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Plasma phenotypes of protein S Lys196Glu and protein C Lys193del variants prevalent among young Japanese women

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Protein S Tokushima (p.Lys196Glu) and two protein C gene variants (p.Arg189Trp, p.Lys193del) are hereditary thrombophilia in Japanese and Chinese populations, respectively; however, their diagnosis by plasma analyses is difficult because of the type II deficiency phenotype. Three gene variant genotypes were examined in young Japanese women ($n = 231$). Plasma total protein S activity and total protein S antigen levels were measured using a total protein S assay system, protein C and protein S activities by clot-based methods, and protein C and free protein S antigen levels by latex agglutination methods. protein S Tokushima (p.Lys196Glu) and protein C p.Lys193del variants were prevalent among participants with allele frequencies of 1.08 and 0.86%, respectively, whereas any carrier of protein C p.Arg189Trp variant was not identified. The plasma phenotype of the type II deficiency of protein S Tokushima heterozygotes was demonstrated by decreased total protein S activity with a normal total protein S antigen level; however, the protein C activities of protein C p.Lys193del heterozygotes were within reference intervals, whereas their protein C antigen levels were elevated. We compared the diagnostic accuracy of the total protein S activity/total protein S antigen ratio for identifying protein S Tokushima heterozygotes with that of the clot-based protein S activity/ free protein S antigen ratio and found that sensitivity and specificity of 100% each was only achieved by the former. Protein S Tokushima and protein C p.Lys193del are prevalent among young Japanese women, and a plasma analysis using the total protein S assay system is more accurate than the clot-based protein S activity/free protein S antigen ratio for diagnosing protein S Tokushima carriers. *Blood Coagul Fibrinolysis* 30:393–400 Copyright © 2019 Wolters Kluwer Health, Inc. All rights reserved.

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Introduction

Venous thromboembolism (VTE), consisting mainly of deep vein thrombosis (DVT) and pulmonary embolism, is a typical multifactorial disease and genetic and environmental factors both contribute to the risk of developing this disease [1]. Genetic risk factors for VTE affect natural anticoagulant mechanisms and result in a hypercoagulable state, which include rare gene variants of anticoagulant factors as well as common gene variants, such as factor V Leiden and prothrombin G20210A. The latter two gene variants are prevalent among Caucasian populations, but have yet to be identified among non-Caucasians [2].

A high prevalence of dysfunctions in the natural anticoagulant factors, protein S and protein C, have been reported among Japanese and Chinese VTE patients [3]. By systematical gene analyses, we identified a protein S gene (*PROS1*) variant with a plasma phenotype of qualitative (type II) deficiency, protein S Tokushima (p.Lys196Glu, rs121918474), as a genetic risk factor for DVT with an allele frequency of 0.8% in the Japanese population and a 3.7-fold higher risk of DVT among heterozygotes [4]. Similar findings were independently reported in two other case–control studies [5,6]. Two protein C gene (*PROC*) variants, p.Arg189Trp (rs146922325) [7,8] and p.Lys193del (rs199469469) [9], have been identified as genetic risk factors for VTE in the Chinese population with allele frequencies of 0.4 and 1.2%, and five-to-seven-fold and three-fold higher risks of VTE among heterozygotes, respectively. The plasma phenotype of these protein C gene variants is also a type II deficiency.

Protein S and protein C are both vitamin K-dependent glycoproteins and function as the main components of the protein C anticoagulant system [10]. Thrombin complexed with thrombomodulin rapidly activates protein C, and activated protein C (APC) inhibits the coagulation pathway by the proteolysis of factors Va and VIIIa. In plasma, approximately 40% of protein S circulates in its free form (free protein S), with the remaining 60% being noncovalently bound to the β chain of C4b-binding protein (C4BP), an important regulator of the complement system.

Free protein S serves as a cofactor to APC, whereas the protein S-C4BP complex loses its cofactor activity. The dissociation of the protein S-C4BP complex is extremely slow in the presence of a physiological concentration of Ca^{2+}, whereas its affinity is significantly decreased in the absence of Ca^{2+} [11].

The laboratory screening of a type II deficiency in anticoagulant factors is currently achieved by measuring plasma activities and compared them to antigen levels [12,13]. Protein C activity is measured by either a clot-based assay, mostly based on activated partial thromboplastin time (APTT), or a chromogenic-based assay; however, the latter is not able to detect abnormalities in cofactor binding, surface binding, and other substrate-binding regions, such as the protein C p.Lys193del variant [9]. The measurement of protein S levels is more complicated because of its cofactor function and partitioning into two forms in plasma. Protein S activity is commonly measured by a clot-based assay, mostly based on APTT or prothrombin time (PT), and is compared with the free protein S level for diagnosing type II protein S deficiency. However, the clot-based assays are considered not to be specific enough to diagnose protein S deficiency because of many preanalytical variables [12–15]. Thus, we established a total protein S assay system in which the undesired effects of the dissociation of the protein S-C4BP complex are avoided and protein S activity is measured by a chromogenic-based assay, thereby enabling the accurate diagnosis of a type II protein S deficiency, such as protein S Tokushima [16].

Plasma protein S levels are lower in women than in men, as are levels in premenopausal women than in postmenopausal women [17,18], and they significantly decrease during pregnancy and oral contraceptive use [19,20]. The aim of the present study was to examine the prevalence of protein S Tokushima and two *PROC* variants, p.Arg189Trp and p.Lys193del, in young Japanese women, and to perform plasma phenotype analyses in order to diagnosing the carriers of these gene variants.

Materials and methods
Participants and blood sampling
The present study was approved by the Ethics Committee of Nakamura Gakuen University. In April 2013 and 2014, female Japanese students ($n = 231$, 20.0 ± 0.6 years) of the Department of Nutritional Sciences of Nakamura Gakuen University who participated in health check-ups were recruited and all target participants provided written informed consent. Following an overnight fast, venous blood was collected early in the morning into tubes containing a one-tenth volume of 0.105 mol/l sodium citrate and were kept on ice for no longer than 3 h, centrifuged at $1500 \times g$ at $4\,^{\circ}C$ for 10 min, and platelet-poor plasma samples were stored in aliquots and frozen at $-70\,^{\circ}C$ for later analyses. Genomic DNA was purified from the buffy coat of citrated blood samples using

NucleoSpin Blood QuickPure (TaKaRa Bio Inc., Shiga, Japan) and stored at $-20\,^{\circ}C$.

Genotyping of *PROS1* and *PROC* variants
The genotypes of protein S Tokushima (p.Lys196Glu, c.586A>G) and protein C p.Arg189Trp (c.565C>T) were assessed by a combination of real-time PCR and Cycling probe method using Cycleave PCR Reaction Mix (Takara Bio Inc.). The primers and probes used for protein S p.Lys196Glu genotyping were 5′-CTCCTGAAAAGTTCTCTGCA/5′-AAATTTCTC-TAACTGGGATT and 5′-Eclipse-GCTTTCAAATa-AG-FAM (the A allele)/5′-Eclipse-AATCTTTCTcATT-ROX (the G allele), respectively, and those for protein C p.Arg189Trp genotyping were 5′-AAGACAGGAGGG-CAGTCTCG/5′-AGCCGCGGATCTACTTGGTC and 5′-Eclipse-GAAGcGGAT-FAM (the C allele)/5′-Eclipse-GGAAGtGGAT-ROX (the T allele), respectively. The genotype of protein C p.Lys193del (c.574_576delAAG) was assessed by the TaqMan method using Premix Ex Taq (Probe qPCR) (Takara Bio Inc.) and the primers and probes used were 5′-AAGACAGGAGGGCAGTCTCG/5′-AGCCGCGGATCTACTTGGTC and 5′-Eclipse-GAT-GGAGAAGAAGCGCAGTC-ROX (the AAG allele)/5′-Eclipse-GATGGAGAAGCGCAGTCACC – FAM (the delAAG allele), respectively. PCR reactions were performed using a Thermal Cycler Dice Real Time System II (Takara Bio Inc.).

Analyses of plasma protein S and protein C phenotypes
Frozen plasma samples that had never thawed before analyses were thawed in a water bath at $37\,^{\circ}C$ within 5 min. In the total protein S assay system, total protein S activity and antigen levels were measured by the chromogenic method and latex agglutination method, respectively, using a Hitachi 7180 automated analyzer (Hitachi High-Technologies, Tokyo, Japan), as described in our pervious study [16] with a slight modification to improve applicability. Briefly, in total protein S activity measurements, the protein S-C4BP complex in plasma was initially dissociated by diluting samples 126 times with dilution buffer containing APC and FXa, and 3 μl of diluted plasma was then mixed with 150 μl of the reagent containing FVa, liposome, and $CaCl_2$. After incubating for 5 min, 50 μl of the reagent containing prothrombin and S-2238, a chromogenic substrate for thrombin, was added and the absorbance change was evaluated at the main wavelength of 405 nm with an auxiliary wavelength of 450 nm for 5 min. In total protein S antigen measurements, free protein S in plasma was initially combined with C4BP by incubating 3 μl of samples with 100 μl of the reagent containing an excess amount of purified human C4BP. After incubating for 5 min, 100 μl of the reagent containing monoclonal anti-protein S antibody (9H6)-bound latex particles was added and the absorbance change was assessed at 700 nm for 5 min. Total protein S activity and antigen levels were expressed as IU/dl using the WHO second International

Standard for protein S (03/228, NIBSC, Hertfordshire, UK). The total protein S activity/total protein S antigen ratio, protein S-specific activity, was then calculated. The intraassay and interassay coefficients of variations ($n = 5$) of the total protein S activity assay, determined using three plasma samples of different protein S levels, were 0.4–1.9 and 1.5–2.8%, respectively, whereas those of the total protein S antigen assay were 0.7–1.8 and 1.2–1.6%, respectively. The reference intervals [mean $\pm 2 \times$ standard deviation (SD)] were assessed by sex using the data of healthy Japanese individuals ($n = 194$, women 40.7%): those of total protein S activity and total protein S antigen levels for women, 74–134 and 74–127 IU/dl, respectively; those for men, 82–138 and 80–131 IU/dl, respectively. The total protein S activity/total protein S antigen ratio showed no sex difference, and its reference interval was 0.86–1.18 for both sexes.

The clot-based activity assays of protein S and protein C and the latex agglutination-based antigen assays of free protein S and protein C were performed in the laboratory of SRL, Inc. (Tokyo, Japan) using commercial kits. PT-based protein S activity was measured using HemosIL protein S clot (Instrumentation Laboratory Co., Bedford, USA), APTT-based protein C activity using HemosIL professional clot (Instrumentation Laboratory Co.), free protein S antigen levels using STA-Liatest Free protein S (Diagnostica Stago, Inc. Parsippany, New Jersey, USA), and protein C antigen levels using LPIA-ACE protein C (LSI Medience Co., Tokyo, Japan). Data were expressed as U/dl using normal pooled plasma as a standard: protein S activity using HemosIL Calibration Plasma (Instrumentation Laboratory Co.) and protein C activity, free protein S antigen, and protein C antigen levels using local pooled plasma obtained from approximately 100 healthy Japanese individuals including both sexes. The protein S activity/free protein S antigen ratio and protein C activity/protein C antigen ratio were then calculated. The reference interval of protein S activity was 56–126 U/dl for women and 67–164 U/dl for men, and those of free protein S antigen, protein C activity, and protein C antigen were 60–150, 64–146, and 70–150 U/dl, respectively, for both sexes.

Statistical analysis

Data were expressed as the mean $\pm$ SD and compared using unpaired Student's t-tests. Univariate linear regression analyses were performed and Pearson's correlation coefficients were calculated. The diagnostic accuracy of the parameters of plasma analyses was assessed from the receiver-operating characteristic (ROC) curves, and the areas under the curve (AUC) with their 95% confidence intervals (CIs) were assessed using a logistic regression analysis. The corresponding sensitivity and specificity values were calculated using the value closest to the lower limit of the reference interval of each parameter as a cut-off value. Statistical analyses were performed using PASW Statistics ver. 23 (SPSS Inc., Chicago, Illinois, USA), and a P value of less than 0.05 indicated significance.

Results

Genotyping of *PROS1* and *PROC* variants

Five (2.2%) out of 231 participants were identified as heterozygous carriers for protein S Tokushima (p.Lys196Glu) with an allele frequency of 1.08%. Four (1.7%) were heterozygous carriers for protein C p.Lys193del with an allele frequency of 0.86%; however, any carrier of protein C p.Arg189Trp was not found. Neither a homozygote nor a double heterozygote of protein S Tokushima and protein C p.Lys193del was identified. Therefore, 1 out of every 25 target individulas were found to have a genetic risk factor for VTE.

Plasma phenotypic analyses

We then compared plasma protein S and protein C levels according to the genotypes of these variants. Before analyses, six individuals were excluded: four for taking oral contraceptives and two with amenorrhea. The remaining 225 individuals, including 9 heterozygous carriers for either protein S Tokushima (p.Lys196Glu) or protein C p.Lys193del variant, did not have a disease or take medication that influences plasma protein S and protein C levels. As shown in Table 1, the total protein S activities of heterozygous individuals for protein S p.Lys196Glu variant (Lys/Glu, $n = 5$) were significantly lower than those of wild-type individuals (Lys/Lys, $n = 220$), whereas no significant difference was observed in total protein S antigen levels between two groups. As a result, the total protein S activity/total protein S antigen ratios of the heterozygotes were significantly lower than those of wild-type individuals. As expected, no significant difference was observed in protein C activity or protein C

Table 1   Protein S and protein C activities and antigen levels in wild-type and heterozygous individuals for the *PROS1 and PROC* variants

	Protein S p.Lys196Glu			Protein C p.Lys193del		
	Lys/Lys ($n = 220$)	Lys/Glu ($n = 5$)	P value	Lys/Lys ($n = 221$)	Lys/del ($n = 4$)	P value
Total protein S activity (IU/dl)	97 ± 14	67 ± 6	<0.001	96 ± 14	99 ± 13	0.734
Total protein S antigen (IU/dl)	97 ± 11	99 ± 10	0.706	97 ± 11	100 ± 11	0.579
Total protein S activity/total protein S antigen ratio	1.00 ± 0.06	0.69 ± 0.03	<0.001	0.99 ± 0.08	1.01 ± 0.05	0.626
Clot-based protein C activity (U/dl)	111 ± 23	123 ± 20	0.251	112 ± 23	90 ± 17	0.062
Protein C antigen (U/dl)	102 ± 17	107 ± 9	0.535	102 ± 16	121 ± 23	0.021
Clot-based protein C activity/protein C antigen ratio	1.09 ± 0.17	1.15 ± 0.12	0.468	1.10 ± 0.17	0.75 ± 0.06	<0.001

Data are expressed as means ± SD and differences between two groups were compared using an unpaired Student's t-test.

antigen levels between two groups. Regarding protein C p.Lys193del variants, the clot-based protein C activities of heterozygous individuals (Lys/del, $n = 4$), all being within the reference interval, were not significantly different from those of wild-type individuals (Lys/Lys, $n = 221$). However, the protein C antigen levels of heterozygotes were significantly higher than those of wild-type individuals, resulting in markedly lower clot-based protein C activity/protein C antigen ratios for heterozygotes than for wild-type individuals. As expected, no significant difference was noted in protein S levels between two groups. These results suggest the high sensitivity of the activity/antigen ratio (specific activity) for diagnosing a type II deficiency with moderately decreased or normal activity, such as protein S Tokushima (p.Lys196Glu) and protein C p.Lys193del variants and that the heterozygous carriers of protein C p.Lys193-del variant cannot be identified by the clot-based protein C activity assay.

Diagnostic accuracy of plasma phenotype analyses for the protein S Tokushima variant

Kimura *et al.* [21] previously reported that the clot (APTT)-based protein S activity assay was not sufficiently sensitive for identifying heterozygous carriers of protein S Tokushima (p.Lys196Glu) variant. In order to validate the diagnostic accuracy of protein S activity assays for the protein S Tokushima variant, we further measured PT-based protein S activity and free protein S antigen levels in the plasma samples of wild-type (Lys/Lys, $n = 130$) and heterozygous (Lys/Glu, $n = 9$) individuals for protein S Tokushima; the latter group included an additional four heterozygous female students, with none taking oral contraceptives, identified in 2018.

Figure 1 shows the distributions of the parameters of plasma protein S phenotypic analyses. The PT-based protein S activities of wild-type individuals were significantly higher than those of heterozygous individuals (87 ± 14 vs. 64 ± 9 U/dl, $P < 0.001$); however, were distributed over a wide range (40–136 U/dl) and overlapped completely with the range of heterozygous individuals (51–81 U/dl) (Fig. 1d). Three wild-type individuals had significantly decreased PT-based protein S activities of 40, 46, and 52 U/dl. The PT-based protein S activity/free protein S antigen ratios of wild-type individuals were significantly higher than those of heterozygous individuals (0.93 ± 0.12 vs. 0.66 ± 0.07, $P < 0.001$); however, were widely distributed (0.40–1.32) and overlapped completely with those of the heterozygotes (0.58–0.78) (Fig. 1f). In contrast, the total protein S activities of wild-type individuals were distributed within a narrower range (67–136 IU/dl) than that of PT-based protein S activities, but partially overlapped with the levels of heterozygous individuals (64–89 IU/dl) (Fig. 1a). The distribution of the total protein S activity/total protein S antigen ratios of wild-type individuals (0.78–1.17) did not overlap with

that of heterozygous individuals (0.65–0.77) (Fig. 1c). As expected, the distributions of total protein S antigen and free protein S antigen levels completely overlapped between the two groups (Fig. 1b and e). The total protein S activities and total protein S antigen levels of all target individulas ($n = 139$) correlated well with PT-based protein S activities ($r = 0.626$, $P < 0.001$) and free protein S antigen levels ($r = 0.681$, $P < 0.001$), respectively.

The diagnostic accuracy of differentiating heterozygous individuals for protein S Tokushima variant from wild-type individuals was then compared with the value closest to the lower limit of the reference interval (mean-$2 \times$ SD) of each parameter being selected as a cut-off value (Table 2). The cut-off value for total protein S activity, 74.5 IU/dl, was higher than that for PT-based protein S activity, 56.5 U/dl; however, the discriminatory power of total protein S activity was stronger than that of PT-based protein S activity, particularly for sensitivity, 77.8 vs. 22.2%. The AUC value of the total protein S activity/total protein S antigen ratio (protein S-specific activity) was the highest among the six parameters at 1.000, and sensitivity and specificity were 100.0 and 96.9%, respectively. Furthermore, by setting the cut-off value of the total protein S activity/total protein S antigen ratio to 0.78, mean-$3 \times$ SD, sensitivity and specificity both further improved to 100%. As the reference interval of the PT-based protein S activity/free protein S antigen ratio was not provided, its cut-off value was temporarily set as the same value as that for the total protein S activity/total protein S antigen ratio; however, its diagnostic accuracy was inferior to that of the total protein S activity/total protein S antigen ratio, with an AUC value, sensitivity, and specificity of 0.972, 100%, and 87.7%, respectively.

Discussion

Hereditary dysfunctions in the protein C anticoagulant system represent the main thrombophilia in Asia [3,22]: the *PROS1* variant, protein S Tokushima (p.Lys196Glu), and two *PROC* variants, protein C p.Arg189Trp and protein C p.Lys193del, are both genetic risk factors for VTE and prevalent among Japanese [4–6] and Chinese populations [7–9], respectively. The plasma phenotypes of these three gene variants are a qualitative (type II) deficiency, and therefore their diagnosis by plasma analyses is difficult [12–15]. Furthermore, plasma protein S levels show marked differences related to sex and age [17,18], being the lowest in premenopausal young women, and thus we conducted genotype and plasma phenotype analyses on young Japanese women of approximately 20 years old.

In genotype analyses of participants ($n = 231$), we found protein S Tokushima and protein C p.Lys193del variants to be prevalent with allele frequencies of 1.08 and 0.86%, respectively; however, no carrier of the protein C p.Arg189Trp variant was identified. Therefore, one out

Fig. 1
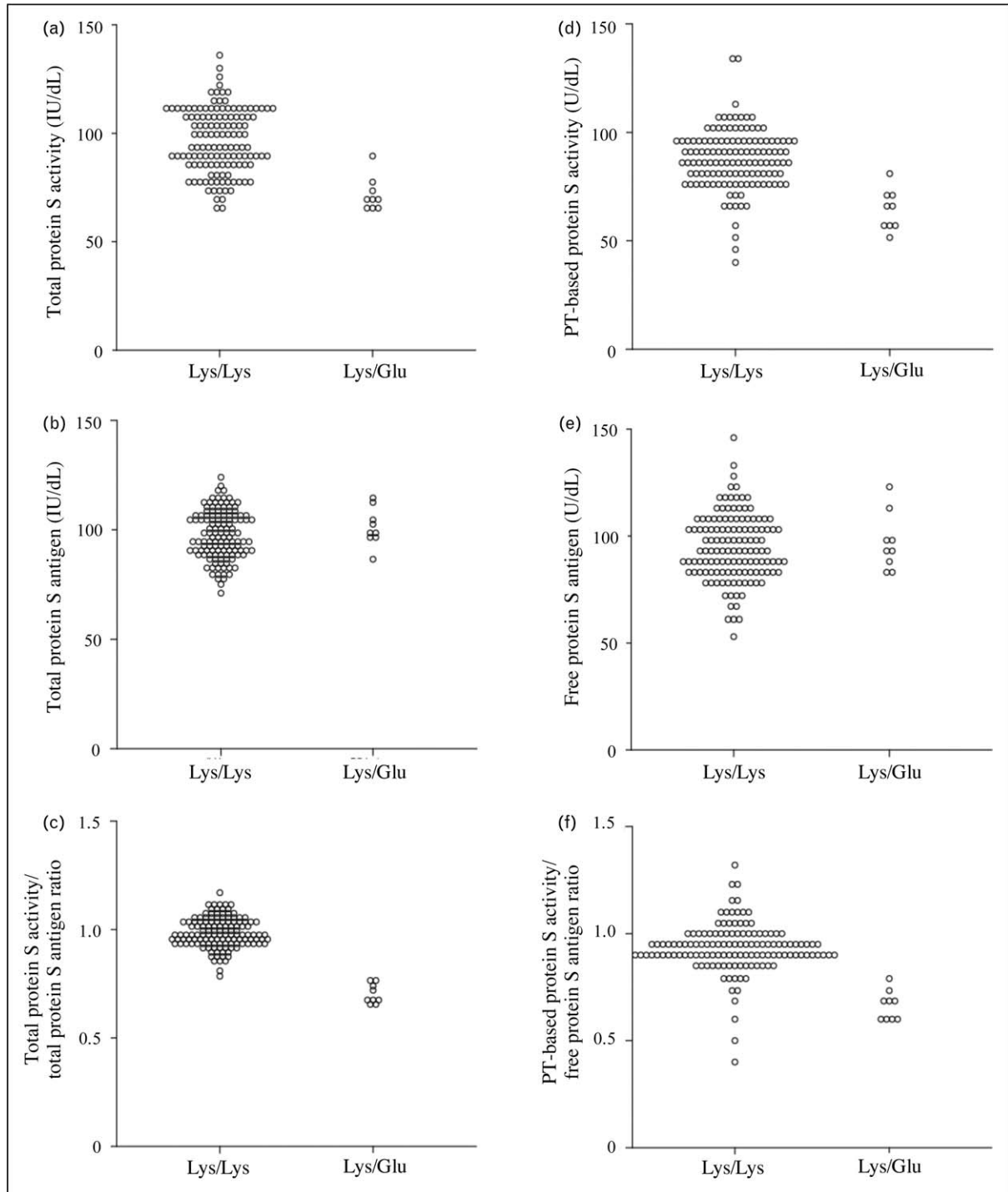
Distributions of levels of total protein S activity (a), total protein S antigen (b), total protein S activity/total protein S antigen ratio (c), PT-based protein S activity (d), free protein S antigen (e), and PT-based protein S activity/free protein S antigen ratio (f) in wild-type (Lys/Lys, $n = 130$) and heterozygous individuals (Lys/Glu, $n = 9$) for the protein S Tokushima (p.Lys196Glu) variant. Total protein S activity and total protein S antigen levels were measured using the total protein S assay system, whereas those of PT-based protein S activity and free protein S antigen were assessed using clot-based method and latex agglutination method, respectively. PT, prothrombin time.

Table 2   Diagnostic accuracy of parameters of plasma protein S phenotypic analyses for heterozygous individuals for the protein S Tokushima (p.Lys196Glu) variant

	Cut-off	Sensitivity (%)	Specificity (%)	AUC (95% CI)	P value[a]
Total protein S activity	74.5 IU/dl	77.8	94.6	0.941 (0.871−1.000)	<0.001
Total protein S antigen	76.0 IU/dl	0.0	98.5	0.402 (0.236−0.568)	0.327
Total protein S activity/total protein S antigen ratio	0.855	100.0	96.9	1.000 (1.000−1.000)	<0.001
PT-based protein S activity	56.5 U/dl	22.2	97.7	0.924 (0.858−0.989)	<0.001
Free protein S antigen	59.5 U/dl	0.0	98.5	0.456 (0.282−0.630)	0.659
PT-based protein S activity/free protein S antigen ratio	0.855	100.0	87.7	0.972 (0.946−0.999)	<0.001

AUC, area under the curve; CI, confidence interval; PT, prothrombin time. [a] Logistic regression analysis.

of every 25 target individuals were found to have a genetic risk factor for VTE. The allele frequency of protein S Tokushima in the present study was slightly higher than that reported previously for the Japanese general population at 0.6–0.9% [4–6]. Matsumoto et al. [23] recently showed that the allele frequency of the protein C p.Lys193del variant was 0.24% among 637 healthy Japanese individuals. A predisposition for the protein C p.Lys193del variant among Japanese DVT patients has been reported: two heterozygotes in 85 patients [4] and four heterozygotes in 173 patients [24], indicating an allele frequency in DVT patients of approximately 1.2%. This value was significantly lower than that in Chinese VTE patients (3.4%) [9]; however, the protein C p.Lys193del variant may also be a common genetic risk factor for DVT among the Japanese population. Recently, the protein C p.Lys193del variant was reported to be prevalent among Japanese neonatal thrombosis patients with a protein C deficiency [25,26].

We then analyzed the plasma phenotypes of the heterozygous carriers of the protein S Tokushima or protein C p.Lys193del variant. The plasma phenotype of type II deficiency of the heterozygotes for protein S Tokushima was clearly demonstrated by moderately decreased levels of total protein S activity, measured by a chromogenic-based assay, with normal total protein S antigen levels relative to those of wild-type individuals, resulting in a significantly decreased total protein S activity/total protein S antigen ratio (protein S-specific activity). A previous study demonstrated that the protein C activities of the protein C p.Lys193del variant measured by a clot-based assay were significantly decreased, whereas their protein C activities measured by a chromogenic assay were not different from those of normal individuals [9]. In the present study, however, the clot-based protein C activities of heterozygotes for the protein C p.Lys193del variant were all within the reference interval, and their protein C antigen levels were significantly higher than those of wild-type individuals, resulting in a moderately decreased protein C activity/protein C antigen ratio. In a previous study on healthy Japanese individuals, the clot-based protein C activities of two out of three heterozygotes for protein C p.Lys193del variants were also within the reference interval [23]. Thus, it appears that the heterozygous carriers of protein C p.Lys193del variant

may not be identified either by clot-based or chromogenic protein C activity assay. In an in-vitro expression experiment, the transcription level of protein C p.Lys193del mutant was found to be approximately 1.3-fold higher than that of wild-type protein C, whereas the concentration of mutant protein C in culture media, having moderately decreased anticoagulant activity, was similar to that of the wild type [9]. Another in-vitro expression experiment reported a relatively low anticoagulant activity of the protein C p.Lys193del mutant only in the presence of protein S [27]. Further experiments need to be performed in order to clarify whether this deletion of a lysine in the sequence Lys-Lys-Arg-Ser-His-Leu upstream of the C terminus of the light chain of the protein C molecule affects secretion of protein C.

The APC cofactor activity of protein S has commonly been measured using clot-based assays, mainly PT-based and APTT-based assays [12,13]. We compared the diagnostic accuracy of a PT-based assay for protein S Tokushima (p.Lys196Glu) heterozygous carriers to that of the chromogenic-based assay of the total protein S assay system. The PT-based protein S activity levels of wild-type individuals were distributed over a wide range and overlapped completely with the range of heterozygous individuals, as observed in an APTT-based protein S activity assay [21], and thus, sensitivity was found to be 22.2% when the cut-off value was set to the lower limit of the reference interval. Three wild-type individuals had significantly decreased PT-based protein S activities; a propensity for spuriously low clot-based protein S activities in normal individuals has also been reported [28]. In contrast, the total protein S activity levels of wild-type individuals were distributed within a narrower range, but partially overlapped with those of heterozygous individuals, resulting in a higher discriminatory power, a sensitivity of 77.8%, even though the cut-off value was higher than that of PT-based protein S activity. The distribution of the PT-based protein S activity/free protein S antigen ratios of wild-type individuals and that of the heterozygotes completely overlapped, whereas that of the total protein S activity/total protein S antigen ratios of wild-type individuals did not overlap with that of heterozygous individuals. The sensitivity and specificity of the total protein S activity/total protein S antigen ratio were 100 and 96.9%, respectively, when the cut-off value was set to

the lower limit of the reference interval, but improved to 100% each when the cut-off value was 0.78, mean-$3 \times$ SD. These results clearly demonstrate that total protein S activity is superior as a screening examination and the total protein S activity–total protein S antigen ratio as a diagnostic examination of a type II protein S deficiency with moderately decreased protein S activity, such as protein S Tokushima.

In plasma, protein S circulates in its free form and in the protein S-C4BP complex, and the latter loses its APC cofactor activity [10]. The dissociation of the protein S-C4BP complex is extremely slow ($K_d = 0.1$ nmol/l) in the presence of a physiological concentration of Ca^{2+}; however, affinity was shown to decrease ($K_d = 6.5$ nmol/l) in the absence of Ca^{2+}, such as in citrated plasma [11]. The dissociation of the protein S-C4BP complex increases in time-dependent, temperature-dependent, and dilution-dependent manners, and affects the measurement of free protein S antigen [29,30] and possibly protein S activity levels. Furthermore, the protein S-C4BP complex has been reported to retain cofactor activity for APC-catalyzed proteolysis at Arg^{306} in factor Va, but gain inhibitory activity for proteolysis at Arg^{506}, which might be responsible for the inhibitory activity of C4BP added to plasma in clot-based assays [31]. These characteristics of the protein S-C4BP complex may contribute to the analytical variables of clot-based assays. In the total protein S assay system, the undesired effects of the dissociation of the protein S-C4BP complex were avoided: by diluting samples with a high dilution ratio and adding liposomes with high affinity for protein S in the total protein S activity assay; by adding an excess amount of purified human C4BP to make free protein S combine with protein S in the total protein S antigen assay [16]. Total protein S activity is then measured by a chromogenic-based method in which the degradation of factor Va in the prothrombinase complex is evaluated by the thrombin production rate; and total PA antigen levels by a latex-agglutination method. Both assays may be performed using an automated analyzer, and, thus, rapid performance and excellent reproducibility have been achieved. Clot-based protein S assays were previously reported to overestimate protein S activity in patients being treated with direct oral anticoagulants (DOACs) [32,33]. We recently demonstrated that the total protein S activity assay is not interfered with DOACs, including dabigatran, rivaroxaban, apixaban, and edoxaban [34].

This study has some limitations. The number of participants, particularly of the carriers of variant alleles, was small, and, thus, large-scale population studies are needed to confirm the findings on genotypes and plasma phenotype analyses. Furthermore, as we only analyzed healthy individuals, case–control studies need to be performed in order to confirm the accuracy of the total protein S assay system for diagnosing carriers of protein S Tokushima variants with thrombosis. In addition, we did not analyze the whole sequences of *PROS1* and *PROC*, and thus the effects of variants other than those examined have yet to be assessed. Moreover, we have not yet investigated the effects of the factor V Leiden protein in the total protein S activity assay because of its absence among the Japanese population [3].

In conclusion, the present study demonstrated that the protein S Tokushima (p.Lys196Glu) and protein C p.Lys193del variants are prevalent in young Japanese women, whereas the protein C p.Arg189Trp variant is not. The plasma phenotype of a type II deficiency in protein S Tokushima heterozygotes was clearly demonstrated by decreased total protein S activity with a normal total protein S antigen level; however, the clot-based protein C activities of protein C p.Lys193del heterozygotes were all within the reference interval, whereas their protein C antigen levels were elevated. Diagnostic accuracy assessed from ROC curves indicated that a plasma analysis using the total protein S assay system, measuring total protein S activity and total protein S antigen levels, is superior to that evaluating clot-based protein S activity and free protein S antigen levels for diagnosing protein S Tokushima carriers.

Acknowledgements

Conflicts of interest
The authors declare that they have no conflicts of interest, except for T.T. and X.J. being employees of Shino-Test Corporation.

References
1   Wolberg AS, Rosendaal FR, Weitz JI, Jaffer IH, Agnelli G, Baglin T, Mackman N. Venous thrombosis. *Nat Rev Dis Primers* 2015; **1**:1–17.
2   Roberts LN, Patel RK, Arya R. Venous thromboembolism and ethnicity. *Br J Haematol* 2009; **146**:369–383.
3   Hamasaki N, Kuma H, Tsuda H. Activated protein C anticoagulant system dysfunction and thrombophilia in Asia. *Ann Lab Med* 2013; **33**:8–13.
4   Kinoshita S, Iida H, Inoue S, Watanabe K, Kurihara M, Wada Y, *et al.* Protein S and protein C gene mutations in Japanese deep vein thrombosis patients. *Clin Biochem* 2005; **38**:908–915.
5   Kimura R, Honda S, Kawasaki T, Tsuji H, Madoiwa S, Sakata Y, *et al.* Protein S-K196E mutation as a genetic risk factor for deep vein thrombosis in Japanese patients. *Blood* 2006; **107**:1737–1738.
6   Ikejiri M, Wada H, Sakamoto Y, Ito N, Nishioka J, Nakatani K, *et al.* The association of protein S Tokushima-K196E with a risk of deep vein thrombosis. *Int J Hematol* 2010; **92**:302–305.
7   Tsay W, Shen MC. R147W mutation of PROC gene is common in venous thrombotic patients in Taiwanese Chinese. *Am J Hematol* 2004; **76**:8–13.
8   Tang L, Guo T, Yang R, Mei H, Wang H, Lu X, *et al.* Genetic background analysis of protein C deficiency demonstrates a recurrent mutation associated with venous thrombosis in chinese population. *PLoS One* 2012; **7**:e35773.

9   Tang L, Lu X, Yu JM, Wang QY, Yang R, Guo T, et al. PROC c.574_576del polymorphism: a common genetic risk factor for venous thrombosis in the Chinese population. J Thromb Haemost 2012; 10:2019–2026.

10   Dahlback B. Advances in understanding pathogenic mechanisms of thrombophilic disorders. Blood 2008; 112:19–27.

11   He X, Shen L, Malmborg AC, Smith KJ, Dahlback B, Linse S. Binding site for C4b-binding protein in vitamin K-dependent protein S fully contained in carboxy-terminal laminin-G-type repeats. A study using recombinant factor IX-protein S chimeras and surface plasmon resonance. Biochemistry 1997; 36:3745–3754.

12   Marlar RA, Gausman JN. Laboratory testing issues for protein C, protein S, and antithrombin. International journal of laboratory hematology 2014; 36:289–295.

13   Bereczky Z, Gindele R, Speker M, Kallai J. Deficiencies of the natural anticoagulants - novel clinical laboratory aspects of thrombophilia testing. EJIFCC 2016; 27:130–146.

14   Persson KE, Dahlback B, Hillarp A. Diagnosing protein S deficiency: analytical considerations. Clin Lab 2003; 49:103–110.

15   Rodger MA, Carrier M, Gervais M, Rock G. Normal functional protein S activity does not exclude protein S deficiency. Pathophysiol Haemost Thromb 2003–2004; 33:202–205.

16   Tsuda T, Jin X, Tsuda H, Ieko M, Morishita E, Adachi T, Hamasaki N. New quantitative total protein S-assay system for diagnosing protein S type II deficiency: clinical application of the screening system for protein S type II deficiency. Blood Coagul Fibrinolysis 2012; 23:56–63.

17   Henkens CM, Bom VJ, Van der Schaaf W, Pelsma PM, Sibinga CT, de Kam PJ, van der Meer J. Plasma levels of protein S, protein C, and factor X: effects of sex, hormonal state and age. Thromb Haemost 1995; 74:1271–1275.

18   Otsuka Y, Ueda M, Nakazono E, Tsuda T, Jin X, Noguchi K, et al. Relationship between plasma protein S levels and apolipoprotein C-II in Japanese middle-aged obese women and young nonobese women. Blood Coagul Fibrinolysis 2018; 29:39–47.

19   Comp PC, Thurnau GR, Welsh J, Esmon CT. Functional and immunologic protein S levels are decreased during pregnancy. Blood 1986; 68:881–885.

20   Kemmeren JM, Algra A, Meijers JC, Tans G, Bouma BN, Curvers J, et al. Effect of second- and third-generation oral contraceptives on the protein C system in the absence or presence of the factor VLeiden mutation: a randomized trial. Blood 2004; 103:927–933.

21   Kimura R, Sakata T, Kokubo Y, Okamoto A, Okayama A, Tomoike H, Miyata T. Plasma protein S activity correlates with protein S genotype but is not sensitive to identify K196E mutant carriers. J Thromb Haemost 2006; 4:2010–2013.

22   Yin T, Miyata T. Dysfunction of protein C anticoagulant system, main genetic risk factor for venous thromboembolism in northeast Asians. J Thromb Thrombolysis 2014; 37:56–65.

23   Matsumoto S, Koga Y, Noda N, Watanabe K, Fujimoto A, Hotta T, et al. Gene frequency analysis of PROC p.Lys193del in the Japanese Population, Jpn J Clin Pathol 2018; 66:833–838.

24   Miyata T, Sato Y, Ishikawa J, Okada H, Takeshita S, Sakata T, et al. Prevalence of genetic mutations in protein S, protein C and antithrombin genes in Japanese patients with deep vein thrombosis. Thromb Res 2009; 124:14–18.

25   Inoue H, Terachi SI, Uchiumi T, Sato T, Urata M, Ishimura M, et al. The clinical presentation and genotype of protein C deficiency with double mutations of the protein C gene. Pediatr Blood Cancer 2017; 64:.

26   Ichiyama M, Inoue H, Ochiai M, Ishimura M, Shiraishi A, Fujiyoshi J, et al. Diagnostic challenge of the newborn patients with heritable protein C deficiency. J Perinatol 2019; 39:212–219.

27   Ding Q, Yang L, Hassanian SM, Rezaie AR. Expression and functional characterisation of natural R147W and K150del variants of protein C in the Chinese population. Thromb Haemost 2013; 109:614–624.

28   Marlar RA, Potts RM, Welsh CH. Accuracy of diagnosis of protein S deficiency by protein S activity and antigen assays. J Clin Ligand Assay 2005; 28:130–135.

29   Persson KE, Hillarp A, Dahlback B. Analytical considerations for free protein S assays in protein S deficiency. Thromb Haemost 2001; 86:1144–1147.

30   Tsuda T, Tsuda H, Yoshimura H, Hamasaki N. Dynamic equilibrium between protein S and C4b binding protein is important for accurate determination of free protein S antigen. Clin Chem Lab Med 2002; 40:563–567.

31   Maurissen LF, Thomassen MC, Nicolaes GA, Dahlback B, Tans G, Rosing J, Hackeng TM. Re-evaluation of the role of the protein S-C4b binding protein complex in activated protein C-catalyzed factor Va-inactivation. Blood 2008; 111:3034–3041.

32   Adcock DM, Gosselin R. Direct Oral Anticoagulants (DOACs) in the Laboratory: 2015 Review. Thromb Res 2015; 136:7–12.

33   Smock KJ, Plumhoff EA, Meijer P, Hsu P, Zantek ND, Heikal NM, Van Cott EM. Protein S testing in patients with protein S deficiency, factor V Leiden, and rivaroxaban by North American Specialized Coagulation Laboratories. Thromb Haemost 2016; 116:50–57.

34   Kuma H, Matsuda R, Nakashima A, Motoyama K, Takazaki S, Hatae H, et al. Protein S-specific activity assay system is not affected by direct oral anticoagulants. Thromb Res 2018; 168:60–62.