

Inherited Anti-Thrombin Deficiency in A Malay-Malaysian Family: A Missense Mutation at Nucleotide g.13267C>A aka anti-thrombin Budapest 5 (p.Pro439Thr) of the SERPINC 1 gene

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SUMMARY

Objective: Inherited anti-thrombin deficiency is an autosomal dominant disorder which is associated with increased risk for venous thromboembolism (VTE). This condition is very rare in Malaysia and there has been no documented report. Thus, the aim of the present study is to investigate the type of an inherited anti-thrombin deficiency mutation in a 25-year-old Malay woman who presented with deep vein thrombosis in her first pregnancy.

Methods: DNA was extracted from the patient's blood sample and buccal mucosal swabs from family members. Polymerase chain reaction (PCR) assays were designed to cover all seven exons of the serpin peptidase inhibitor, clade C (antithrombin), member 1 (SERPINC1) gene; and the products were subjected to DNA sequencing. Sequences were referred to NCBI Reference Sequence: NG_012462.1.

Results: A heterozygous substitution mutation at nucleotide position 13267 (CCT->ACT) was identified in the patient and two other family members, giving a possible change of codon 439 (Pro->Thr) also known as anti-thrombin Budapest 5. The genotype was absent in 90 healthy controls.

Conclusion: The study revealed a heterozygous anti-thrombin Budapest 5 mutation in SERPINC 1 giving rise to a possible anti-thrombin deficiency in a Malay-Malaysian family.

KEY WORDS:

Inherited anti-thrombin deficiency, Mutation, SERPINC 1, Venous thromboembolism

INTRODUCTION

The prevalence of venous thromboembolism (VTE) is lower in Asian populations than Caucasian ones¹. Even in a large cohort study involving multi-ethnic patients who settled permanently in the United States, Asians were found to have a very low risk for VTE². Lack of a genetic predisposition towards VTE is believed to be the possible cause for this disparity³.

Inherited anti-thrombin deficiency is an autosomal dominant disorder which is associated with increased risk for VTE. Anti-thrombin is a naturally occurring anticoagulant. It is a serine protease inhibitor that is principally synthesised by the liver and vascular endothelial cells. Its function is to inactivate thrombin and to some extent FIXa, FXa, FXIa and FXIIIa. Anti-thrombin deficiency is classified based on anti-thrombin plasma activity and antigen levels into Type I, a quantitative defect, and Type II, which is a qualitative defect⁴. The gene encoding anti-thrombin (SERPINC1) is mapped to chromosome 1q23-25. It has seven exons and six introns and is 13.5 kilobases long (NCBI Gene ID: 462). Expression and observational studies have indicated that mutations in the SERPINC1 gene, especially in the coding regions, are responsible for anti-thrombin deficiency^{5,6}. More than 200 heterogenous mutations have been documented in the SERPINC 1 gene. The mutations are mostly point mutations and small deletions/insertions^{4,7}.

Inherited anti-thrombin deficiency has been estimated to occur in 0.15% of individuals in healthy Japanese populations⁸, a figure that is relatively similar to that for Caucasian populations⁹. For Asian populations, case reports on inherited anti-thrombin deficiency have confined to Japanese⁷, Chinese¹⁰ and Korean¹¹ families. There has been no documented report from South-East Asia, including amongst Malay-Malaysians.

The present research describes the clinical features and results of molecular genetic studies of a Malay family with possible inherited anti-thrombin deficiency. The proband was a Malay woman who presented with deep vein thrombosis (DVT) at 17 weeks of pregnancy.

MATERIALS AND METHODS

Subjects and sample preparations

The patient was a 25-year-old Malay woman (Gravida 1, Para 0). She was hospitalised during the 17th week of pregnancy for severe groin pain and right lower limb swelling. An urgent Doppler ultrasound scan revealed extensive venous thrombosis involving the right

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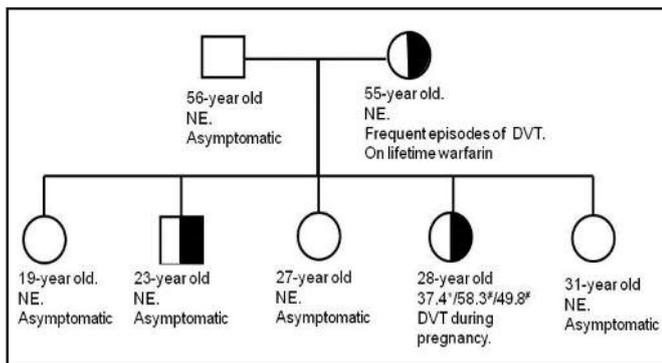


Fig. 1 : Family tree of proband (indicated by an arrow); family members with AT Budapest 5 (p.Pro439Thr) are shaded. Unexplored anti-thrombin assays are indicated by NE. The proband's three repeated anti-thrombin assays are as shown; * was done while proband was on anti-coagulant therapy and pregnant. # Free from thrombosis and anti-coagulant therapy.

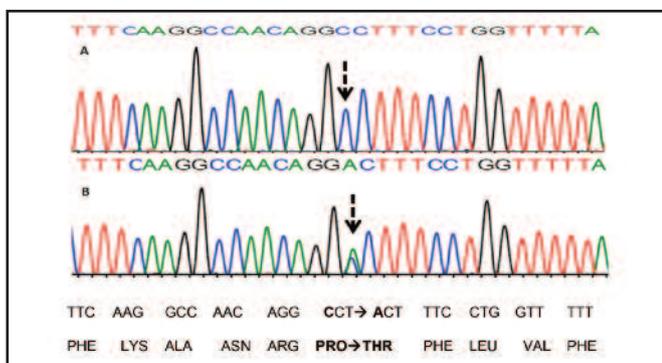


Fig. 3 : Direct sequencing of Exon 7 of SERPINC1 gene with A shows normal allele and B with heterozygous CCT →ACT missense mutation, also known as anti-thrombin Budapest 5 (p.Pro439Thr) as indicated by the arrow.

saphenofemoral junction up to the external iliac vein. A subsequent thrombophilia workup was sent to a tertiary centre, specifically the National Blood Centre (Pusat Darah Negara). This showed an isolated low level of antithrombin activity (37.4%; normal value: 70–142%). The lupus anticoagulant, anti-phospholipid antibody, protein C and S activities and activated protein C resistance assay were within the normal range. The patient was started on low molecular weight heparin (LMWH), which was continued throughout her pregnancy. She had an uneventful spontaneous vaginal delivery at 40 weeks of gestation. The LMWH was subsequently discontinued and repeat Doppler ultrasound showed clearance of the thrombosis. Repeated thrombophilia workups at 4 months and 1 year post-delivery exhibited persistently low levels of anti-thrombin activity, at 58.3% and 49.8%, respectively. All other thrombophilia markers were negative. Doppler ultrasound at the pelvic and lower limbs during these intervals showed no thrombosis.

A family history revealed that the patient's 55-year-old mother had been put on life-long warfarin therapy following several episodes of DVT which began when she was in her late 40s. The underlying diagnosis of the thrombosis was

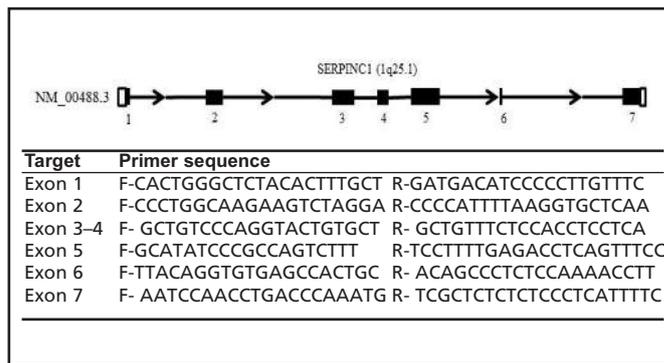


Fig. 2 : Primers used for PCR and direct nucleotide sequencing of the SERPINC 1 gene.

never established. The patient's four other siblings had no history suggestive of VTE. The patient's family tree is as illustrated in Figure 1. The patient and family members provided written informed consent for venous blood sample collection and buccal mucosal swab sample collection, respectively. Samples were collected according to standard procedures. Both molecular analysis and plasma anti-thrombin assay were performed for the patient. As for the family members, only molecular genetic analysis was carried out, as we could not obtain the blood samples for anti-thrombin assay.

Genomic DNA Extraction

Blood was purified for DNA using a QIAmp® DNA Blood kit (Qiagen, Germany) following the manufacturer's instructions. The buccal swab samples were purified using a modified protocol of the above kit.

Polymerase chain reaction

Primers were designed using Primer 3 (v.0.4.0), which is available online (<http://frodo.wi.mit.edu/>) to cover the flanking regions of all seven exons of the SERPINC 1 gene (Figure 2). Polymerase chain reaction (PCR) was run with a total reaction of 50 µL consisting of 1 X PCR buffer, 0.2 mmol/L dNTPs, 0.25 µM forward and reverse primers, 1 U Taq DNA polymerase (Qiagen) and 100–200 ng of DNA template. The cycle conditions included initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 30 s, 60°C for 30 s and 70°C for 30 s, followed by a final extension at 70°C for 10 min.

Direct nucleotide sequencing

PCR products were purified using a standard protocol, cycle sequenced with an ABI BigDye Terminator Kit (Applied Biosystems, US) and analysed using the ABI3130 Genetic Sequencer (Applied Biosystems). PCRs for all seven exons were amplified first on the proband's DNA sample. Once the mutation was identified, the PCR amplification of the involved exon was carried out on the rest of the family members. NCBI Reference Sequences NG_012462.1 and NM_000488.3 are the basis for interpretation of the sequences. The sequences were also blasted against the NCBI database (blast.ncbi.nlm.nih.gov/).

Restriction fragment length polymorphism

We analysed the occurrence of p.Pro439Thr in 90 healthy controls using PCR– restriction fragment length polymorphism (RFLP). These healthy controls were recruited from previous study¹². Primers (F: TGAAGAAGGCAGTGAAGCAG, R: GGCTACTCTGCCCATGAAGA) were used in the PCR reaction to amplify a 154 bp region flanking the mutation site. The PCR products were digested with Stu1 restriction enzyme (New England Biolabs) and resolved by 3.5% agarose gel electrophoresis with ethidium bromide to give products of 154 bp, 91 bp and 63 bp in the presence of this variant.

RESULTS

The results revealed a heterozygous substitution C-to-A transversion on exon 7 of SERPINC1 gene (NM_000488.3) at nucleotide position 13267 (g.13267C>A) in the proband, her mother and her brother (Figure 3). The nomenclature is based on the recommendation by the Human Genome Variation Society (<http://www.hgvs.org/>). The mutation gives a possible change of CCT→ACT at codon position 439 (p.Pro439Thr) and was previously identified as anti-thrombin Budapest 513. The mutation was not found in the 90 healthy individuals.

DISCUSSION

This study describes a Malay family with anti-thrombin deficiency mainly based on the finding of anti-thrombin Budapest mutation in three of the family members, including the proband. The proband was a 25-year-old Malay woman who presented with extensive DVT in the 17th week of pregnancy. Isolated deficiency of anti-thrombin activity in the thrombophilia workup suggested that pregnancy as the sole underlying cause of the thrombotic event was less likely. Moreover, there was a positive family history of several episodes of thrombosis in the mother. The diagnosis of anti-thrombin deficiency was established following repeated thrombophilia workups, including after the completion of subcutaneous low molecular heparin (Clexane) therapy and remission of the thrombosis, taking into account that an active veno-occlusive event may cause temporarily low anti-thrombin activity. The patient's anti-thrombin activity ranged from 37.4% to 58.3%. The reference level formulated by Pusat Darah Negara, who gathered specimens from all over Malaysia for thrombophilia workup, is between 70% and 142% activity. Unfortunately, no anti-thrombin antigen assay was done to practically determine the type of AT deficiency and further affirm the diagnosis. As per the clinical diagnosis, studies have shown that most patients with inherited, heterozygous anti-thrombin deficiency have anti-thrombin activity levels in the range of 40–60%⁶, and this fit the description of our proband.

As inherited anti-thrombin deficiency is extremely rare in Malaysia, molecular genetic analysis was undertaken to investigate a possible molecular defect of SERPINC1, the gene encoding anti-thrombin. In the proband, a p.Pro439Thr missense mutation was identified in the SERPINC1 gene. This heterozygous mutation was also discovered in the patient's mother, who is on long term oral warfarin therapy, and in her male sibling, who is asymptomatic. The p.Pro439Thr

mutation in the SERPINC1 gene was identified as anti-thrombin Budapest 5. The present of a significant history of thrombosis in the proband and her mother, laboratory findings of low anti-thrombin activity and the absence of a p.Pro439Thr missense mutation in 90 healthy individuals indicated that this non-synonymous transversion mutation is a mutation rather than a genetic variation.

This p.Pro439Thr missense mutation was previously reported as pleiotropic due to alteration of the reactive site and its heparin-binding properties¹³. Thus, traditionally, anti-thrombin Budapest 5 would have been considered a Type 2 anti-thrombin deficiency, in which the anti-thrombin antigen is of normal level as opposed to evidencing slightly decreased anti-thrombin activity in plasma¹⁴. However, a more recent study has proposed intermediate features of Type 1 and Type 2 anti-thrombin deficiency based on the proteomic expression finding of a recombinant p.Pro439Thr¹⁵. In the current study, the exact type of anti-thrombin was not determined, as the antigen level of anti-thrombin was not measured.

In prospective studies among patients with inherited anti-thrombin, 60% of the cases had unprovoked first venous thrombotic events, whilst the remaining 40% were related with non-permanent risk factors¹⁶. Our proband belongs to the second group of patients, in which pregnancy was identified as the risk factor for DVT. Without anticoagulant prophylaxis, pregnant women with anti-thrombin deficiency have a significantly high the risk of developing VTE¹⁷. Vicente *et al.*¹⁸ reported that the risks for VTE in pregnant women with anti-thrombin deficiency with and without previous history of thrombosis were as high as 49% and 31%, respectively. Therefore, prophylactic anticoagulant therapy during the patient's subsequent pregnancies is indicated and she should receive adequate counselling on contraception.

It is important to note that the proband's mother, who was also identified as having anti-thrombin Budapest 5, was free from VTE until her late 40s, when she presented with frequent episodes of unprovoked DVT. This conforms with the report that in anti-thrombin deficiency, the risk of VTE increases significantly after the age of 20, and by the age of 50 years old, 50% of individuals with anti-thrombin deficiency will have had an episode of VTE¹⁹. The patient's teenaged brother, who was also positive for a similar mutation, was asymptomatic.

The study has several limitations. Firstly, the anti-thrombin antigenic assay was not measured in the patient. The assay is important to exactly determine the specific type of the anti-thrombin deficiency although the test is not crucial for making the clinical diagnosis of anti-thrombin deficiency. Secondly, since we could not obtain the blood samples, no anti-thrombin activity is measured in the family members. Thus, the definite genotype affect on the phenotype could not be assessed. However, since anti-thrombin Budapest 5 (p.Pro439Thr), is a known mutation, assessing the available anti-thrombin mutation database may able to predict the phenotype nature of this genotype.

CONCLUSION

In conclusion, a young Malay patient with anti-thrombin deficiency was identified to have a non-synonymous missense mutation at nucleotide 13267 (g.13267C>A), also known as anti-thrombin Budapest 5 (p.Pro439Thr), of the SERPINC1 gene. As inherited anti-thrombin deficiency is rare in Malaysia, molecular study was undertaken to establish the diagnosis and to illustrate the inherited nature of the anti-thrombin deficiency in this patient. Although genotype testing generally does not affect management of thrombosis, the genetic findings might be helpful in making some important future clinical decision and counselling of the patient.

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