Original article

Genetic analysis of antithrombin deficiency among Thai patients with thrombosis

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Abstract:

Introduction: *Hereditary antithrombin (AT) deficiency, primarily caused by mutations of SERPINC1 mutations, is a strong risk factor for occurrences and recurrences of venous thrombosis. While AT activity level measurements may be interfered by several factors, genetic analysis is more definitive for diagnosis.* ***Objective:*** *This study aims to address the knowledge gap of genetic basis of AT deficiency among Thai patients.* ***Materials and methods:*** *Samples were collected from patients with thrombosis and low AT activities. Cases with acquired causes of low AT were excluded. All 7 exons of the SERPINC1 gene were PCR amplified and subjected to Sanger sequencing.* ***Results:*** *The study included 15 patients and 6 controls with normal AT activity levels. The results revealed the presence of 6 benign single nucleotide polymorphisms (SNPs) located in intron 1, 2, exon 5 and intron 5. Two of these SNPs in intron 2 were first described here. Notably, a novel SNP in exon 3, SERPINC1 c.570 C>G (p.Y190X), was discovered. This is likely pathogenic because the premature stop codon led to a non-functional protein.* ***Conclusion:*** *The results suggest that pathogenic SERPINC1 variants detectable by DNA sequencing are uncommon among Thai patients with low AT activity levels underscoring the role of genetic analysis for definitive diagnosis. Further studies involving larger numbers of patients are required.*

Keywords : ● *Antithrombin deficiency* ● *SERPINC1 gene mutations* ● *Thrombosis* ● *Thai population* ● *Genetic variations*

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Introduction

Antithrombin (AT), predominantly produced by the liver, plays the key role in the anticoagulation system. As a member of the serine protease inhibitor (Serpin) superfamily, AT inhibits mainly thrombin and factor Xa. The AT protein structure contains 432 amino acids comprising four glycosylation sites.[1] It features the reactive center loop (RCL)[2,3] and the heparin-binding domain, enhancing AT's inhibitory activity upon heparin binding.[4]

AT regulates coagulation by binding to glycosaminoglycans on intact endothelium, preventing inappropriate clot formation and averting pathologic conditions, such as deep vein thrombosis (DVT), pulmonary embolism (PE) and stroke.[5] Egeberg et al. reported the first case of familial AT deficiency, associated with recurrent venous thrombosis in 1965.[6] Hereditary AT deficiency is an autosomal dominant disorder caused by mutations in the *SERPINC1* gene on chromosome 1 containing 7 exons.[6] Homozygous mutations of this gene cause potentially fatal disseminated thrombosis in neonates, termed purpura fulminans. Heterzygotes typically experience venous thrombosis since young adults. The International Society on Thrombosis and Haemostasis (ISTH) classifies AT deficiency in Type I (quantitative) and Type II (qualitative) disorders. The latter type is involved in defects in the reactive site, heparin-binding site or pleiotropic effects.[7]

The levels of AT activity are usually determined using the chromogenic method. However, the test can be interfered by various conditions, such as acute thrombosis, liver dysfunction, kidney diseases and the use of heparin anticoagulants. Genetic testing provides a more accurate diagnosis, crucial for managing this condition.

There has been no comprehensive database on *SERPINC1* gene mutations in the Thai population. The incidence of thrombosis in Asia is lower than that in Western nations, but it is increasing.[8] This study aims to investigate *SERPINC1* mutations in AT-deficient samples from Thai patients, filling the knowledge gap. Identification of common genetic variants among Thais would be helpful to design a cost-effective DNA sequencing strategy and interpret the sequencing results in our population.

Material and methods

Sample selection

This study included patients with thrombosis and low AT activity levels from King Chulalongkorn Memorial Hospital. All patients with low AT activity levels were enrolled, excluding those with conditions that could cause falsely low AT activity levels. The exclusion criteria included patients with disseminated intravascular coagulation (DIC), liver cirrhosis, nephrotic syndrome or treated with heparin/low molecular weight heparin (LMWH) from September 2022 to December 2023. Six controls were healthy volunteers with their AT activity levels falling within the normal range of 75 to 125 U/dL without thrombosis or related conditions. The controls were used to determine if the new variants were pathogenic. (Figure 1)

Antithrombin assay

Venous blood (5.4 mL) was drawn in tubes containing 0.105 mM trisodium citrate (9:1, v/v). Samples were centrifuged at 2,500g for 15 minutes, and the supernatant was collected. The SIEMENS BERICHROM AT III chromogenic activity assay used heparin cofactor-independent lyophilized reagents and a bovine thrombin substrate. The test exhibited no interference by anti-FXa anticoagulants, e.g. rivaroxaban. External quality assurance (EQA) was provided by UK NEQAS Blood Coagulation.

Primer Design

The reference sequence for the *SERPINC1* gene was accessed through the National Institutes of Health website (https://www.ncbi.nlm.nih.gov/nuccore/NG_012462.1). Eight primer pairs were designed to amplify DNA fragments encompassing both exonic and intronic regions (Table 1).

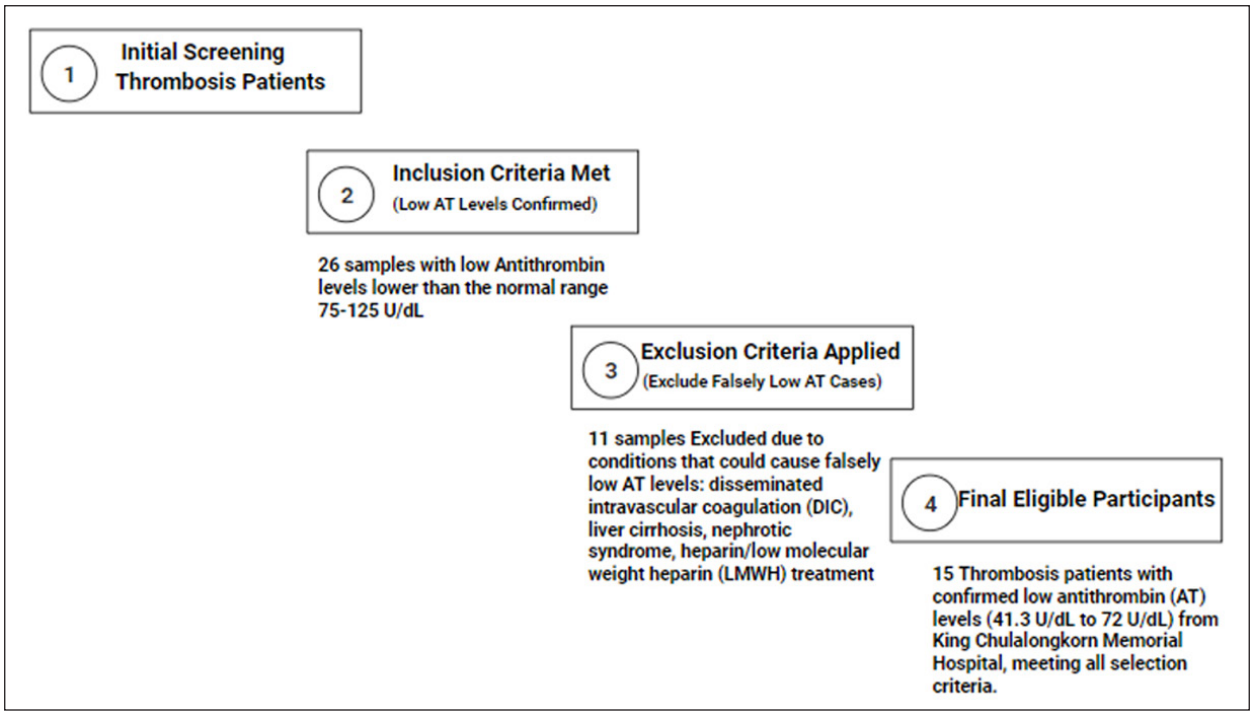

Figure 1 Flow diagram showing the 15 participants with the inclusion and exclusion criteria

Table 1 Primer pairs for *SERPINC1* gene amplification

	Forward primer	Reverse primers
Exon 1	CAACACTGGGCTCTACACTTTGCT	GATGACATCCCCCTTGTTTCTTAGGC
Intron 2	CTGCTTCCAAACCACACATGTT	TTTTCCTGATTTTGCTCCAATAGGCAC
Exon 2	GCACATAATACTCCAAATAACCATCAGAT	AAAGGGAATCGTAATGCATAATGAGC
Exon 3	ACTGACCAGCATGTGCTCA	TAACTTTTAGTCAGCCCTCCAGC
Exon 4	ATGGCTTCTTAATCAAATGGTGGGA	GCACATAATACTCCAAATAACCATCAGAT
Exon 5	CAGCCATTGTTAACCTTTTTGTGC	TCCTTTCTATTCTTTCTCCAA
Exon 6	ATTCATTGTGAGAGTATGATTAGGTGAAGA	TCCCTAAATGTTTGTGTTCCCG
Exon 7	TTTGAGGAATTGCTGTGTCTGTG	AGCATGTTTCCCCTTTCCTTG

DNA Preparation, PCR Amplification and Sanger Sequencing

The remaining blood from the AT activity assay was used for DNA extraction using the silica method (QIAamp DNA Blood Mini Kit by QIAGEN). Polymerase chain reaction (PCR) amplification was performed using the EconoTaq® PLUS PCR Optimization Kit by LGC, following the manufacturer's instructions and in-lab optimized conditions detailed in Table 2. PCR products underwent electrophoresis on 1% agarose gel. Purification was performed using GeneJET PCR Purification Kits (#K0701 and #K0702) by Thermo Fisher Scientific. The OD260/280 ratio and NanoDropTM Spectrophotometer were used to determine the purity of the PCR products. Sequencing was carried out using the Sanger method. DNA variations in the *SERPINC1* gene were analyzed by comparing sequencing chromatograms with the reference sequence from the GenBank database using BioEdit Software.

Results

The clinical characteristics of 15 patients are shown in Table 3. Eight (53.3%) were female with a mean age of 49 years ranging from 15 to 87 years and an mean AT activity level of 56 U/dL ranging from 41 to 72 U/dL (normal range 75 to 125 IU/dL) Venous thrombosis accounted for 67% of cases, while arterial thrombosis accounted for 33%.

Table 2 The PCR condition optimized for the 8 pairs of primers

PCR Condition:			
Time	**Temperature (°C)**	**Condition**	**Step**
3 min	94	Pre-denaturation (1 round)	1
Target gene amplification (35 rounds)			2
30 sec	94	1 Denaturation	
30 sec	60	2 Annealing	
30 sec	72	3 Extension	
10 min	72	The final extension (1 round)	3

Table 3 Clinical characteristics of the patients in this study

ID	AT activity (75-125 U/dL)	Diagnosis	Sex	Age (Years)
1	43	Deep vein thrombosis	Male	71
2	55	Pulmonary embolism	Male	36
3	57	Stroke	Female	79
4	60	Stroke in the young	Male	15
5	72	Pulmonary embolism	Male	18
6	66	Stroke and ischemic colitis	Female	87
7	72	Stroke in the young	Female	46
8	52	Mesenteric vein thrombosis	Male	74
9	56	Pulmonary embolism	Female	68
10	41.3	Deep vein thrombosis. His father also had thrombosis	Male	29
11	70	Stroke in the young	Female	33
12	51	Pulmonary embolism and deep vein thrombosis	Female	25
13	71	Pulmonary embolism	Male	78
14	50	Portal vein thrombosis	Female	43
15	58	Mesenteric vein thrombosis. Her mother had DVT.	Female	31

The PCR products were displayed in gel electrophoresis (Figure 2). All PCR products were sequenced. Analysis of collected data revealed 3 SNPs in intron 1, 2, one in exon 3, two in exon 5 and one in intron 5.

The summary of variants is listed in Table 4. The variant RS2227589 was described as benign and was found in controls. Deletional and insertional variants were noted in intron 2, g.9610delA and g.9623_9624insA, respectively, which have not been previously described.

Sample No. 12 was from a young female diagnosed with unprovoked PE at 25. She received anticoagulant therapy for six months but then was lost to follow-up. Five years later, at the age of 30, she developed DVT in her left leg during her 36th week of pregnancy. The analysis of this sample revealed the mutation c.570 C>G in exon 3 (Figure 3), resulting in a premature stop codon (p.Y190X).

Two synonymous mutations, RS5877 and RS5878, were identified in exon 5. Neither altered the protein sequence. These 2 SNPS were also detectable in a proportion of the controls (Table 4). Finally, another previously benign SNPs, RS2759328, was found in intron 5.

Table 4 Summary of the variants found in this study

Location	Variant	Patient (n = 15)	Control (n = 6)	Total (n = 21)
INTRON1	RS2227589 (g.5301G>A)	4 (26.7%)	2 (33.3%)	6
INTRON2	Not reported (g.9610delA)	1 (6.7%)	0 (0.0%)	1
INTRON2	Not reported (g.9623_9624insA)	1 (6.7%)	0 (0.0%)	1
EXON3	Not reported (p.Y190X)	1 (6.7%)	0 (0.0%)	1
EXON5	RS5877 (g.12655A>G or p.V333V)	11 (73.3%)	3 (50.0%)	14
EXON5	RS5878 (g.12665A>G or Q336Q)	7 (46.7%)	3 (50.0%)	10
INTRON5	RS2759328 (g.14812G>A)	5 (33.3%)	2 (33.3%)	7

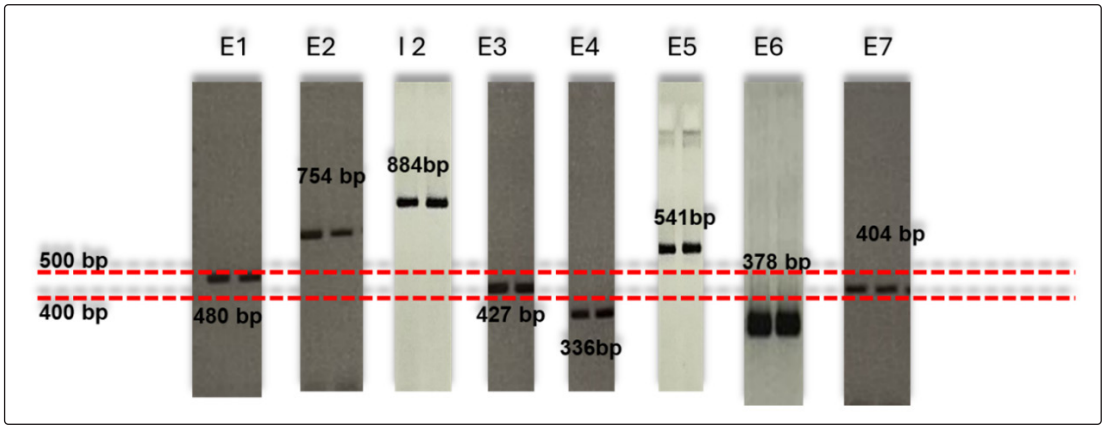

Figure 2 Gel electrophoresis showing the PCR products of all 7 exons and intron 2. E1-E7: Exon 1 to Exon 7, I2: Intron 2. The molecular weight markers are labelled as dotted lines.

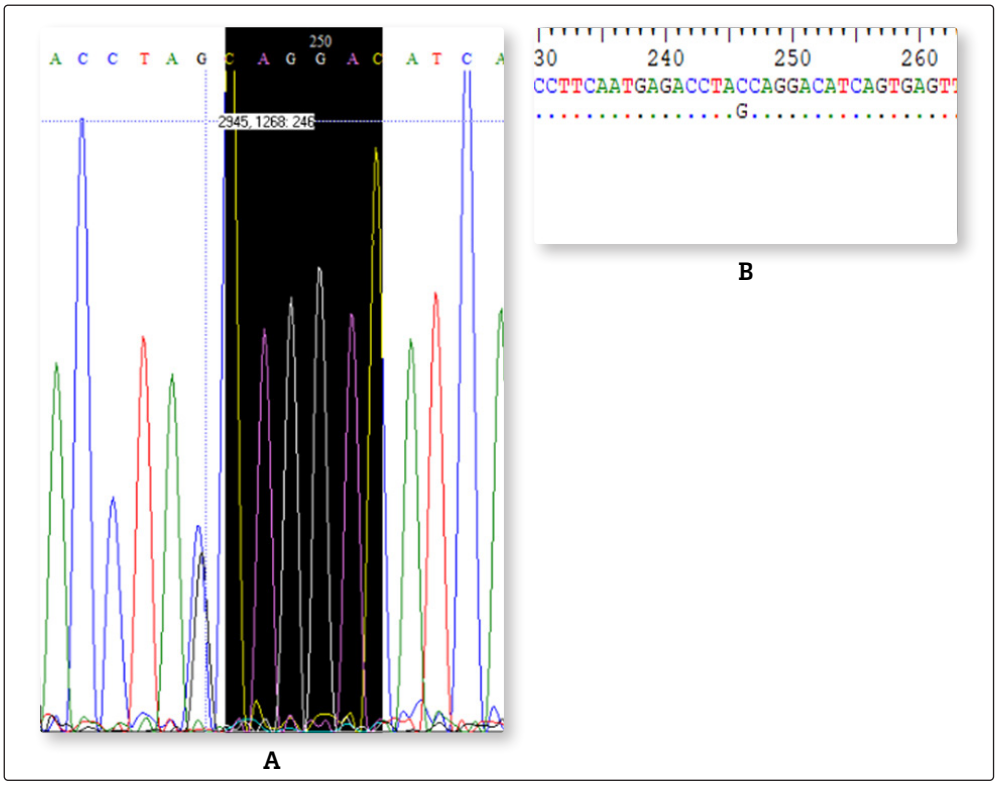

Figure 3 The BioEdit program displaying the alignment diagram (**A**) of the Exon 3 SNP (c.570 C>G) as shown at the crossline position and the SNP sequence (**B**). The upper line represents the consensus sequence and the lower line is the patient sequence.

Discussion

This study found only one likely pathogenic mutation (6.7%) using Sanger sequencing among 15 patients clinically diagnosed as AT deficiency. The causes of low AT in the remaining 14 cases remained to be determined, either congenital or acquired. This demonstrated the significance of genetic testing for definitely diagnosing hereditary AT deficiency.

This study discovered a novel mutation *SERPINC1*p.Y190X in exon 3, resulting in a truncated and likely nonfunctional protein. This comprised the young patient who had recurrent VTE suggesting the presence of thrombophilia. This underscores the importance of genetic analysis for the diagnostic accuracy and management of this condition. Additionally, Deletional (g.9610delA) and insertional (g.9623_9624insA) variants located in intron 2 were identified for the first time. These variants were found to be distant from the splicing regions, suggesting they are likely nonpathogenic.

We used ClinVar as the primary reference to identify genetic variants. The benign variants found included rs2227589 (C>A/C>T) in intron 1; rs5877 (T>A/T>C), a benign synonymous variant; rs5878 (T>A/T>C), a benign missense variant and rs2759328 (C>A/C>T), a benign intron 5 variant. This information may be helpful to interpret *SERPINC1* sequencing data in the Thai population in the future.

Four cases (26.6%) contained the SNP rs2227589 variants, identified in a related study conducted in a Chinese population. The Chinese study demonstrated a significant association between rs2227589 SNP in the promoter region of intron 1, described as a mild thrombotic risk factor and an increased risk of PE. This SNP is significantly associated with lower anticoagulant activity. However, we also detected this variant in a similar proportion of the controls questioning its importance in regulating AT activity levels.[9]

Wang et al. investigated the correlation between SNPs of the AT gene (*SERPINC1*) and peri-operative sensitivity to heparin among Han, Uighur and Kazakh patients undergoing heart surgery. The study found that Han patients exhibited a higher frequency of g.981A>G (rs5877) and g.1011A>G (rs5878) variants, present in 11 and 7 samples, respectively. Although these mutations did not alter protein structure, they might be associated with longer coagulation times and higher dosages of heparin and protamine required among Han patients. Anyway, we also found these SNPs in controls with normal AT activity levels suggesting that they were nonpathogenic.

Cases without detectable pathogenic mutations might have low AT from acquired conditions that did not increase the risk of thrombosis, although these conditions were not written in their medical records. In addition, acute extensive VTE may cause acquired AT deficiency. This constitutes a limitation of this study as patients with acute thrombosis were not excluded. Alternatively, Sanger sequencing might have missed some pathogenic variants, e.g., large structural variants (deletions and duplications).[10] or variants in non-coding regions critical for gene regulation. Furthermore, defects in glycosylation with no amino acid change have been reported to cause AT deficiency.[10,11] These require more advanced technologies, e.g., long-read sequencing, for detection in the future.[12]

In summary, pathogenic mutation in *SERPINC1* is uncommon among Thai patients with a clinical diagnosis of AT deficiency, emphasizing the need for continued genetic investigations. Future studies should be performed using other methods to detect structural variants and/or glycosylation defects for patients with unidentified variants using Sanger sequencing. Additionally, a larger cohort is required to better characterize the genetic basis of AT deficiency in our population.

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