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Natural anticoagulant deficiencies in Thais: A population-based study

Ponlapat Rojnuckarin*, Rung Settapiboon, Benjaporn Akkawat, Sudawadee Teocharoen, Amornchai Suksusut, Noppacharn Uaprasert

Research Collaborations in Hematologic Malignancies and Hematopoietic Stem Cell Transplantation, Division of Hematology, Department of Medicine, Chulalongkorn University and King Chulalongkorn Memorial Hospital, Bangkok, Thailand

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ABSTRACT

Introduction: In contrast to Caucasians, hereditary anticoagulant deficiencies are more common in Asians and mutations are heterogeneous among different countries. This study aimed to determine the prevalence and genetic basis of protein C and protein S deficiencies in Thai population.

Methods: Healthy volunteers were tested for protein C activity and free protein S antigen. Subjects with low values were requested for repeated testing and exclusion of acquired causes. Cases with persistently low levels were assayed for respective gene mutations using direct sequencing and multiplex ligation-dependent probe amplification (MPLA) when *PROS1* point mutation was undetectable.

Results: For protein C activities ($N = 5234$), the values of men were lower than those of post-menopausal women ($P < 0.001$). In 17 of 18 subjects, there were 7 types of *PROC* mutations, 64.7% of which were p.R189W and 2 were previously unreported. Protein S levels ($N = 5242$) were highest in men, followed by post-menopausal and pre-menopausal women, respectively ($P < 0.001$). The repeatedly low protein S was mostly in female (88.2%). Among 29 subjects with protein S below the sex-specific 2.5 percentile cut-points, 4 types of mutations were found in 5 subjects (17.2%) with one previously unreported mutation. Free protein S levels were below 30 U/ml in all cases with mutations. The estimated prevalence of *PROC* and *PROS1* mutations in Thai population was 0.69% and 0.22%, respectively.

Conclusions: PROC mutations, especially p.R189W, are common in Thais. However, mildly depressed protein S levels were frequently found in women with undetectable *PROS1* mutation.

1. Introduction

Studies on venous thromboembolism (VTE) in Asian countries reveal the disease characteristics that are distinct from those of Western patients. Firstly, the VTE incidences in general Chinese and Korean populations are 5–6 folds lower than those of Western countries [1]. However, the VTE rates in high risk patients are within the ranges reported in Caucasians, *e.g.* 30–40% after major orthopedic surgery as detected by venography [2] and approximately 2–3% symptomatic VTE in high risk medical patients [3]. Furthermore, Asian patients who are treated with warfarin show higher risks of bleeding complications compared with Caucasians at similar INR levels [4,5].

As the disease is multifactorial in etiology, approximately 30% of VTE is attributed to thrombophilia. In Caucasians, the most common heritable hypercoagulable states, Factor V Leiden and prothrombin G20210A mutation, are found in 18.8% and 7.1% of VTE patients and 4.8 and 2.7% of general population, respectively [6]. However, both genetic polymorphisms are very rare in Asian general populations and Asian VTE patients [7]. On contrary, protein C, protein S and antithrombin deficiencies are more common in Chinese, Japanese and Korean patients. Each was found in approximately 10% of Asian VTE patients [7]. This disparity may have clinical implications. As factor V and prothrombin mutations are weaker thrombophilia [8], their presence is unrelated to VTE recurrences and, therefore, laboratory testing in every patient is discouraged [9]. However, anticoagulant deficiencies are stronger risk factors for thrombosis [8] and may be helpful to predict VTE recurrences in Asians [10].

Notably, the prevalence and types of natural anticoagulant deficiencies are different among Asian ethnicities. Furthermore, gene mutations of the same anticoagulant factor in Asians are heterogeneous, unlike factor V and prothrombin mutations in Caucasians. For example, *PROS1* p.K196E mutation is detectable in 0.9–1.8% of Japanese population [11,12], but it is not found in Chinese or Korean population. *PROC* p.R189W mutation is reported to be common in Taiwanese [13]

* Corresponding author at: Department of Medicine, Faculty of Medicine, Chulalongkorn University and King Chulalongkorn Memorial Hospital, Bangkok 10330, Thailand.

E-mail address: Ponlapat.R@Chula.ac.th (P. Rojnuckarin).

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and Thai [14] populations, while it is not observed in Japanese. On the other hand, antithrombin p.R76C and p.S148P mutations are reported exclusively in Korean population [15]. Furthermore, there are many other individual mutations. These data underscore the importance of the race-specific studies in Asian populations.

A study in Thai subjects revealed a high protein S deficiency prevalence of up to 3.7% [16]. However, the genetic test was not performed. Another investigation discovered that *PROC* p.R189W mutation is common in Thais [14], but other types of mutations have not been analyzed. Therefore, this study aimed to investigate the prevalence of protein C and protein S deficiency in a large number of healthy Thai volunteers. The subjects with low levels were analyzed for mutations to characterize the genetic profile of natural anticoagulant protein deficiencies in our population.

2. Materials and methods

This is a cross-sectional observation study. Adult subjects from Bangkok and 4 other provinces (Saraburi, Samutprakan, Chachoengsao and Cholburi) were asked to participate in the study as a part of community health checkups. The volunteers with pregnancy, hemorrhagic disorders or antithrombotic uses were excluded. The research was approved by the institutional review board of Chulalongkorn University. Informed consents were given by all participants.

The sample size was calculated by the estimated prevalence of anticoagulant deficiency in Asians of 0.5% [11,13]. The precision of at least 0.2% requires > 4778 subjects.

Demographic information, past medical history including thromboembolism, family history of thrombosis, current medications including female hormones, and menstrual history were recorded. Blood specimens were then obtained from all consenting subjected and sent to the laboratory within 4 h. Citrated blood was centrifuged and plasma samples were frozen at −70 °C until assay.

Protein C activities were determined by a Chromogenic assay kit (Dade Behring, Germany) and free protein S antigen levels were measured by an in-house ELISA as previously described [17]. One normal control and one abnormal control plasma samples (Dade Behring, Germany) were utilized in each run to ensure that both values were within the defined ranges. Each sample was tested in duplicate, using 2 different dilutions.

Subjects with low protein C or protein S values according to the laboratory reference ranges (Protein C < 70 IU/dL or Free Protein S antigen < 53 IU/dL) were asked for repeated blood samplings. The laboratory reference ranges were defined by the means ± 2 standard deviations (SD) of the measured values in over 100 healthy Thai subjects. For the volunteers who agreed to give second specimens, the acquired causes of anticoagulant deficiencies, *i.e.* an anticoagulant use, estrogen use, liver diseases, autoimmune diseases or HIV infection, were ruled out by history, physical examination and laboratory evaluations that included liver function and anti-HIV tests.

Cases with persistently low protein C or protein S levels were subjected to genetic analysis of the respective gene. The 15 exons including the regulatory region of *PROS1* gene were polymerase chain reaction (PCR) amplified and directly sequenced using ABI BigDye system (Applied Biosystems, CA, USA). The amplification primers were synthesized according to Reitsma *et al* [18], except for the exon 1 [19] and the amplicon for exon 5/6 [20]. Multiplex ligation-dependent probe amplification (MPLA) of *PROS1* (MRC Holland, Netherlands) was performed when *PROS1* gene mutation was not found. All 9 exons of the *PROC* gene were PCR amplified and sequenced using the primers according to Tang *et al* [21].

Novel mutations were analyzed *in silico* using PolyPhen-2 [22] for missense mutations and BDGP neural network for splice site mutations [23].

The means and SD were used for describing continuous data with normal distribution by the Kolmogorov-Smirnov test. The Pearson's chi-squared test and independent *t*-test were applied to determine differences of proportions and continuous variables, respectively. The SPSS® Statistics 20.0 (IBM® Corporation, New York, USA) was used for calculations. The levels of $P < 0.05$ were considered statistical significance.

3. Results

3.1. Study population

There were a total of 5243 Thai subjects included in the study. The mean age was 44.4 ± 13.8 yrs., ranging from 15 to 99 yrs. Female accounted for 59.2% ($N = 3102$) of the population and 66.4% of women were pre-menopausal. History of liver diseases, coronary disease, stroke and venous thrombosis was reported in 3.9%, 1.0%, 0.6% and 0.4%, respectively.

Protein C and protein S level assays were performed in 5234 and 5242 participants revealing the levels below the laboratory reference ranges in 1.07 and 4.85%, respectively. There were 46.4% and 43.3% of those subjects, whose repeated tests of protein C and protein S were available, respectively. There was no subject exclusion in the low protein C group. However, 2 subjects of the low protein S group were excluded due to the possible causes of acquired deficiency, *i.e.* HIV seropositivity and oral contraceptive use. There was neither personal nor family history of thromboembolism in the subjects with repeatedly low levels.

Finally 69.2% (18/26) protein C and 47.2% (51/108) of protein S test results were repeatedly low. The flow diagram of the study volunteers was shown in Fig. 1.

3.2. Protein C and protein S levels in Thai population

The mean protein C activity in Thai population was 125.2 ± 27.13 IU/dL. Post-menopausal women had higher protein C activities compared with male subjects.

There was no statistically significant difference between those of men and pre-menopausal women ($P = 0.08$) as demonstrated in Table 1.

The mean free protein S antigen level in Thai population was 91.01 ± 26.63 IU/dL. The values were significantly different between sexes and menstrual statuses. Protein S levels were highest in men followed by post-menopausal women and pre-menopausal women, respectively. The means and 2.5 percentiles of protein S were listed in Table 1. In our population, hormonal uses, for either oral contraception

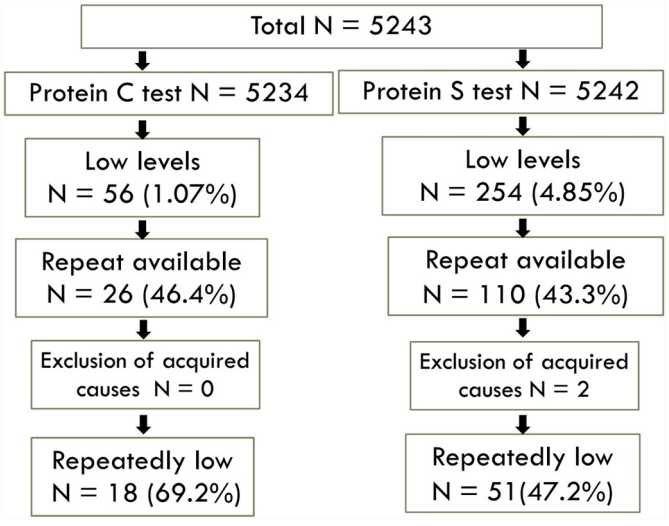

Fig. 1. Flow Diagram of the study population.

Table 1
Protein S and protein C levels in Thai population.

	Mean ± SD (IU/dL)	2.5 percentile (IU/dL)	*P* values
Protein S levels (*N* = 5242)			
Male	100 ± 25.6	55	
Female	84.6 ± 25.4	46	P < 0.001 Compared to male
Post-menopause	88.9 ± 25.4	47.5	*P* < 0.001 Compared to male
Pre-menopause	82.4 ± 25.1	44	P < 0.001 Compared to post-menopausal female
Protein C levels (N = 5234)			
Male	122.7 ± 26.7	74	
Pre-menopausal female	121.5 ± 25.3	76	P = 0.08 Compared to male
Post-menopausal female	138.0 ± 27.8	86	P < 0.001 Compared to male

SD, Standard deviation.

or post-menopausal replacement therapy, were reported in 17.25% of female population and were not associated with lower protein S levels (data not shown).

3.3. Cases with repeatedly low protein C levels

There were 18 cases with repeatedly low protein C activities. The mean age was 44.4 ± 19.4 yrs. and 61% of them were female. All were subjected to molecular analysis and mutations were discovered in 94.4% (17/18 cases) using direct sequencing.

The most common mutation was *PROC* p.R189W which was found in 11 cases (64.7%) and 5 of these were homozygous mutations. The mean protein C levels of subjects with heterozygous and homozygous p.R189W were 63.0 and 48.5 IU/dL, respectively. The majority of mutations were within the exon 7 (70.6%). There were 5 subjects with concomitant missense and regulatory mutations.

There were 2 previously unreported nucleotide changes. One case had p.F118 L from TTC > TTG transition, while the previously reported p.F118 L was due to TTC > CTC transversion [24]. The other had a splice site mutation in IVS-4 as shown in Table 2. This mutation was predicted to be deleterious by BDGP Neural Network for splice site mutations [23].

If the subjects without repeated tests were assumed to have the same rate of mutations, the estimated prevalence of protein C mutations in Thai population was approximately 0.69% (95% confidence interval [CI] 0.50–0.95).

3.4. Cases with repeatedly low protein S levels

There were a total of 51 subjects with persistently low free protein S antigen levels. The mean age was 42.7 ± 14.6 yrs. and 88% of them were female. The samples from subjects with average levels below the sex-specific 2.5 percentiles were subjected to molecular analysis. There were 29 volunteers in this group. Twenty five (86.2%) of them were female.

Using direct sequencing, mutations were discovered in 17.2% of subjects (5/29) as shown in Table 3. There was no large deletion or duplication detectable by MLPA (Multiplex Ligation-dependent Probe Amplification).

Among 5 cases with mutations, only one was male. Two females had had a similar mutation in intron 12 (c.1492 + 5G > C). This previously unreported splice site mutation was predicted to be deleterious [23]. The means ± SDs of protein S levels were 17.0 ± 8.85 IU/dL *vs.* 35.5 ± 5.21 IU/dL in mutation-positive *vs.* mutation-negative cases, respectively ($P = 0.007$).

Table 2
Mutations of *PROC* gene detectable in healthy Thai volunteers.

No.	Age	Sex	Levels[a]	Sites	Mutations	References
1	61	M	46	Regulatory	c.-1657 T > C	[33]
2	64	M	59.75	Regulatory	Homozygous c.-1657 T > C	[33]
3	55	M	46.5	IVS4	c. 263-2A > T	Novel
4	70	M	42.8	Exon 5	c.354C > G, p. F118 L	Novel
5	25	F	62.75	Exon 7	c.565C > T, p.R189W	[34]
6	23	M	62.75	Exon 7	c.565C > T, p.R189W	
7	35	F	68.75	Exon 7	c.565C > T, p.R189W	
8	33	M	61	Exon 7	c.565C > T, p.R189W[b]	
9	26	F	65	Exon 7	c.565C > T, p.R189W[b]	
10	40	F	57.75	Exon 7	c.565C > T, p.R189W[b]	
11	45	F	48	Exon 7	Homozygous c.565C > T, p.R189W	
12	22	F	49.5	Exon 7	Homozygous c.565C > T, p.R189W	
13	22	F	41.5	Exon 7	Homozygous c.565C > T, p.R189W	
14	32	F	54	Exon 7	Homozygous c.565C > T, p.R189W	
15	66	M	49.5	Exon 7	Homozygous c.565C > T, p.R189W	
16	45	F	53.75	Exon 7	c.632G > A, p.R211Q[c]	[35]
17	88	F	56	Exon 9	c.889G > C, p.D297H[b]	[36]

M, Male; F, Female.
[a] Average levels between repeated tests (IU/dL).
[b] Concomitant with c.-1657 T > C,
[c] Concomitant with homozygous c.-1657 T > C.

Table 3
Mutations of *PROS1* gene detectable in healthy Thai volunteers.

No.	Age	Sex	Levels[a]	Site	Mutations	References
1	39	M	27	Promoter	c.-168C > T	[37]
2	22	F	12.5	Exon 12	c.1351C > T p.R451Ter	[38]
3	24	F	16.5	IVS12	c.1492 + 5G > C	Novel
4	75	F	24	IVS12	c.1492 + 5G > C	Novel
5	52	F	< 5	Exon 14	c.1680 T > G p.Y560Ter	[39]

M, Male; F, Female
[a] Average levels between repeated tests (IU/dL).

If we assumed that the subjects who did not come for repeated test had a similar rate of mutation, the estimated prevalence of hereditary protein S deficiency in Thailand was approximately 0.22% (95% Confidence interval [CI] 0.12–0.39).

4. Discussion

This study demonstrates the uniqueness of thrombophilia in Asians. The prevalence of *PROC* mutations in Thai population (0.69%) was higher than a report in Western population of 0.145% [25]. The prevalence of hereditary protein S deficiency in Thais of 0.22% was also relatively higher than the study in Caucasians that showed the frequencies of 0.03–0.13% [26]. Similarly, investigations in Chinese, Japanese and Korean populations also revealed higher prevalence of natural anticoagulant deficiencies compared with those of Western countries. Notably, the deficient factors and common mutations are heterogeneous among Asian countries as summarized in Table 4. From previous and current studies, *PROC* p.R189W mutation is common in both Chinese [13] and Thai population, while c.574_576del mutation is prevalent only in Chinese [21], but not in Thais. We did not investigate antithrombin levels because antithrombin deficiency is not common in Thai patients with VTE [7]. Thus, prevalence in our population is expected to be very low.

In Thai general population, protein S levels were highest in male, followed by post-menopausal female and pre-menopausal female,

Table 4
Comparison of hereditary thrombophilia in Asian populations from current and previous studies [11–13,15,21].

	Chinese	Japanese	Korean	Thai (current study)
Protein C deficiency				
-Prevalence	0.4%	0.13%	0.29%	0.69%
-Recurrent mutations	p. R189W c.574_576del	None	p.R211W	p.R189W c.-1657 T > C
Protein S deficiency				
-Prevalence	0.057%	0.9–1.8%	0.16%	0.22%
-Recurrent mutations	None	p.K196E	None	c.1492 + 5G > C
Antithrombin deficiency				
-Prevalence	0.086%	0.15%	0.48%	Not done
-Recurrent mutations	None	None	p.R76C p.S148P	

respectively (Table 1). The result was similar to the previous studies in Italian [27] and Chinese [28] populations. Therefore, sex-specific normal values of protein S should be applied. Furthermore, menstrual status of the patients should also be considered while interpreting the laboratory results. On the other hand, protein C levels were significantly elevated in post-menopausal women. As thrombophilia testing is usually indicated in younger patients with thrombosis, the clinical importance of age- and sex-specific reference range for protein C assay is not clear.

Our study discovered that low protein S levels were common in young female subjects with undetectable *PROS1* mutations, especially when protein S levels are mildly depressed (30–50 IU/dL). A previous large case-control study in Western population also revealed that low protein S levels were infrequently caused by *PROS1* mutation and they were not associated with thrombosis [29]. Therefore, the risk for thrombosis in mildly depressed protein S subjects without *PROS1* mutation is probably low. These data may suggest that genetic analysis is critical for diagnosis of hereditary protein S deficiency. However, the costs of sequencing and MLPA of *PROS1* are high and the results may not change the treatment decisions. Therefore, selection of appropriate cases for gene mutation studies is critical to ensure cost-effectiveness. The level of < 30 IU/dL may be used as a cut-off indication for genetic assay in our patients.

In contrast, *PROC* mutations are usually detectable in subjects with low protein C. Therefore, protein C assay is a good test to indicate gene mutations. In addition, over 70% of mutations in Thais are within exon 7 especially *PROC* p.R189W. Sequencing of this particular exon can detect most of the mutations making genetic analysis of *PROC* is less costly in Thais. A study in Thailand [14] found that p.R189W was very common and found up to 5.9% (41/690) of healthy subjects. We found that p.R189W homozygotes had lower protein C activities compared with heterozygotes (Table 2). These data suggest that some heterozygous *PROC* p.R189W cases may have protein C activities within normal range. This protein C variant was shown to have normal enzymatic activity, but impaired cofactor (Endothelial protein C receptor) binding [30]. It is still unclear whether the activity assay or genetic analysis is the better predictor for thrombosis.

Apart from age and sex, several factors may interfere with the measurements of natural anticoagulants, including acute thrombosis, anticoagulant uses, liver diseases, disseminated intravascular coagulation (DIC), hormone intakes, pregnancy, HIV infection and autoimmune disease [31]. Therefore, genetic testing may be more specific to make diagnosis of thrombophilia and anticoagulant level determinations can be used for screening. Next generation sequencing (NGS) is becoming accessible to the clinics with more affordable prizes. Thrombophilia tests using NGS technology may be applicable to practices in the future [32].

In order to eliminate falsely low protein S and protein C levels, we selected only reproducible abnormalities without a known cause for genetic tests. This resulted in a limitation of the study as second samples were sometimes not available. Our estimated prevalence needed to be corrected for missing cases. The other limitation of this study is the use of protein S antigen assay because our laboratory could not find a protein S activity test with reproducible results. Therefore, the type 2 protein S defects might have been missed.

In conclusion, hereditary protein C and protein S deficiencies are relatively common in Thai population compared with Caucasians and the rates are similar to other Asian countries. Notably, the mutations are heterogeneous among different races and among individual subjects. In addition, many subjects with persistently low protein S levels showed no detectable genetic mutations in *PROS1* gene underscoring the importance of the genetic analysis for definite diagnosis.

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Conflict of interests

None declared.

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