL	13.1±1.8	13.6±1.9	0.33
Platelets, ×10^3/μL	239±70	226±45	0.43
B-type natriuretic peptide, pg/mL	311±437	235±268	0.46
Hemodynamic data			
RAP, mm Hg	6.8±3.6	6.7±3.0	0.91
Systolic PAP, mm Hg	74.0±24.6	79.9±15.7	0.31
Diastolic PAP, mm Hg	24.2±7.0	27.5±6.9	0.10
Mean PAP, mm Hg	40.9±10.9	45.8±9.0	0.10
PCWP, mm Hg	9.0±3.1	10.1±3.5	0.26
Systolic BP, mm Hg	118.6±16.8	124.1±19.1	0.29
Diastolic BP, mm Hg	70.8±10.8	75.7±13.0	0.17
PVR, Wood unit	8.4±3.8	10.8±4.8	0.06
CI, L/min per m^2	2.57±0.49	2.44±0.45	0.33

Results are expressed as mean±SD, unless otherwise indicated. APE indicates acute pulmonary embolism; BP, blood pressure; CI, cardiac index; CTEPH, chronic thromboembolic pulmonary hypertension; NYHA, New York Heart Association; PAP, pulmonary arterial pressure; PCWP, pulmonary capillary wedge pressure; PVR, pulmonary vascular resistance; and RAP, right arterial pressure.

c.1418C>T in *THBD* Enhanced the Activation of TAFI

TAFI is activated by the thrombin-thrombomodulin complex on the surface of the pulmonary endothelium.[11] Serum levels of TAFIa are significantly higher in patients with CTEPH than in healthy controls.[10,11] Moreover, the interaction between TAFI and thrombomodulin plays a role in the development of CTEPH.[11] It has been reported that this variant increased the expression levels of thrombomodulin in endothelial cells and reduced those of soluble thrombomodulin.[40] Indeed, plasma levels of soluble thrombomodulin in CC, CT, and TT were 2752±611, 2613±537, and 2016±113 pg/mL, respectively (Figure 2). Patients with CTEPH with the genotype TT tended to have lower plasma levels

Table 6. Nonsynonymous Variants Associated With APE in Patients With CTEPH With No History of APE

Gene	Variation, cDNA	LJB-SIFT Score	LJB-Phylop Score	Allele Frequency in 3.5KJPN (n=3554), %	Allele Frequency in CTEPH With APE, %	*P* Value (vs 3.5KJPN)	Allele Frequency in CTEPH Without APE, %	*P* Value (vs 3.5KJPN)
*THBD**	c.1418C>T*	0.85*	0.96*	27.6*	17.3*	0.38	50.0*	0.003*
F2	c.494C>T	0.92	0.97	62.0	70.8	0.38	62.5	1.00
*F5**	c.6665A>G*	0.99*	1.00*	8.5*	21.7*	0.024*	7.1*	1.00
*F5**	c.3980A>G*	0.94*	0.99*	6.2*	21.7*	0.003*	7.1*	1.00
MTHFR	c.665C>T	0.99	0.99	38.1	39.1	0.88	33.9	1.00
*VWF**	c.4585G>C*	0.99*	0.98*	0.1*	2.1*	0.028*	0	0.65

APE indicates acute pulmonary embolism; and CTEPH, chronic thromboembolic pulmonary hypertension.
*The variants of which frequency was significantly higher in patients with CTEPH with or without a history of APE compared with those of general population.

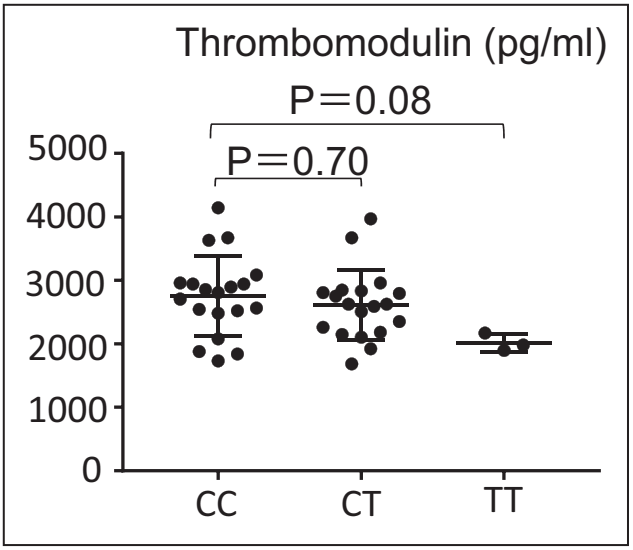

Figure 2. Plasma levels of soluble thrombomodulin in patients with chronic thromboembolic pulmonary hypertension (CTEPH) with or without the *THBD* c.1418C>T variant.
Quantification of plasma thrombomodulin in patients with CTEPH with or without the *THBD* c.1418C>T variant is shown. Statistical significance was determined with Kruskal-Wallis test.

of soluble thrombomodulin compared with those with the genotype CC (*P*=0.08). These results suggest that the c.1418C>T in *THBD* might be related to the lower plasma levels of soluble thrombomodulin in patients with CTEPH. Thus, this variant in *THBD* might alter the activation levels of TAFI in patients with CTEPH. We measured plasma levels of TAFI and TAFIa by Western blotting. Although the plasma levels of TAFI were comparable among the patients with CTEPH harboring the genotypes CC, CT, and TT, the plasma levels of TAFIa were significantly higher in patients with the genotypes CT and TT than in patients with genotype CC (Figure 3). Moreover, receiver operating characteristic analysis revealed that plasma levels of TAFIa could discriminate patients with CTEPH with c.1418C>T from patients with CTEPH with the genotype CC, with the area under the curve of 0.804 (95% CI, 0.687–0.921) (Figure 4). We determined 1.3 as the cutoff point of the plasma levels of TAFIa. The prevalence of patients with CTEPH in the genotypes CC, CT, and TT among those with plasma levels of TAFIa >1.3 was 9.1%, 59.1%, and 57.1%, respectively (*P*<0.05). Thus, the prevalence of patients with CTEPH with increased TAFIa was significantly higher in patients with CTEPH with c.1418C>T compared with those with the genotype CC. These results suggest that the c.1418C>T variant in *THBD* might be related to the activation of TAFI in patients with CTEPH.

DISCUSSION

The novel findings of the present study are as follows: (1) there was no PAH-associated variant identified in patients with CTEPH, (2) there were APE-associated variants in patients with CTEPH, (3) patients with CTEPH without a history of APE had the variants of thrombomodulin and those with a history of APE had the variants of factor V, and (4) TAFIa was higher in patients with CTEPH with the c.1418C>T variant in *THBD*. Taken together, these results indicated that the genetic background of CTEPH differed from that of PAH but may partially overlap with that of APE.

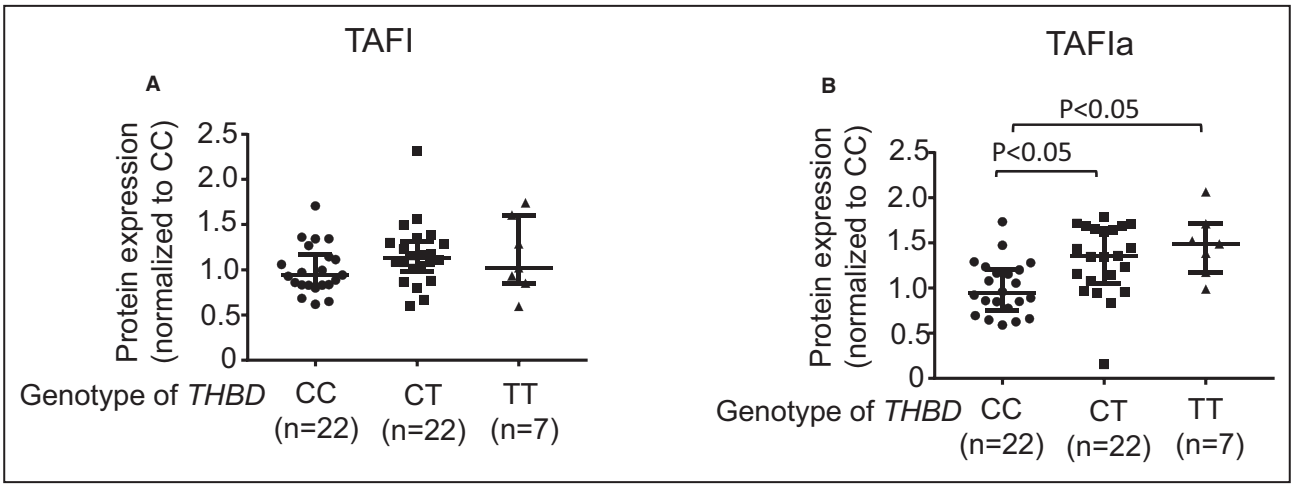

Figure 3. Plasma levels of thrombin-activated fibrinolysis inhibitor (TAFI) and activated TAFI (TAFIa) in patients with chronic thromboembolic pulmonary hypertension (CTEPH) with or without the *THBD* c.1418C>T variant.
A, Quantification of plasma TAFI in patients with CTEPH with or without the *THBD* c.C1418T variant is shown. **B**, Quantification of plasma TAFIa in patients with CTEPH with or without the *THBD* c.1418C>T variant is shown. The line represents the median value. The protein expression levels of TAFI and TAFIa of each group were expressed as median (interquartile range). Statistical significance was determined with Kruskal-Wallis test.

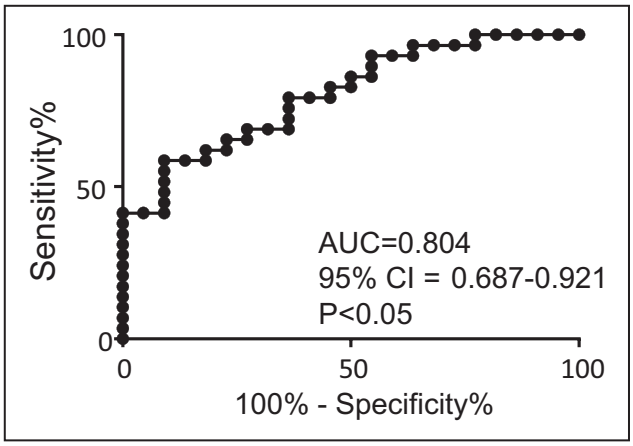

Figure 4. Receiver operating characteristic curve analysis of plasma levels of activated thrombin-activated fibrinolysis inhibitor in patients with chronic thromboembolic pulmonary hypertension with the genotypes CC and any T.
The area under the curve (AUC) was 0.804, and 95% CI was 0.687 to 0.921.

PAH-Associated Variants in CTEPH

CTEPH is considered to develop following APE. The pathogenesis of CTEPH after APE remains to be elucidated. Moreover, it has been reported that endothelial dysfunction is commonly involved in the pathogenesis of both CTEPH[5] and PAH.[2] Furthermore, distal pulmonary artery remodeling is distributed in both no-flow and normal-flow lung tissues.[46] Thus, there might be some potential overlap in the pathogenesis of CTEPH and PAH. The genetic background of PAH has been reported previously.[13,31–36] Most common variants in PAH occur in the gene *BMPR2*.[13] BMPR2 belongs to the transforming growth factor-β cell signaling superfamily, and the *BMPR2* variants result in downregulation of Smad signaling in pulmonary arterial smooth muscle cells and in enhancement of proproliferative and antiapoptotic effects, thus promoting the development of PAH.[47] Furthermore, it is known that other gene variants associated with *BMPR2* signaling occur in PAH, and include *ACVRL1*,[33] *ENG*,[31] and *SMAD9*.[32] Moreover, the variants of *CAV1*,[34] *KCNK3*,[35] and *CBLN2*[36] have been found in patients with PAH using whole exome screening.

There have been few reports describing the correlation between the variants associated with PAH and CTEPH.[19–21] Although variants of *BMPR2* were not identified in 16 patients with CTEPH examined in the first report,[21] several PAH-associated variants were subsequently identified in 47 patients with CTEPH.[20] In the latter report, the allele frequencies of the identified variants in patients with CTEPH were compared with those in patients with APE without pulmonary hypertension. The present study comprised a large prospective genome cohort in the Tohoku area of northeast Japan. As all patients with CTEPH in the present study were enrolled from the same area, we were able to precisely compare the allele frequency of the variants in patients with CTEPH with that of the general population.[48] In the present study, any specific variants associated with PAH were not detected in patients with CTEPH compared with the general population.

Taken together, the genetic backgrounds of patients with CTEPH appear to be different from those of patients with PAH.

APE-Associated Variants in CTEPH

CTEPH is believed to be caused primarily by APE. Recently, it was reported that 79.8% of patients with CTEPH had a history of APE.[48] On the other hand, it was reported that the frequency of a history of APE was different between Japan and other countries (37.2% versus 74.8%, respectively).[49] In the present study, 45.1% of patients with CTEPH had a history of APE.[3] Thus, a limited percentage of patients with CTEPH have a history of APE. Moreover, only a few specific thrombophilic factors, such as antiphospholipid antibodies,[6] factor V Leiden,[7] and von Willebrand factor,[7] are associated with CTEPH. Indeed, it remains to be examined whether patients with CTEPH, especially those without a history of APE, have some APE-associated variants. In the present study, we were able to identify APE-associated variants in patients with CTEPH. Among these, several variants of factor V were identified especially in patients with CTEPH with a history of APE. Factor V, which consists of A1, A2, B, A3, C1, and C2 domains, plays a key role in blood coagulation.[50] Domain B is removed from factor V with prothrombinase, which allows factor V to be activated (factor Va). Factor Va promotes the coagulation cascade, which is inactivated by APC (activated protein C). Factor V Leiden, which is associated with APE, is considered a risk factor of CTEPH.[7] In the present study, factor V Leiden was not found in the Japanese population, as described in a previous report.[22] Nevertheless, we identified 2 variants (c.3980A>G and c.6665A>G), which might affect the function of factor V based on in silico analysis. The variant c.3980A>G is located in the B domain of factor V, which is removed on activation of factor V. Moreover, this variant was not located in the conserved structure domain. Thus, this variant may not affect the function of factor V.[50] Conversely, c.6665A>G is located in the C2 domain of factor V,[50] and this domain is essential for membrane binding of factor V and promotes APC resistance.[50] Moreover, this variant was located in the conserved structure domain. Indeed, it has been reported that the

frequency of the variants in this domain was higher in patients with venous thromboembolism.[51,52] Thus, the present study demonstrates that patients with CTEPH, especially those with a history of APE, share the same genetic background of APC resistance with patients with APE.

Variants of Thrombomodulin and Activation of TAFI

Thrombomodulin, which is encoded by *THBD*, is a transmembrane protein that is constitutively expressed on the luminal surface of vascular endothelial cells.[53] Although thrombomodulin plays a role in anticoagulation by APC, it also exerts antifibrinolytic effects by activation of TAFI.[11] TAFI is activated by the thrombin-thrombomodulin complex on the surface of endothelial cells, and TAFIa attenuates fibrinolysis by removing the C-terminal lysines from fibrin. Thus, thrombomodulin plays a crucial role in the activation of TAFI and antifibrinolysis. Recently, we reported that the serum levels of TAFIa were significantly increased in patients with CTEPH than in healthy controls.[10,11] Moreover, TAFIa enhanced organized thrombus formation in the pulmonary arteries, promoting wall thickening of the distal pulmonary arteries in a mouse model of CTEPH.[11] Thus, the interaction between TAFI and thrombomodulin plays a role in the development of CTEPH. In the present study, we also found that the allele frequency of one *THBD* variant (c.1418C>T) was significantly higher in patients with CTEPH without a history of APE. As this variant was located in the conserved structure domain, it may affect the function of thrombomodulin. Interestingly, it was reported that the allele frequency of this variant was higher in patients with deep vein thrombosis.[49] Although we were unable to investigate the association of this variant and plasma levels of soluble thrombomodulin in a separate cohort, it has recently been reported that this variant increased the expression levels of thrombomodulin in endothelial cells and reduced those of soluble thrombomodulin.[40] Consistently, in the present study, the plasma levels of TAFIa were significantly higher in patients with CTEPH with this variant than in those without it. As TAFI is activated by thrombin-thrombomodulin complex on the surface of endothelial cells, this variant may enhance the activation of TAFI and promote the development of CTEPH.

It is known that the cause of Japanese CTEPH is different from that in the rest of the world.[54,55] It was reported that Japanese patients with CTEPH were predominantly women and had less history of APE compared with the rest of the world. Indeed, in the present study, 80% of patients were women and 45.1% of patients had a history of APE. These ratios are similar to those of the previous report from Japan.[54] Thus, the genetic background in Japanese patients might be different from that in the rest of the world. It remains to be examined whether patients with CTEPH in other countries have these variants.

Taken together, we identified variants associated with APE in patients with CTEPH, even in patients with no history of APE, suggesting that CTEPH and APE may have a similar genetic background. Moreover, the present results suggest that the variant of *THBD* leads to activation of TAFI in patients with CTEPH and that TAFIa impairs fibrinolysis in patients with CTEPH. Moreover, patients with CTEPH have some variants that are risk factors of APE. Thus, asymptomatic pulmonary embolism may occur, associated with impaired fibrinolysis with resultant development of CTEPH.

Study Limitations

Several limitations should be mentioned for the present study. First, we were unable to detect all the variants associated with APE or PAH. Second, it was a small population in this study. Third, we were unable to examine the association of long-term prognosis and these variants. The prognosis of CTEPH has been improved by the development of balloon pulmonary angioplasty.[56] A total of 51 patients with CTEPH were treated with balloon pulmonary angioplasty, and all of them survives so far. Fourth, we were unable to compare the read depth of the candidate variants between cases and controls, as individual data of controls were unavailable.

CONCLUSIONS

In the present study, we demonstrated that patients with CTEPH had some variants associated with APE, regardless of the presence or absence of a history of APE. Furthermore, the genetic background might be different between patients with CTEPH with and without a history of APE.

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Disclosures

None.

REFERENCES

1. Pengo V, Lensing AW, Prins MH, Marchiori A, Davidson BL, Tiozzo F, Albanese P, Biasiolo A, Pegoraro C, Iliceto S, et al. Incidence of chronic thromboembolic pulmonary hypertension after pulmonary embolism. *N Engl J Med*. 2004;350:2257–2264.
2. Lang IM, Madani M. Update on chronic thromboembolic pulmonary hypertension. *Circulation*. 2014;130:508–518.
3. Ribeiro A, Lindmarker P, Johnsson H, Juhlin-Dannfelt A, Jorfeldt L. Pulmonary embolism: one-year follow-up with echocardiography Doppler and five-year survival analysis. *Circulation*. 1999;99:1325–1330.
4. Matthews DT, Hemnes AR. Current concepts in the pathogenesis of chronic thromboembolic pulmonary hypertension. *Pulm Circ*. 2016;6:145–154.
5. Sakao S, Tatsumi K. Crosstalk between endothelial cell and thrombus in chronic thromboembolic pulmonary hypertension: perspective. *Histol Histopathol*. 2013;28:185–193.
6. Wolf M, Boyer-Neumann C, Parent F, Eschwege V, Jaillet H, Meyer D, Simonneau G. Thrombotic risk factors in pulmonary hypertension. *Eur Respir J*. 2000;15:395–399.
7. Wong CL, Szydlo R, Gibbs S, Laffan M. Hereditary and acquired thrombotic risk factors for chronic thromboembolic pulmonary hypertension. *Blood Coagul Fibrinolysis*. 2010;21:201–206.
8. Delcroix M, Kerr K, Fedullo P. Chronic thromboembolic pulmonary hypertension: epidemiology and risk factors. *Ann Am Thorac Soc*. 2016;13(suppl 3):S201–S206.
9. Yaoita N, Shirakawa R, Fukumoto Y, Sugimura K, Miyata S, Miura Y, Nochioka K, Miura M, Tatebe S, Aoki T, et al. Platelets are highly activated in patients of chronic thromboembolic pulmonary hypertension. *Arterioscler Thromb Vasc Biol*. 2014;34:2486–2494.
10. Yaoita N, Satoh K, Satoh T, Sugimura K, Tatebe S, Yamamoto S, Aoki T, Miura M, Miyata S, Kawamura T, et al. Thrombin-activatable fibrinolysis inhibitor in chronic thromboembolic pulmonary hypertension. *Arterioscler Thromb Vasc Biol*. 2016;36:1293–1301.
11. Satoh T, Satoh K, Yaoita N, Kikuchi N, Omura J, Kurosawa R, Numano K, Al-Mamun E, Siddique MA, Sunamura S, et al. Activated TAFI promotes the development of chronic thromboembolic pulmonary hypertension: a possible novel therapeutic target. *Circ Res*. 2017;120:1246–1262.
12. Bouma BN, Meijers JC. Thrombin-activatable fibrinolysis inhibitor (TAFI, plasma procarboxypeptidase B, procarboxypeptidase R, procarboxypeptidase U). *J Thromb Haemost*. 2003;1:1566–1574.
13. International PPH Consortium, Lane KB, Machado RD, Pauciulo MW, Thomson JR, Phillips JA III, Loyd JE, Nichols WC, Trembath RC. Heterozygous germline mutations in BMPR2, encoding a TGF-β receptor, cause familial primary pulmonary hypertension. *Nat Genet*. 2000;26:81–84.
14. Ma L, Chung WK. The genetic basis of pulmonary arterial hypertension. *Hum Genet*. 2014;133:471–479.
15. Austin ED, Loyd JE. The genetics of pulmonary arterial hypertension. *Circ Res*. 2014;115:189–202.
16. Hemnes AR, Zhao M, West J, Newman JH, Rich S, Archer SL, Robbins IM, Blackwell TS, Cogan J, Loyd JE, et al. Critical genomic networks and vasoreactive variants in idiopathic pulmonary arterial hypertension. *Am J Respir Crit Care Med*. 2016;194:464–475.
17. Kataoka M, Momose Y, Aimi Y, Fukuda K, Gamou S, Satoh T. Familial chronic thromboembolic pulmonary hypertension in a pair of Japanese brothers. *Chest*. 2016;150:748–749.
18. Desmarais J, Elliott CG. Familial chronic thromboembolic pulmonary hypertension. *Chest*. 2016;149:e99–e101.
19. Newnham M, South K, Bleda M, Auger WR, Barberà JA, Bogaard H, Bunclark K, Cannon JE, Delcroix M, Hadinnapola C, et al. The ADAMTS13-VWF axis is dysregulated in chronic thromboembolic pulmonary hypertension. *Eur Respir J*. 2019;28:53.
20. Xi Q, Liu Z, Zhao Z, Luo Q, Huang Z. High frequency of pulmonary hypertension-causing gene mutation in Chinese patients with chronic thromboembolic pulmonary hypertension. *PLoS One*. 2016;11:e0147396.
21. Ulrich S, Szamalek-Hoegel J, Hersberger M, Fischler M, Garcia JS, Huber LC, Grünig E, Janssen B, Speich R. Sequence variants in BMPR2 and genes involved in the serotonin and nitric oxide pathways in idiopathic pulmonary arterial hypertension and chronic thromboembolic pulmonary hypertension: relation to clinical parameters and comparison with left heart disease. *Respiration*. 2010;79:279–287.
22. Tanabe N, Kimura A, Amano S, Okada O, Kasahara Y, Tatsumi K, Takahashi M, Shibata H, Yasunami M, Kuriyama T. Association of clinical features with HLA in chronic pulmonary thromboembolism. *Eur Respir J*. 2005;25:131–138.
23. Kominami S, Tanabe N, Ota M, Naruse TK, Katsuyama Y, Nakanishi N, Tomoike H, Sakuma M, Shirato K, Takahashi M, et al. HLA-DPB1 and NFKBILL may confer the susceptibility to chronic thromboembolic pulmonary hypertension in the absence of deep vein thrombosis. *J Hum Genet*. 2009;54:108–114.
24. Chen S, Luan H, Li L, Zeng X, Wang T, Li Y, Yuan H. Relationship of HLA-B51 and HLA-B52 alleles and TNF-α-308A/G polymorphism with susceptibility to Takayasu arteritis: a meta-analysis. *Clin Rheumatol*. 2017;36:173–181.
25. Hoeper MM, Bogaard HJ, Condliffe R, Frantz R, Khanna D, Kurzyna M, Langleben D, Manes A, Satoh T, Torres F, et al. Definitions and diagnosis of pulmonary hypertension. *J Am Coll Cardiol*. 2013;62:D42–D50.
26. Katsuoka F, Yokozawa J, Tsuda K, Ito S, Pan X, Nagasaki M, Yasuda J, Yamamoto M. An efficient quantitation method of next-generation sequencing libraries by using MiSeq sequencer. *Anal Biochem*. 2014;466:27–29.
27. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, Altshuler D, Gabriel S, Daly M, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res*. 2010;20:1297–1303.
28. Naumnik B, Borawski J, Pawlak K, Myśliwiec M. Renal function, proteinuria and ACE-inhibitor therapy as determinants of plasma levels of endothelial markers. *Nephrol Dial Transplant*. 2002;17:526–528.
29. Ohdama S, Takano S, Miyake S, Kubota T, Sato K, Aoki N. Plasma thrombomodulin as a marker of vascular injuries in collagen vascular diseases. *Am J Clin Pathol*. 1994;101:109–113.
30. Nagasaki M, Yasuda J, Katsuoka F, Nariai N, Kojima K, Kawai Y, Yamaguchi-Kabata Y, Yokozawa J, Danjoh I, Saito S, et al. Rare variant discovery by deep whole-genome sequencing of 1,070 Japanese individuals. *Nat Commun*. 2015;6:8018.
31. Chaouat A, Coulet F, Favre C, Simonneau G, Weitzenblum E, Soubrier F, Humbert M. Endoglin germline mutation in a patient with hereditary haemorrhagic telangiectasia and dexfenfluramine associated pulmonary arterial hypertension. *Thorax*. 2004;59:446–448.
32. Drake KM, Dunmore BJ, McNelly LN, Morrell NW, Aldred MA. Correction of nonsense BMPR2 and SMAD9 mutations by ataluren in pulmonary arterial hypertension. *Am J Respir Cell Mol Biol*. 2013;49:403–409.
33. Seki T, Hong KH, Yun J, Kim SJ, Oh SP. Isolation of a regulatory region of activin receptor-like kinase 1 gene sufficient for arterial endothelium-specific expression. *Circ Res*. 2004;94:e72–e77.
34. Austin ED, Ma L, LeDuc C, Berman Rosenzweig E, Borczuk A, Phillips JA III, Palomero T, Sumazin P, Kim HR, Talati MH, et al. Whole exome sequencing to identify a novel gene (caveolin-1) associated with human pulmonary arterial hypertension. *Circ Cardiovasc Genet*. 2012;5:336–343.
35. Kuhr FK, Smith KA, Song MY, Levitan I, Yuan JX. New mechanisms of pulmonary arterial hypertension: role of Ca^{2+} signaling. *Am J Physiol Heart Circ Physiol*. 2012;302:H1546–H1562.
36. Germain M, Eyries M, Montani D, Poirier O, Girerd B, Dorfmüller P, Coulet F, Nadaud S, Maugenre S, Guignabert C, et al. Genome-wide

association analysis identifies a susceptibility locus for pulmonary arterial hypertension. *Nat Genet*. 2013;45:518–521.
37. de Jesus Perez VA, Yuan K, Lyuksyutova MA, Dewey F, Orcholski ME, Shuffle EM, Mathur M, Yancy L Jr, Rojas V, Li CG, et al. Whole-exome sequencing reveals TopBP1 as a novel gene in idiopathic pulmonary arterial hypertension. *Am J Respir Crit Care Med*. 2014;189:1260–1272.
38. Welch CL, Chung WK. Genetics and other omics in pediatric pulmonary arterial hypertension. *Chest*. 2020;157:1287–1295.
39. Corral J, Hernandez-Espinosa D, Soria JM, Gonzalez-Conejero R, Ordonez A, Gonzalez-Porras JR, Perez-Ceballos E, Lecumberri R, Sanchez I, Roldan V, et al. Antithrombin Cambridge II (A384S): an underestimated genetic risk factor for venous thrombosis. *Blood*. 2007;109:4258–4263.
40. Navarro S, Medina P, Bonet E, Corral J, Martínez-Sales V, Martos L, Rivera M, Roselló-Lletí E, Alberca I, Roldán V, et al. Association of the thrombomodulin gene c.1418C>T polymorphism with thrombomodulin levels and with venous thrombosis risk. *Arterioscler Thromb Vasc Biol*. 2013;33:1435–1440.
41. Eichinger S, Heinze G, Jandeck LM, Kyrle PA. Risk assessment of recurrence in patients with unprovoked deep vein thrombosis or pulmonary embolism: the Vienna prediction model. *Circulation*. 2010;121:1630–1636.
42. Austin H, De Staercke C, Lally C, Bezemer ID, Rosendaal FR, Hooper WC. New gene variants associated with venous thrombosis: a replication study in White and Black Americans. *J Thromb Haemost*. 2011;9:489–495.
43. Morange PE, Oudot-Mellakh T, Cohen W, Germain M, Saut N, Antoni G, Alessi MC, Bertrand M, Dupuy AM, Letenneur L, et al. KNG1 Ile581Thr and susceptibility to venous thrombosis. *Blood*. 2011;117:3692–3694.
44. Nizankowska-Mogilnicka E, Adamek L, Grzanka P, Domagala TB, Sanak M, Krzanowski M, Szczeklik A. Genetic polymorphisms associated with acute pulmonary embolism and deep venous thrombosis. *Eur Respir J*. 2003;21:25–30.
45. Medina P, Navarro S, Bonet E, Martos L, Estellés A, Bertina RM, Vos HL, España F. Functional analysis of two haplotypes of the human endothelial protein C receptor gene. *Arterioscler Thromb Vasc Biol*. 2014;34:684–690.
46. Moser KM, Bloor CM. Pulmonary vascular lesions occurring in patients with chronic major vessel thromboembolic pulmonary hypertension. *Chest*. 1993;103:685–692.
47. Yang X, Long L, Southwood M, Rudarakanchana N, Upton PD, Jeffery TK, Atkinson C, Chen H, Trembath RC, Morrell NW. Dysfunctional Smad signaling contributes to abnormal smooth muscle cell proliferation in familial pulmonary arterial hypertension. *Circ Res*. 2005;96:1053–1063.
48. Mayer E, Jenkins D, Lindner J, D'Armini A, Kloek J, Meyns B, Ilkjaer LB, Klepetko W, Delcroix M, Lang I, et al. Surgical management and outcome of patients with chronic thromboembolic pulmonary hypertension: results from an international prospective registry. *J Thorac Cardiovasc Surg*. 2011;141:702–710.
49. Sugiyama S, Hirota H, Kimura R, Kokubo Y, Kawasaki T, Suehisa E, Okayama A, Tomoike H, Hayashi T, Nishigami K, et al. Haplotype of thrombomodulin gene associated with plasma thrombomodulin level and deep vein thrombosis in the Japanese population. *Thromb Res*. 2007;119:35–43.
50. Vos HL. Inherited defects of coagulation factor V: the thrombotic side. *J Thromb Haemost*. 2006;4:35–40.
51. van der Neut KM, Dirven RJ, Vos HL, Bertina RM. The R2-haplotype associated Asp2194Gly mutation in the light chain of human factor V results in lower expression levels of FV, but has no influence on the glycosylation of Asn2181. *Thromb Haemost*. 2003;89:429–437.
52. Alhenc-Gelas M, Nicaud V, Gandrille S, van Dreden P, Amiral J, Aubry ML, Fiessinger JN, Emmerich J, Aiach M. The factor V gene A4070G mutation and the risk of venous thrombosis. *Thromb Haemost*. 1999;81:193–197.
53. Esmon CT, Owen WG. Identification of an endothelial cell cofactor for thrombin-catalyzed activation of protein C. *Proc Natl Acad Sci USA*. 1981;78:2249–2252.
54. Tanabe N, Sugiura T, Tatsumi K. Recent progress in the diagnosis and management of chronic thromboembolic pulmonary hypertension. *Respir Investig*. 2013;51:134–146.
55. Pepke-Zaba J, Delcroix M, Lang I, Mayer E, Jansa P, Ambroz D, Treacy C, D'Armini AM, Morsolini M, Snijder R, et al. Chronic thromboembolic pulmonary hypertension (CTEPH): results from an international prospective registry. *Circulation*. 2011;124:1973–1981.
56. Aoki T, Sugimura K, Tatebe S, Miura M, Yamamoto S, Yaoita N, Suzuki H, Sato H, Kozu K, Konno R, et al. Comprehensive evaluation of the effectiveness and safety of balloon pulmonary angioplasty for inoperable chronic thromboembolic pulmonary hypertension: long-term effects and procedure-related complications. *Eur Heart J*. 2017;38:3152–3159.