

# Analysis of the genetic variants associated with recurrent thromboembolism in a patient with hemoglobin H disease following splenectomy: A case report

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**Abstract.** Reports of recurrent thromboembolism in thalassemia, particularly in hemoglobin H (HbH) disease associated with congenital thrombophilic mutations, are scarce. However, several mutations were detected in a 22-year-old woman with HbH disease. The patient experienced the first thrombotic event at the age of 20 years and had four recurrent thromboses in a short time interval, despite receiving anticoagulant treatment. The present study reports a case with six nucleotide substitutions, including a missense 565C>T (Arg189Trp) mutation and two synonymous mutations, 66T>C (Pro22Pro) and 423G>T (Ser141Ser), identified in the protein C gene. The other three mutations, 947G>A (Arg316His), 981A>G (Val327Val), and 775C>A (rs13146272), were identified in the protein S, antithrombin and cytochrome P450, family 4, subfamily V, polypeptide 2 genes, respectively. These findings suggest that if thrombotic events repeatedly occur in a patient with thalassemia, not only the risk factors associated with a hypercoagulable state, but the acquired and congenital thrombophilia should be screened for.

## Introduction

Although splenectomy is always followed by a significant elevation in hemoglobin (Hb) levels in hemoglobin H (HbH) disease, also known as  $\alpha$ -thalassemia intermedia, this procedure is not generally recommended as the majority of patients do not experience adverse effects with Hb levels in a steady state. However, in certain circumstances, such as a baseline Hb <7 g/dl, significant hepatosplenomegaly with or without hypersplenism, abdominal discomfort or growth retardation requiring frequent transfusions (10-12 transfusions per year), splenectomy may be considered (1). Thromboembolic events (TEE) are frequently reported in patients with thalassemia following splenectomy. In a survey involving 56 tertiary referral centers in eight countries, venous thromboembolism (VTE) was observed in 146 of 8,860 patients with thalassemia, of which 134 underwent splenectomy (2). These pathogenic mechanisms could explain the chronic hypercoagulable state observed in thalassemia patients without a spleen, including thrombocytosis and chronic platelet activation, perturbation of the red blood cell membrane, and protein C (PC) and protein S (PS) deficiencies that increase following splenectomy (3,4). Several studies have demonstrated that genetic mutations do not appear to have an important role in the pathogenesis of thrombosis observed in thalassemia (5-8). However, PC and PS mutations are frequent genetic risk factors involved in VTE in the Chinese population. Therefore, the presence of these mutations may aggravate the risk of thrombosis in thalassemia. In support of this hypothesis, the present study reports a case of a patient with HbH disease bearing mutations associated with thrombosis who experienced recurrent VTE.

## Case report

The patient was a 22-year-old Chinese female of Han nationality with no siblings, and was diagnosed with HbH disease at 0.5-years old. In recent years, the Hb level of the patient fluctuated between 40 and 60 g/l, and thus the red blood cell transfusions were frequent, approximately once every 1-2 weeks. The ferritin level ranged from 3,500-6,800 ng/ml (female normal range: 13-150 ng/ml), and therefore, iron chelation therapy was also applied periodically. Since 2012, the patient experienced inflammation pain on the epigastrium and an abdominal ultrasonogram

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*Abbreviations:* Hb, hemoglobin; HbH, hemoglobin H; TEE, thromboembolic events; VTE, venous thromboembolism; PC, protein C; PS, protein S; APTT, activated partial thromboplastin time; PT, prothrombin time; PROC, protein C gene; PROS1, protein S gene; SERPINC1, antithrombin gene; SNPs, single-nucleotide polymorphisms; DVT, deep-vein thrombosis

*Key words:* hemoglobin H disease, recurrent thromboembolism, hypercoagulable state, thrombophilic mutations, Arg189Trp mutation

Table I. Summary of the clinical features of patients with postsplenectomy TE.

Interval between splenectomy and each TE	Type of TE	Postsplenectomy PLT	Postsplenectomy RBC
1 month	Portal vein thrombus	710x10 <sup>9</sup> /l	5.28x10 <sup>12</sup> /l
1 year and 3 months	PE	619x10 <sup>9</sup> /l	5.04x10 <sup>12</sup> /l
1 year and 7 months	Superficial thrombosis of the left long saphenous vein	5.02x10 <sup>12</sup> /l 420x10 <sup>9</sup> /l	
1 year and 9 months	Superficial thrombosis of both long saphenous vein; recurrent PE	357.3x10 <sup>9</sup> /l	5.05x10 <sup>12</sup> /l
1 year and 10 months	Thrombosis of the right cephalic vein and thrombophlebitis	330x10 <sup>9</sup> /l	4.96x10 <sup>12</sup> /l

TE, thromboembolism; PLT, platelet; RBC, red blood cells; PE, pulmonary embolism.

repeatedly showed apparent splenomegaly. Subsequently, the patient underwent a splenectomy in 2013. Following this, the Hb level ranged from 90 to 110 g/l postoperation. However, recurrent infection, particularly cellulitis of the lower limbs, appeared following the surgery. The first thrombotic event, portal vein thrombus, occurred in 2013 at the age of 20 years, only 1 month after the splenectomy. Despite receiving anticoagulant treatment, the patient suffered from another four thrombotic events at set intervals. The details of each TEE are presented in Table I.

Computed tomography of the different sections of the body showed thrombosis of the portal vein, bilateral pulmonary artery, both long saphenous veins and the right cephalic vein (Fig. 1; certain sections are not shown). Recent laboratory studies demonstrated that the PC antigen and activity were 68.2% (normal range, 70-150%) and 51.0% (normal range, 60-140%), and the corresponding values for PS were 61.4% (normal range, 65-135%) and 50.2% (normal range, 76-135%), respectively. Activated partial thromboplastin time, prothrombin time, antithrombin activity, fibrinogen and anticardiolipin were normal, and only D-dimer was elevated (Table II). PC and PS antigens were measured by an enzyme-linked immunosorbent assay using the Human Protein C ELISA kit (AssayPro, St. Charles, MO, USA) and Zymutest Protein S ELISA kit (Hyphen Biomed, sur-Oise, France). PC and antithrombin activities were measured by a substrate method using STA-Stachrom Protein C and STA-Stachrom AT III (Beckman Coulter, Fullerton, CA, USA). PS activity was tested by coagulation method using STA-Staclot Protein S (Beckman Coulter). Other parameters were all detected by automatic coagulation Analyzer in Grade A Tertiary Hospital.

Initial intravenous administration of low molecular weight heparin calcium was followed by warfarin sodium. Subsequently, an oral anticoagulant was continued to maintain the International normalized ratio within a range of 2-3.

Genetic analysis was performed. Isolation of genomic DNA from whole blood was conducted using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and was followed by polymerase chain reaction (PCR) sequencing analysis of the PC gene (PROC), PS gene (PROS1), antithrombin gene (SERPINC1), and several candidate single-nucleotide polymorphisms (SNPs) associated with deep-vein thrombosis (DVT) identified by genome-wide association studies. The detected mutations were confirmed by

Table II. Laboratory data of the patient.

Variables	Patient	Normal range
Protein C activity, %	51	60-140
Protein C antigen, %	68.2	70-150
Protein S activity, %	50.2	76-135
Protein S antigen, %	61.4	65-135
Antithrombin (AT-III), %	125	83-128
Fibrinogen, g/l	4.84	2.00-5.00
D-dimer, ng/ml	856	0-450
APTT, sec	40	25.0-45.0
PT, sec	12.6	10-15
Anticardiolipin antibody	(-)	(-)

APTT, activated partial thromboplastin time; PT, prothrombin time.



Figure 1. Computed tomography of the chest showing thrombosis of the right pulmonary artery (blue arrow).

reverse sequencing and were verified using a second amplicon. The amplified fragments were sequenced on an ABI 3730XL DNA automated sequencer (Applied Biosystems, Thermo Fisher Scientific, Inc., Waltham, MA, USA). The variants were designated according to current nomenclature and the recommendations of the Human Genome Variation Society (HGVS, <http://www.hgvs.org/mutnomen/>). These SNPs were rs6025 (FV c.1691G>A), rs1799963 (FII g.20210G>A), rs5361 (SELE), rs1613662 (GP6) and rs13146272 [cytochrome P450,

Table III. Sequence analysis of the various SNPs.

Gene	Chromosome	Region	SNP	Nucleotide position and mutation	Amino acid change	Functional consequence
PROC	2	Exon 2	rs14430087	66 T>C	Pro22=	Synonymous
	2	Exon 6	rs5936	423 G>T	Ser141=	Synonymous
	2	Exon 7	rs146922325	565 C>T	Arg189Trp	Missense
PROS1	3	Exon 9	rs747259055	947 G>A	Arg316His	Missense
SERPINC1	1	Exon 5	rs5877	981 A>G	Val327=	Synonymous
CYP4V2	4	-	rs13146272	775 C>A	Gln259Lys	Missense

SNP, single-nucleotide polymorphism; PROC, protein C; PROS1, protein S; SERPINC1, antithrombin; CYP4V2, cytochrome P450, family 4, subfamily V, polypeptide 2.

Table IV. Associated citations with the gene variant (rs146922325, c.565C&gt;T, p.Arg189Trp or p.Arg147Trp, R147W) in the protein C gene.

Study, year	No. of deficiencies/total (%)		Odds ratio (95% CI)	P-value	Population	Refs.
	Cases	Controls				
Tsay <i>et al.</i> , 2004	5/116 (4.31)	11/1,292 (0.85)	5.10 (1.7-14.8)	-	Chinese	(10)
Tang <i>et al.</i> , 2012	59/1,003 (5.88)	9/1,031 (0.87)	7.10 (3.50-14.39); adjusted: 7.34 (3.61-14.94) or 7.13 (3.49-14.56)	3.31x10 <sup>-10</sup> ; adjusted: 3.88x10 <sup>-8</sup> or 6.88x10 <sup>-8</sup>	Chinese	(9)
Tang <i>et al.</i> , 2013	68/1,304 (5.21)	12/1,334 (0.90)	6.06 (3.26-11.25)	1.03x10 <sup>-10</sup>	Chinese	(11)

CI, confidence interval.

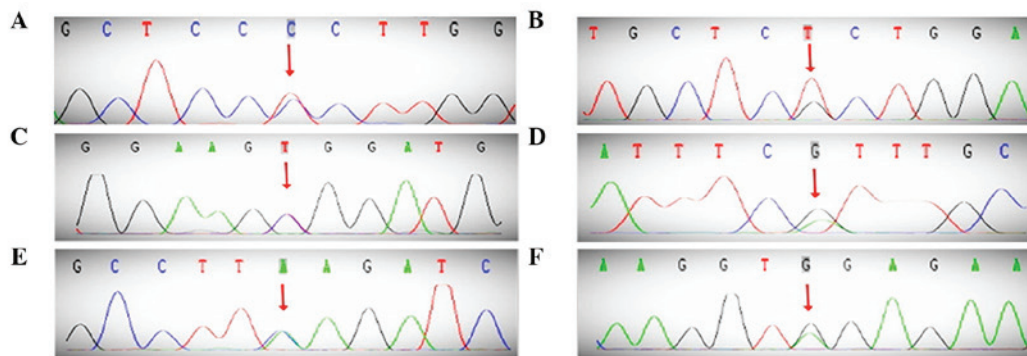


Figure 2. DNA sequence of the PROC, PROS1, SERPINC1 and CYP4V2 genes. (A) 66T>C, (B) 423G>T, (C) 565C>T. (D) PROS1, 947G>A. (E) SERPINC1, 981A>G. (F) CYP4V2, 775C>A. PROC, protein C; PROS1, protein S; SERPINC1, antithrombin; CYP4V2, cytochrome P450, family 4, subfamily V, polypeptide 2.

family 4, subfamily V, polypeptide 2 [CYP4V2]). Subsequent to sequencing all nine exons of PROC, the following three SNPs were identified in exons 2, 6 and 7: rs14430087 (66T>C), rs5936 (423G>T), and rs146922325 (565C>T, Arg189Trp, R147W; Fig. 2). The former two SNPs, rarely reported, are synonymous mutations, while the missense mutation, Arg189Trp, has increasingly been observed in previous studies (9-11). In addition, PROS1 contained an SNP in exon 9, rs747259055 (947G>A, Arg316His). Finally, SERPINC1 had a synonymous

SNP in exon 5, rs5877 (981A>G; Fig. 2). Among the candidate SNPs, rs13146272 was identified in CYP4V2 (Table III).

## Discussion

It is well known that several risk factors have a synergistic effect on the pathogenesis of TEE in thalassemia, however, studies of recurrent TEE in thalassemia, particularly in HbH related to congenital thrombophilic mutations, are limited. In 2002,



Kahn *et al* (12) first described a  $\beta$ -thalassemia major patient with recurrent VTE in association with factor V R506Q and prothrombin G20210A mutations. However, these mutations, which are reported frequently in Caucasians, were not detected in the Chinese Han patient of the present study. PROC, PROS1 and SERPINC1, as well as candidate SNPs, were sequenced in DNA samples from the patient and three missense mutations and three synonymous mutations were identified.

There have been several studies on the inherited anomalies of anticoagulation factors, such as antithrombin, PS and PC, in patients with venous thrombosis in the Chinese population. PC deficiency is associated with an increased risk of VTE, and its genetic background has been analyzed in several populations (13,14). In the present study, rs14430087 (66T>C), rs5936 (423G>T) and rs146922325 (c.565C>T, p.Arg189Trp, R147W) in PROC were identified in the patient. The p.Arg189Trp variant has been described in the Chinese population in patients with PC deficiency (Table IV) (9-11). Tang *et al* (9) described a recurrent mutation (c.565C>T) that appear in 17 of the 34 probands (50%) and was not only the most frequent variant for PC deficiency, but also a significant risk factor for venous thrombosis in Chinese individuals. The study revealed that first-degree relatives bearing this variant had an 8.8-fold increased risk of venous thrombosis (9). In addition, Tsay *et al* (10) tested PROC in the families of 21 unrelated probands with symptomatic PC deficiency, and reported that the heterozygous R147W mutation by itself remained a significant thrombotic risk factor. Furthermore, they discovered that among the 21 families, nine symptomatic probands shared the R147W missense mutation in exon 7 plus a silent T66C mutation in exon 2, which was not identified in other family members or in the healthy control group that did not carry the R147W mutation (10). The present study concurred that this was the case in the patient. A study on the clinical and genetic features of PC deficiency in 23 unrelated subjects in the Chinese population demonstrated that the Arg189Trp mutation occurred in 43.5% of all subjects. This study also illustrated that complex genotypes in PROC deficiency are mainly responsible for the increased risk for VTE, particularly for recurrent VTE (15).

Additionally, the present patient also suffered from PS deficiency. However, only one novel mutation, Arg316His (947G>A), was identified in exon 9 of PROS1. Furthermore, the antithrombin level was normal, however, g.981A>G (rs5877) was detected in exon 5 of SERPINC1. Although silent mutations (such as g.981A>G) do not influence protein structure expression, they may still affect protein function and, therefore, the sensitivity of a patient to heparin (16). In addition, rs13146272 in the CYP4V2 gene, which has been reported to be associated with DVT (17,18), was found in the present patient; however, whether this variant is associated with TEE in the Chinese population remains to be elucidated.

In the present study, the patient experienced the first thrombosis at the age of 20 years, and four recurrent thrombotic events occurred in an extremely short time interval (~2 years). Several risk factors with respect to the hypercoagulable state in thalassemia, as well as acquired and congenital thrombophilia, could result in an increased risk of VTE or recurrent VTE. Therefore, this case illustrates a patient with HbH who underwent a splenectomy with congenital thrombophilic mutation and a requirement for long-term anticoagulant treatment.

In conclusion, when thrombotic events repeatedly occur in a patient with thalassemia, risk factors associated with the hypercoagulable state, as well as acquired and congenital thrombophilia, should be screened for. In addition, prophylactic anticoagulation therapy is also recommended in thalassemia intermedia patients who are undergoing certain types of surgery, as well as those who have a previous history of deep vein thrombosis or pulmonary embolism. In patients with TEE life-long anticoagulation appears to be rational and effective in prevention of recurrent TEE.

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