European Journal of Obstetrics & Gynecology and Reproductive Biology 211 (2017) 90–97

Contents lists available at ScienceDirect

European Journal of Obstetrics & Gynecology and Reproductive Biology

journal homepage: www.elsevier.com/locate/ejogrb

Full length article

Genotyping analysis of protein S-Tokushima (K196E) and the involvement of protein S antigen and activity in patients with recurrent pregnancy loss

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ARTICLE INFO

Article history:
Received 7 November 2016
Received in revised form 27 January 2017
Accepted 30 January 2017

Keywords:
Protein S
Protein S-Tokushima
Recurrent pregnancy loss
Lupus anticoagulant

ABSTRACT

Objective: Preston et al. indicated that Protein S (PS) deficiency was associated with stillbirths but not miscarriages. The PS-Tokushima missense variant was reported to serve as a genetic risk factor for deep vein thrombosis in the Japanese population. A previous cross-sectional study showed no increase in the prevalence of PS-Tokushima in patients with recurrent early pregnancy loss or in patients with intra uterine fetal death and/or fetal growth restriction. There has been limited number of prospective studies examining the pregnancy outcome in patients with both a PS deficiency and recurrent pregnancy loss (RPL). We examined the association between PS deficiency, PS-Tokushima and RPL.

Study design: The study group consisted of 355 Japanese women with two or more consecutive pregnancy losses and 101 parous women. The frequency of PS-Tokushima and the subsequent live birth rate in relation to a PS deficiency defined as low PS-specific activity (total PS activity/total PS antigen) and the carriage of PS-Tokushima were examined.

Results and conclusions: There was no significant difference in the frequency of PS-Tokushima between patients and controls. The 8 patients carriers of PS-Tokushima variant were capable of a subsequent live birth without the use of heparin. There was no significant difference in subsequent live birth rates between patients with low or normal PS-specific activity/PS activity without heparin prophylaxis after excluding miscarriages caused by an abnormal embryonic karyotype using multivariate logistic regression analysis. There was no association between PS-Tokushima and RPL and a PS deficiency or low PS activity was shown not to serve as a reliable clinical predictor of subsequent miscarriage.

Introduction

Established causes of recurrent pregnancy loss (RPL) include antiphospholipid syndrome (APS), uterine anomalies, and parental and embryonic chromosomal abnormalities [1–4]. APS, acquired thrombophilia, are the only treatable cause of RPL, and combined low-dose aspirin and heparin treatment has been shown to improve the live birth rate in patients with APS [5,6]. Heritable thrombophilia has been reported to be associated with RPL [7,8]. A cross-sectional study by Preston et al. indicated that protein S (PS) deficiency was associated with stillbirths (odds ratio(OR) 3.3, 95% confidence interval (CI) 1.0–11.3) but not miscarriages (OR 1.2, 95% CI 0.7–1.9) [7]. A meta-analysis by Rey et al. revealed that PS deficiency is associated with recurrent fetal loss (OR 14.72, 95% CI 0.99–218) and late non-recurrent fetal loss (OR7.39, 95%CI 1.28–42.63) but not with recurrent early miscarriage [8]. An association between PS deficiency and recurrent fetal loss is unclear since sample size might be insufficient because the frequency of PS deficiency is relatively small.

Protein S (PS) is a cofactor for activated protein C (APC) and degrades activated factor (F) V and FVIII [9]. The free form of PS functions as a cofactor because its cofactor activity for APC is lost when PS binds to C4b-binding protein (C4bBP). A congenital PS deficiency is a well-known risk factor for the development of deep vein thrombosis (DVT) [10].

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http://dx.doi.org/10.1016/j.ejogrb.2017.01.056
0301-2115/

The PS-Tokushima (p.Lys196Glu, K196E) missense variant (PS-Tokushima) was identified in the second epidermal growth factor-like (EGF) domain of PS and reported to serve as a genetic risk factor for DVT in the Japanese population [11]. The prevalence of this variant was found to be about 1.65-1.8% in the Japanese general population [12,13] and it has not as yet been identified in Chinese, Koreans, or Caucasians [14–16].

The meta-analysis did not show that a PS deficiency increased the risk of early miscarriage at less than 10 weeks gestation [8]. In addition, the terms early miscarriage and fetal loss should be distinguished. There has been limited number of prospective studies examining the pregnancy outcome in patients with both a PS deficiency and RPL. However, a nation-wide survey found that 43.9% of facilities in Japan examined PS activity in patients with RPL [17]. A similar problem is speculated to exist worldwide.

In the cross-sectional presented here, we examined associations between PS-Tokushima variant carriage, total PS antigen, total PS activity, PS deficiency defined as low PS-specific activity (ratio = activity/antigen) and RPL. In the cohort study, we examined whether PS-Tokushima or a PS deficiency influenced the subsequent live birth rate.

Materials and methods

Patients and controls

All patients were seen at Nagoya City University Hospital between September 2008 and July 2014. The study group consisted of 355 Japanese women with two or more consecutive pregnancy losses [18,19].

All patients underwent a systematic examination, including hysterosalpingography, chromosome analysis of both partners, determination of antiphospholipid antibodies (aPLs), including lupus anticoagulant (LA), by 5×-diluted activated partial thromboplastin time (aPTT), diluted Russell's viper venom time (RVVT) and β2 glycoprotein I-dependent anticardiolipin antibody determination (β2GPI-aCL), as well as blood tests for hypothyroidism and diabetes mellitus, before a subsequent pregnancy [20]. Criteria for exclusion from the analyses included the presence of uterine anomalies and chromosomal abnormalities in either partner.

Subsequent pregnancies of all patients were followed up until December 14, 2014. Gestational age was calculated from basal body temperature (BBT) charts. Ultrasonography was performed once a week from 4 to 8 weeks of gestation. Dilation and curettage was performed on patients diagnosed as having a miscarriage. A part of the villi was cultured, and the cells were harvested after 6–22 days

Figure 1. Flowchart of patients

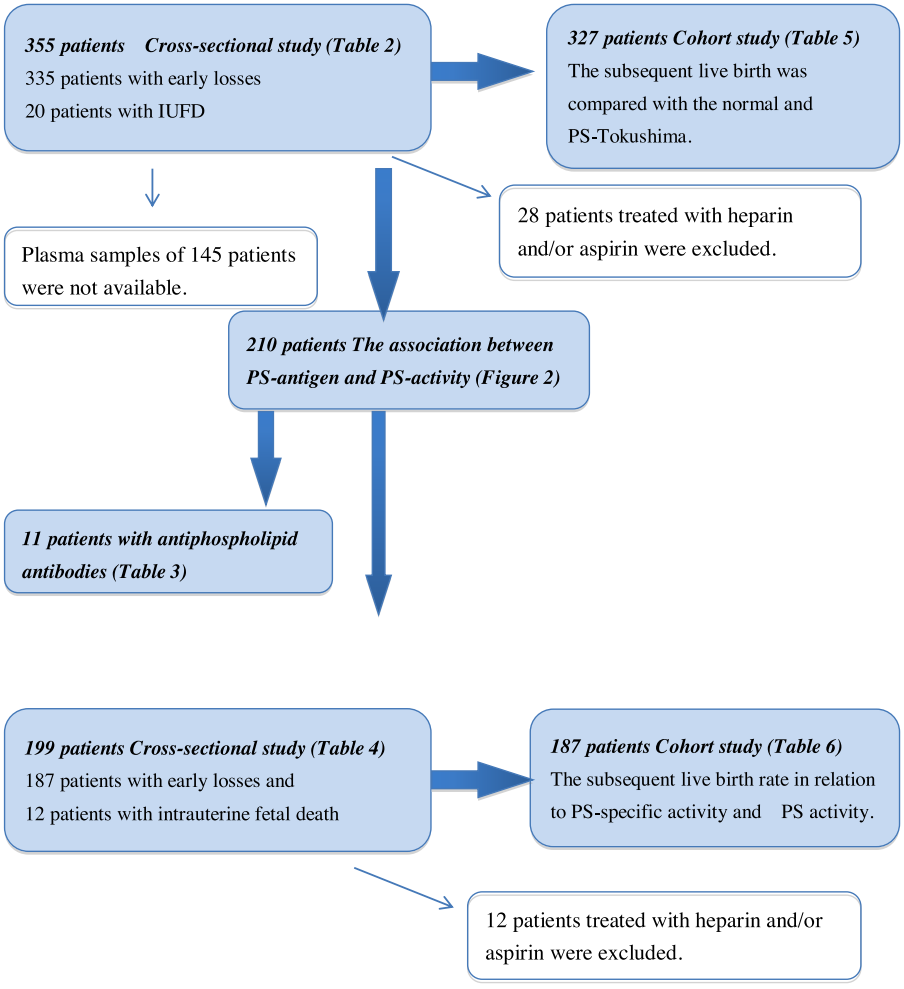

Fig. 1. A flow chart is shown to clarify the exclusion criteria. The frequencies of PS-Tokushima were compared between 355 patients and 101 controls. The total PS antigen, activity, and specific activity were compared between the 210 patients and 101 controls since only 210 patients were available to provide plasma samples. The PS activity was compared between 11 patients with aPLs and 199 patients without aPLs. The 11 patients with aPLs were excluded from further analysis.

of cultivation for chromosomal analysis using the standard G-banding technique.

Control subjects consisted of 101 women with at least one child and no history of infertility or miscarriage, all of whom had been recruited from April to August 2013. None of the patients or controls was pregnant at the time of the study.

This study was conducted with the approval of the Research Ethics Committee of Nagoya City University Graduate School of Medical Sciences. Each patient provided written consent after receiving a thorough explanation concerning the purpose of the study and the methods to be employed.

Cross-sectional study

The frequencies of PS-Tokushima were compared between 355 patients and 101 controls. The total PS antigen, activity, and specific activity were compared between the 210 patients and 101 controls since only 210 patients were available to provide plasma samples. A flow chart is shown in Fig. 1 to clarify the exclusion criteria.

The PS activity was compared between 11 patients with aPLs and 199 patients without aPLs. The 11 patients with aPLs were excluded from further analysis.

Cohort study

In the present cohort study, the subsequent live birth rate was compared among the 327 patients with the normal and PS-Tokushima variant, and 199 patients were examined for the PS antigen, activity, and specific activity.

Twelve patients received heparin plus aspirin in deference to their wishes even after they had been provided with information that aspirin or heparin had, in general, no effect on the live birth rate in cases of unexplained recurrent miscarriage [21]. The cohort study was conducted on a total of 187 patients which did not include these 12 patients.

Statistical analyses

The PS antigen, activity, specific activity between patients with and without aPLs and between patients and controls were compared by Student's *t*-test. The PS-specific activity was compared between subjects with and without PS-Tokushima by Student's *t*-test.

The allele frequency was compared by chi-squared test between patients and controls.

Multivariate logistic regression analyses were performed to examine the association with the subsequent live birth rate, after adjusting for age and the number of previous miscarriages. The same analysis was performed after excluding miscarriage cases with an abnormal embryonic karyotype. PS antigen, activity, and specific activity levels were categorized as normal or low using the 5th and 10th percentiles of values of the parous controls. PS antigen, activity, and specific activity levels of the patients were also categorized into quartiles.

All analyses were carried out using the statistical software SPSS, Version 21. $P < 0.05$ was considered to denote statistical significance.

Genetic analysis

Venous blood samples were collected in tubes containing K2 ethylenediamine tetraacetic acid and applied to genomic deoxyribonucleic acid (DNA) extracting columns (QIAamp DNA Blood Midi; Qiagen, Tokyo, Japan) according to the manufacturer's protocol. Polymerase chain reaction (PCR) was performed on genomic DNA samples using a Phusion High-Fidelity DNA Polymerase (New England BioLabs, Finland). A one μL (about 10 ng) solution (DNA preparation) was used as a template for the PCR. Exon 5, intron E and exon 6 of the *PS* gene were amplified by PCR using the sense and antisense primers 5′-CAATTTTAGAATTC-CATGACATGAGA-3′ and 5′-TGTGTTTGAATTCTACCATCCTGCT-3′, respectively.

After initial denaturation at 98 °C for 30 s, 35 cycles (98 °C for 10 s, 62.3 °C for 30 s, and 72 °C for 15 s) and a final extension at 72 °C for 5 min were used to amplify 434 base pair products.

A one-point variant changes an AAG codon to a GAG codon, and leads to a substitution of Lys 196 by Glu in the second EGF domain of the PS molecule. To confirm the genotype, purified templates were sequenced with a BigDye Terminator v3.1 Cycle Sequencing kit (ABI Prism, Applied Biosystems, Foster City, CA, USA) on an automated sequencer, the 3100 Genetic Analyzer.

PS antigen, activity, and specific activity

Plasma samples were prepared in tubes containing 3.2% sodium citrate by centrifugation at 4 °C at 3000 rpm for 15 min. The plasma samples were then stored at −40 °C until use. The PS antigen, activity, and specific activity were determined with a total PS-assay system (Shino-Test, Japan) [22]. Tsuda et al. developed a new total PS assay system and found that PS-specific activity, which is equal to total PS activity/total PS antigen could be used to detect a PS type II deficiency such as PS-Tokushima.

Briefly, to measure total PS antigen levels, free PS in plasma was first combined with C4bBP by incubation with an excess of purified human C4bBP PS complex, and the PS concentration was measured using a latex agglutination method. After the standard or samples were measured using an automated analyzer, total PS antigen levels were determined by the absorbance using a standard curve. The intra-assay coefficient of variation (CV) for the high antigen control was 0.8%, and that for the low antigen control was 0.6%. The inter-assay CV for the high antigen control was 1.6%, and that for the low antigen control was 1.0%.

We slightly modified the method for measuring total PS activity in order to make the assay more practical. Specifically, each plasma sample was reacted with activated protein C, factor Va (FVa) and factor Xa (FXa) in the presence of phospholipid. After the dissociation of PS-C4bBP complex and degradation of FVa, the resulting solution was mixed with prothrombin and S-2238. The intra-assay coefficient of variability (CV) for the high activity control was 1.3%, and that for the low activity control was 3.2%. The inter-assay CV for the high antigen control was 1.8%, and that for the low antigen control was 3.6%.

In PS gene analyses, healthy controls with a specific activity of 0.69 or less (mean-3 standard deviation (SD)) were revealed to have the PS-Tokushima variant [22].

Table 1
Characteristics of 355 patients and 101 controls.

	Controls	Patients	P-value
No. of patients	101	355	
Mean age (SD)	35.75 ± 4.93	33.77 ± 4.41	0.089
No. of previous miscarriages	0	2.52 ± 0.86	
No. of previous intrauterine fetal deaths	0	0.07 ± 0.29	
0	0	335 (94.4%)	
1–2	0	20 (5.6%)	
No. of previous live births	1.64 ± 0.67	0.21 ± 0.44	<0.0001
Primary	0	283 (79.7%)	
Secondary	101	72 (20.3%)	

Results

The characteristics of patients and controls are shown in Table 1. The mean age of the patients tended to be insignificantly lower than that of the parous controls (p = 0.089). Twenty patients (5.6%) had a history of previous intrauterine fetal death (IUFD) after 12 weeks gestation. Secondary RPL was 20.3%. Secondary RPL was defined as two or more consecutive pregnancy losses after normal delivery.

Nine of 355 (2.5%) patients and one of the 101 (1.0%) controls were positive for PS-Tokushima (Table 2). There was no significant difference in the prevalence of the variant (OR 2.58, 95% CI 0.32–20.60). The statistical power for PS-Tokushima ($1-\beta$) was 0.173. Then we merged our data with the previously published Japanese data [12,13,23]. The combined data also suggested no significant difference ($1-\beta = 0.076$, Table 2).

Eleven (5.2%) of 210 patients whose PS antigen and activity could be measured had aPLs (Table 3). Ten patients were positive for LA-aPTT, 4 were positive for β2GPI-aCL and 3 were strongly positive for LA-aPTT, LA-RVVT and β2GPI-aCL. Of the 11, 7 patients were diagnosed as having APS, based on the fact that aPLs persisted for more than 12 weeks.

There were no significant differences in the mean values of PS antigen, PS activity and PS specific activity among patients with and without aPLs. Two of 11 (18.2%) patients with LA-aPTT and 3 of 199 (1.5%) patients without aPLs showed low PS activity. One of the 2 patients appeared strongly positive for aPLs and exhibited low PS specific activity. No mutation was found in the whole PS gene of the patient No. 10 (Table 3). We marked in red the lower than the reference range value in Table 3.

Ten patients and one control showed low levels of PS-specific activity (Fig. 2). Genetic analysis of 9 patients and one control revealed them heterozygous for PS-Tokushima. One patient had APS. Mean (SD) values of PS-specific activity of subjects with PS-Tokushima (0.65 (0.04)) were significantly lower than those of subjects with the normal allele (0.95 (0.10), <0.001).

We excluded the 11 patients with aPLs from the following comparison. There were no differences in levels of PS antigen, PS activity and PS-specific activity between patients and controls (Table 4). There was also no difference in the frequency of low PS antigen, PS activity and PS-specific activity.

In the cohort study, all 8 patients with PS-Tokushima gave live birth without heparin and two patients had live births with aspirin alone (Table 5). One patient with PS-Tokushima and a history of IUFD miscarried again in spite of heparin use.

There was no difference in the live birth rate between normal and low PS-specific activity, using a cutoff value of 5th or 10th percentile of parous women or by quartile classification according to the results of the logistic regression analysis (Table 6). One hundred and thirty two of 187 (70.6%) patients gave live birth after the 12 patients treated with heparin were excluded.

A total of 24 (43.6%) miscarried conceptuses could be karyotyped, of which 10 (41.7%) had a normal karyotype and 14 (58.3%) had an abnormal karyotype. According to the results of the logistic regression analysis, there was no difference in the live birth rate associated with the low PS-specific activity as compared to that associated with normal PS-specific activity after excluding cases in which there was a miscarriage caused by an abnormal embryonic karyotype.

The subsequent live birth rate in patients with low PS activity was significantly higher than that in patients with normal PS activity according to the results of the logistic regression analysis when the 10th percentile was used as the cutoff value (Table 6). The tendency decreased after excluding cases in which the miscarriage was caused by an abnormal embryonic karyotype. When the PS activity was categorized into quartiles, live birth rates did not differ between quartiles. The result remained essentially the same after excluding cases with an abnormal embryonic karyotype.

The same analysis was performed for PS antigen and no difference was observed (not shown).

Discussion

In the present study, there was no difference in the prevalence of the PS-Tokushima variant between patients with early RPL and control women. The frequencies were similar to that observed in the Japanese general population (1.8%) [13]. A previous cross-sectional study showed no increase in the prevalence of PS-Tokushima in patients with recurrent early pregnancy loss (1.7%, 4 of 233) or in patients with IUFD and/or fetal growth restriction (1.8%, 2 of 114) [23]. The PS-Tokushima was not associated with RPL.

The first cross-sectional study by Preston et al. indicated that PS deficiency was associated with stillbirth (OR 3.3, 95% CI 1.0–11.3) but not with miscarriage (OR 1.2, 95% CI 0.7–1.9) [7].

The meta-analysis showed an association between Factor V Leiden and early (OR 2.01, 95% CI 1.13–3.58) and late (7.83, 2.83–21.67) recurrent fetal loss, and late non-recurrent fetal loss (3.26, 1.82–5.83) as well as an association between PS deficiency and recurrent fetal loss (14.72, 0.99–218.01) and late non-recurrent fetal loss after 22 weeks gestation (7.39, 1.28–42.63)[8]. A cross-sectional study by Gris et al. showed no association between 500 patients with recurrent miscarriage (less than 16 weeks) and 150 controls [24]. The association between a PS deficiency and early miscarriage was not confirmed. One of the problems in these

Table 2
The prevalence of protein S-Tokushima in our study and the previously published Japanese data.

	Control	Patients with 2 or more pregnancy losses	OR (95%CI)	Patients with early RPL	OR (95%CI)	Patients with a history of IUFD	OR (95%CI)
The present study	1.0% (1/101)	2.5% (9/355)	2.58 (0.32–20.60)	2.4% (8/335)	2.47 (0.30–19.79)	5.0% (1/20)	5.00 (0.30–83.31)
Neki et al.	–			1.7% (4/233)		1.8% (2/114)	
Yamazaki et al. Thromb Res 1993	1.65% (3/182)						
Kimura R et al. Blood 2006	1.81% (66/3651)						
Total	1.78% (70/3934)			2.07% (12/579)	1.14 (0.62–2.12)	2.24% (3/134)	1.26 (0.39–4.07)

OR: Odds ratio, CI: confidence interval.
RPL: recurrent pregnancy loss, IUFD: intrauterine fetal death.

Table 3
Levels of antiphospholipid antibodies and PS antigen, PS activity and PS-specific activity in 11 patients with antiphospholipid antibodies.

Patient No.	LA-aPTT	LA-RVVT	b2-GPI dependent aCL	PS Tokushima	Total PS antigen	Total PS activity	PS- specific activity	Subsequent pregnancy		
								Treatment	Outcome	Embryonic karyotype
1	7.4	negative	negative	A1	23.73	21.52	0.91	A	failure	47,XX,+15
2[a]	8.1	negative	negative	A1	23.97	20.06	0.84	A/H	failure	48,XX,+8,+22
3	8.2	negative	negative	A1	22.75	22.99	1.01	A/H	success	
4[a]	8.3	negative	negative	A1	20.1	17.1	0.85	no	failure	46,XX
5	9.8	negative	negative	A1	16.9	14.7	0.83	A	success	
6	8.7	negative	negative	A1	23.48	23.73	1.01	A	failure	47,XX,+16
7[a]	10.9	negative	negative	A1	24.5	25.2	1.03	A	success	
8[a]	23.5	1.46	125	A1	24.0	23.2	0.97	A/H	success	
9[a]	35	1.89	6.6	A1	18.6	17.4	0.93	A/H	success	
10[a]	60.2	2.53	125	A1	16.10	8.60	0.53	A/H	success	
11[a]	negative	negative	5.4	A1	24.22	26.17	1.08	A/H	failure	chemical
Mean (SD)					21.67 (3.16)	20.06 (5.23)	0.91 (0.15)			

The low values were marked in red.
PS: protein S.
LA: lupus anticoagulant, aPTT: activated partial thromboplastin time, RVVT: Russell's viper venom time.
b2-GPI dependent aCL: b2-glycoprotein I dependent anticardiolipin antibody.
A: low dose aspirin, A/H: aspirin and unfractionated heparin.
[a] Patients with antiphospholipid syndrome.

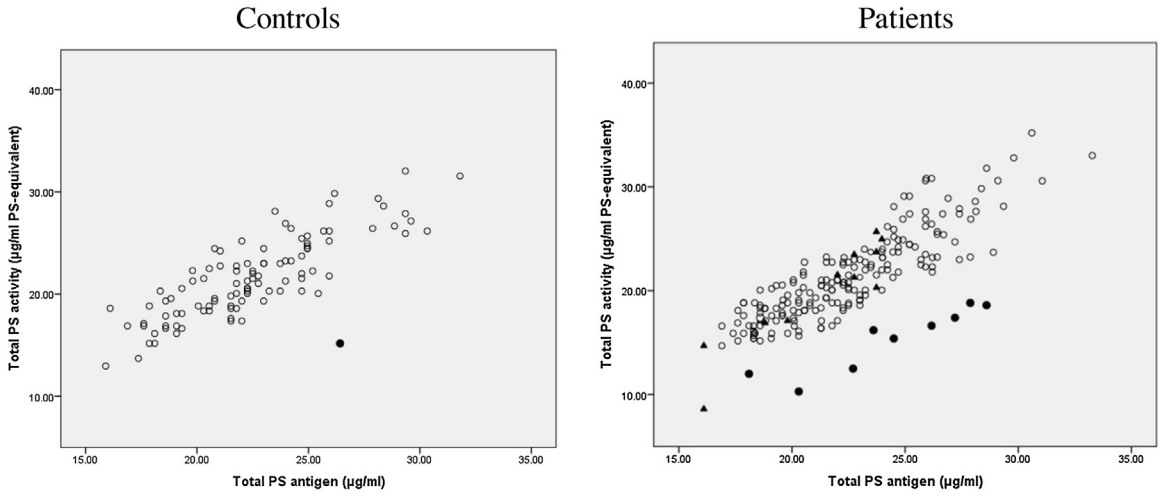

Fig. 2. The association between protein S antigen and protein S activity in 210 patients and 101 controls. Ten patients and one control showed low levels of PS-specific activity. Genetic analysis of 9 patients and one control revealed them heterozygous for PS-Tokushima. One patient had APS.

Table 4
Mean value of Total PS antigen, PS activity and PS specific activity in 199 patients and 101 controls.

	Controls	Patients with 2 or more pregnancy losses		Patients with a history of IUFD	
Total	101	199	P-value	12	P-value
Mean (SD) value of total PS antigen (μg/ml)	22.53 (3.38)	22.53 (3.27)	0.999	23.08 (2.98)	0.592
Normal range (5–95th percentile)*	17.61–29.35				
Mean (SD) value of total PS activity (μg/ml PS-equivalent)	21.59 (3.97)	21.08 (4.34)	0.321	21.28 (4.25)	0.796
Normal range (5–95th percentile)*	15.27–28.84				
Mean (SD) value of PS specific activity	0.96 (0.10)	0.93 (0.11)	0.068	0.92 (0.12)	0.249
Normal range (5–95th percentile)*	0.81–1.14				
Number (%) of patients with low PS antigen	2 (1.98%)	6 (3.02%)	OR(95% CI) 1.54 (0.30–7.75)	0	OR(95% CI)
Number (%) of patients with low PS activity	5 (4.95%)	8 (4.02%)	0.80 (0.26–1.43)	0	–
Number (%) of patients with low PS specific activity	4 (3.96%)	18** (9.05%)	2.12 (0.69–6.54)	1 (8.3%)	2.20 (0.23–21.7)

Levels were determined as low when values were less than the 5th percentile of the fertile controls.
PS: protein S.
* These range was used for low level of PS antigen and activity.
** This result is biased because we examined two additional cases after the patients were found to have PS-Tokushima.

Table 5
Cohort Study: The subsequent live birth rate was compared among the 327 patients with the normal and PS-Tokushima.

Genotype	Live birth rate	Live birth rate excluding abnormal embryonic karyotype	Crude analysis	Multivariable logistic regression
			OR (95%CI)	OR (95%CI)
Normal	72.4% (231/319)	78.3% (231/295)	Reference	Reference
PS-Tokushima	100% (8/8)	100% (8/8)	–	–

OR: Odds ratio, CI: confidence interval.

Table 6
Cohort study: Subsequent live birth rate in relation to PS-specific activity and PS activity in 187 patients with a history of recurrent pregnancy loss without heparin treatment.

PS specific activity						PS activity				
		Live birth rate	Live birth rate excluding abnormal embryonic karyotype	Crude analysis OR (95%CI)	Multivariable logistic regression OR (95%CI)		Live birth rate	Live birth rate excluding abnormal embryonic karyotype	Crude analysis OR (95%CI)	Multivariable logistic regression OR (95%CI)
5th percentile	Normal	69.2% (117/169)	75.0% (117/156)	Reference	Reference	Normal	70.2% (127/181)	75.1% (127/169)	Reference	Reference
	Low	83.3% (15/18)	83.3% (15/18)	1.67 (0.46–6.06)	1.68 (0.46–6.19)	Low	83.3% (5/6)	100% (5/5)	–	–
10th percentile	Normal	69.4% (111/160)	74.5% (111/149)	Reference	Reference	Normal	67.5% (106/157)	73.1% (106/145)	Reference	Reference
	Low	77.8% (21/27)	84.0% (21/25)	1.80 (0.58–5.57)	1.84 (0.59–5.75)	Low	86.7% (26/30)	89.7% (26/29)	3.19 (0.91–1.13)	3.40 (0.96–12.07)
Quartile	1.0-	68.0% (34/50)	72.3% (34/47)	Reference	Reference	23.24-	71.4% (35/49)	76.1% (35/46)	Reference	Reference
	0.94-	75.6% (34/45)	82.9% (34/41)	1.86 (0.66–5.23)	1.77 (0.62–5.05)	20.3–23.2	60.4% (29/48)	67.4% (29/43)	0.65 (0.26–1.65)	0.58 (0.23–1.51)
	0.87-	64.6% (31/48)	67.4% (31/46)	0.79 (0.33–1.92)	0.75 (0.30–1.87)	18.1–20.2	73.3%(33/45)	80.5% (33/41)	1.30 (0.46–3.62)	1.16 (0.43–3.46)
	−0.86	75.0% % (33/44)	82.5% (33/40)	1.80 (0.64–5.08)	1.74 (0.61–4.97)	−18.0	77.8% (35/45)	79.5% (35/44)	1.22 (0.46–3.32)	1.16 (0.42–3.18)

PS: protein S.
OR: Odds ratio, CI: confidence interval.

studies is that the definition of miscarriage or fetal loss has not been established.

Our previous study indicated no increase in the subsequent miscarriage rate in patients with a low level of total PS antigen (65%) compared with patients with a normal PS antigen level [25]. That study had several limitations. We did not consider the influence of lupus anticoagulant (LA). Furthermore, we could not exclude miscarriages caused by an abnormal embryonic karyotype. Thus, from results of the present cross-sectional and cohort study, we confirmed that both a PS deficiency and low PS activity were not a predictor for subsequent miscarriage.

Gris et al. showed an association between late fetal loss and PS deficiency in their cross-sectional study [26] and results of their prospective non-randomized study proved that low molecular weight heparin (LMWH) improved the live birth rate compared with aspirin alone in patients with one fetal loss (79%, 11/14 and 7%, 1/14) [27]. However, sample size was relatively small. In the present study, all 8 patients with PS-Tokushima gave live birth without heparin though the present study included only 5.6% (20 of 355) of patients with a history of IUFD.

As for antithrombotic prophylaxis in patients with both RPL and PS deficiency, several non-controlled studies with small sample size reported the effect of LMWH during the next attempts at pregnancy [28,29]. Our previous study showed live birth rates of 80.8% (21/26) with aspirin alone, 83.7% (41/49) in patients with a PS deficiency and 69.7% (46/66) in patients with a normal PS level with 2 or more RPL [25,30]. Recent RCT indicated that dalteparin had no effect in reducing pregnancy complications such as RPL, preeclampsia, small gestational age and placental abruptio and venous thromboembolism in patients with a history of thrombophilia and pregnancy complications [31]. There is no evidence to support the use of LMWH in patients with both RPL and a PS deficiency.

Current guidelines do not recommend screening for thrombophilia because of obstetric complications and as there is no evidence that the live birth rate can be improved with the anticoagulant [32–35]. However, PS activity was examined in 43.9% of facilities where patients with RPL were being treated, according to a nation-wide Japanese survey [17]. Up to 40% of American physicians are speculated to screen for PS activity [35].

As for an association between PS and aPLs, Parke et al. concluded that 7 of 11 patients with aPLs had low levels of free PS, and total PS levels were within the normal range in all patients [36]. In the present study, only 2 of 11 patients with aPLs who had no PS-Tokushima variant showed a low PS activity and specific activity. The difference in the results might depend on the methods of PS measurement and the kind and titer of the aPLs. Not all, but some aPLs might function as anti-PS antibodies. PS-specific activity has been used to detect a PS type II deficiency in a total PS assay system [22].

We were able to measure total PS antigen and activity in only 210 patients because of the lack of plasma samples. This was one limitation of the present study. Another limitation was that we examined only 20 patients with IUFD. Obstetrical complications such as severe preeclampsia, placental abruption, fetal growth restriction, and stillbirth might be associated with thrombophilia through an intervillous or spiral-artery thrombus and inadequate placental perfusion [26,27,29]. We should distinguish between early RPL and these forms complication.

In conclusion, the PS-Tokushima variant and low PS activity were not risk factors for early RPL. Furthermore, they were not shown to serve as a reliable clinical predictors of subsequent miscarriage. Therefore, we propose that testing for PS antigen and/or activity is not needed, as it is without clinical benefit and constitutes an unnecessary expense. Further study is needed to confirm our findings because the sample size was insufficient given that the frequency of PS-Tokushima was 1.8%.

Author contributions

Tsuda T is employed by a commercial company: Shino-Test Corporation. TT contributed to measure protein S antigen and activities. Shino-Test Corporation covered the cost of the measurement. TT did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests

TT is employed by a commercial company: Shino-Test Corporation. TT contributed to the measurement of protein S (PS) antigen, PS activity and PS-specific activity. Shino-Test Corporation covered the cost of the measurement. TT did not have any role in the study design, data collection or analysis. The funder provided support in the form of salaries for TT, but did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

MS-O has received a research grant and lecture honoraria from Kaken Pharmaceutical Co. Ltd., Kissei Pharmaceutical Co. Ltd., Aska Pharmaceutical Co. Ltd., Sekisui Medical Co. Ltd. and Siemens Japan. The other authors have no competing interests to declare that might be perceived as posing a conflict of interest associated with this study.

Acknowledgement

This study was supported by a Grant-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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