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分担研究報告書

「難治性不育症に関連する遺伝子の網羅的探索」

抗リン脂質抗体症候群患者における全身性エリテマトーデス疾患感受性遺伝子に関する研究

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研究要旨
不育症は抗リン脂質抗体症候群 (APS) の主要症状である。日本人 (APS) 患者において、全身性エリテマトーデス(SLE)の疾患感受性遺伝子を検討した。APS 患者 90 名(原発性 41 名、SLE 合併二次性 APS49 名)、SLE341 名、健常人 428 名から同意を取得しゲノム DNA 抽出し、TaqMan 法により遺伝子多型を解析した。BANK1、BLK、1q25.1 領域の遺伝子多型は SLE と APS のいずれにおいても関連が認められ、APS と SLE に共通の疾患感受性遺伝子があることが示唆された。

A. 研究目的

抗リン脂質抗体症候群(APS)は後天性の血栓性疾患であるとともに、習慣流産などの妊娠合併症の原因となる自己免疫疾患である。APS は全身性エリテマトーデス(SLE)などの自己免疫疾患に合併する二次性 APS と基礎疾患を持たない原発性 APS に大別される。2008 年以降、欧米の SLE のゲノムワイド関連解析により多くの疾患感受性遺伝子が明らかとなった。本研究では日本人 APS において、SLE 疾患感受性遺伝子を検討し、APS と SLE に共通する遺伝的背景を明らかにすることを目的とした。

B. 研究方法

APS 患者 90 名(原発性 41 名、SLE 合併二次性 APS49 名)、SLE341 名、健常人 428 名から文書による同意を取得し、末梢血からゲノム DNA を抽出した。TaqMan 法を用いて、欧米の SLE のゲノムワイド関連解析で明らかとなった感受性遺伝子、遺伝子領域(BANK1、BLK、1q25.1 領域、TNFAIP3、TNFSF4、PXK)の一塩基多型を解析した。

(倫理面への配慮)

研究への参加は、文書による同意を得てから行い、検体は匿名化することで個人情報を保護した。本研究は北海道大学の倫理委員会の審査を得て行った。

C. 研究結果

解析した遺伝子多型のアリル頻度の結果は表に示すとおりであり、BANK1、BLK、1q25.1 領域の一塩基多型は SLE のみならず APS においても優位な関連があることが示された。TNFAIP3 は SLE のみ、TNFSF4 は APS にのみ関連が認められ、PXK は SLE、APS いずれにも有意な関連は認められなかった。

D. 考察

SLE と APS の遺伝的背景はオーバーラップしている可能性が示唆され、両者は共通の遺伝的背景を持つと考えられた。APS と SLE の遺伝的背景の共通性と相違を明らかにすることによって、APS や SLE の病態への理解が深まる可能性が考えられた。また、人種によっても疾患感受性遺伝子が異なることが明らかとなり、多数の日本人患者を対象とした遺伝子多型解析が必要であると考えられた。

E. 結論

SLE と APS は共通の遺伝的背景を持つと考えられた。また、人種間においても疾患感受性遺伝子が異なる可能性が示唆された。

F. 健康危険情報

なし

G. 研究発表

1. 論文発表

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H. 知的財産権の出願・登録状況
（予定を含む。）

1. 特許取得
なし

2. 実用新案登録
なし

3. その他
なし

表１　全身性エリテマトーデス関連遺伝子の抗リン脂質抗体症候群感受性

Gene	SNP_rs	疾患	アリル頻度		OR	95%CI
			疾患	対照		
BANK1	rs10516487	SLE	0.92	0.89	1.48	1.04-2.11
		APS	0.94	0.89	2.07	1.06-4.06
BLK	rs13277113	SLE	0.75	0.70	1.30	1.04-1.63
		APS	0.79	0.70	1.70	1.15-2.50
(1q25.1)	rs10798269	SLE	0.75	0.70	1.28	1.02-1.61
		APS	0.80	0.70	1.74	1.18-2.59
PXK	rs6445975	SLE	0.26	0.23	1.19	0.94-1.50
		APS	0.21	0.23	0.91	0.61-1.34
TNFAIP3	rs2230926	SLE	0.12	0.06	1.91	1.33-2.73
		APS	0.09	0.06	1.52	0.86-2.68
TNFSF4	rs844644	SLE	0.35	0.32	1.16	0.94-1.44
		APS	0.41	0.32	1.47	1.06-2.05

研究成果の刊行に関する一覧表

書籍

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杉浦真弓	抗リン脂質抗体と不育症	日本血栓止血学会編集委員		日本血栓止血学会	東京	2011	in press
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Midline uterine defect size is correlated with miscarriage of euploid embryos in recurrent cases

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Objective: To compare subsequent pregnancy outcomes after two or more miscarriages in patients with and without congenital uterine anomalies.
Design: Case-control study.
Setting: Nagoya City University Hospital.
Patient(s): A total of 42 patients with a bicornuate or septate uterus and 1528 with normal uteri.
Intervention(s): No surgery.
Main Outcome Measure(s): The cumulative success rate for birth, abnormal chromosome karyotype rate in aborted concepti, and the predictive values of the height of the defect/length of the remaining uterine cavity ratio (D/C ratio).
Result(s): Of the total of 1676 patients, 54 (3.2%) had congenital uterine anomalies; 25 (59.5%) of the 42 patients with a bicornuate or septate uterus had a successful first pregnancy after examination, while this was the case for 1096 (71.7%) of the 1528 with normal uteri. There was no difference in the cumulative live-birth rate (78.0% and 85.5%) within the follow-up period. However, the rates for an abnormal chromosome karyotype in aborted concepti in cases with and without uterine anomalies were 15.4% (two of 13) and 57.5% (134 of 233), respectively, with the latter being significantly higher. The D/C ratio in the miscarriage group was also significantly greater than that for the live-birth group.
Conclusion(s): Congenital uterine anomalies have a negative impact on reproductive outcome in couples with recurrent miscarriage and are associated with further miscarriage with a normal embryonic karyotype. The D/C ratio was found to have a predictive value for further miscarriages in recurrent cases. (Fertil Steril® 2010;93:1983–8. ©2010 by American Society for Reproductive Medicine.)

Key Words: Bicornuate uterus, congenital uterine anomaly, recurrent miscarriage, septate uterus

Established causes of recurrent miscarriages are antiphospholipid antibodies (aPL), uterine anomalies, and chromosomal abnormalities in the embryo (1–3). Abnormal chromosomes in either partner, particularly translocations, are also risk factors (4). Regarding uterine anomalies, Raga et al. reported that patients (6.3%, 54 of 868; $P<.05$) with a history of two or more miscarriages had a significantly elevated incidence of Mullerian anomalies compared with fertile (3.8%, 49 of 1289) and sterile (2.4%, 25 of 1024) cases (2). The frequency of congenital uterine anomalies has been reported to be between 1.8% and 37.6% in women with a history of recurrent miscarriage, the variation largely depending on the methods of selection and criteria for diagnosis (5–7).

Thus, affected patients are offered surgery in an attempt to restore the uterine anatomy (8–16). The conclusion is that operations can increase successful pregnancies, but to our knowledge there have been no prospective studies comparing pregnancy outcomes between cases with and without surgery in patients with a history of recurrent miscarriage. Lee et al. reported a preoperative pregnancy loss rate of 77.4%, a 18.2% miscarriage rate, and a 77.3% uncomplicated delivery rate after hysteroscopic septum resection (14). However, it is inappropriate to simply make comparisons before and after surgery because the miscarriage rate before examination might be 100% but the subsequent success rate is never 0. The subsequent live-birth rate is expected to be 72% in recurrent miscarriage patients without abnormal chromosomes in either partner (17) and decreases with the number of previous miscarriages (3).

Information concerning the prognosis in women with congenital uterine anomalies with a history of recurrent miscarriage is limited. The present study was therefore conducted to assess the subsequent live-birth rate, comparing pregnancy outcome between cases with and without bicornis or septum in individuals with a history of recurrent miscarriage.

PATIENTS AND METHODS

We conducted a case-control study. We studied 1676 patients with a history of two or more (2–12) consecutive miscarriages whose subsequent pregnancies were ascertained at least once in our medical records. Hysterosalpingography (HSG), chromosome analysis for both partners, determination of aPL,

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including lupus anticoagulant and β2-glycoprotein I dependent anticardiolipin antibodies (18), and blood tests for hyperthyroidism, diabetes mellitus, and hyperprolactinemia were performed for all patients before subsequent pregnancy. All patients were examined between 1986 and 2007 at Nagoya City University Hospital.

Laparoscopy/laparotomy and/or magnetic resonance imaging (MRI) were performed to ascertain the type of anomaly (investigating both the uterine cavity and the external uterine contours) in accordance with the American Fertility Society classification of Mullerian anomalies (19–21). Tompkin's index was used to distinguish between arcuate uterus and mild septate or bicornuate uterus (22). A Tompkin's index >25% was the criterion for septate or bicornuate uterus. Patients desiring surgical treatment before subsequent pregnancies underwent a Jones metroplasty, Strassman metroplasty, or hysteroscopic transcervical resection (TCR; 8–10).

All pregnancy outcomes of 1676 patients were examined. Patients with at least one kind of aPL were treated with low-dose aspirin and heparin combined therapy. Gestational age was calculated from basal body temperature charts. Ultrasound was performed once or twice a week from 4 to 8 weeks' gestation. Dilation and curettage was performed on all patients diagnosed with miscarriage, and the karyotypes of aborted conceptuses were determined with the use of a standard G-banding technique. The study was approved by the Research Ethics Committee at Nagoya City University Medical School.

In the present study, [1] the prevalence of clear congenital uterine malformations such as septate uterus, bicornuate uterus, unicornuate uterus, and didelphys was examined; [2] the first pregnancy outcome after systematic examination for recurrent miscarriage was determined for both septate and bicornuate uterus cases, comparing patients with or without anomalies; [3] all pregnancy outcomes after systematic examination were also assessed, and the final live-birth rate/patient was calculated; [4] abnormal karyotype rates for aborted concepti at the first miscarriage after the ascertainment of uterine abnormalities were also compared between patients with and without congenital uterine anomalies; [5] the height of the defect/length of the remaining uterine cavity (D/C) ratios were calculated in cases with bicornuate and septate uterine and compared between miscarriages and live birth at the subsequent first pregnancy. We also ascertained whether the D/C ratio has predictive value for further miscarriage in recurrent miscarriage cases.

The analysis was carried out using the SAS system (SAS Institute Inc., Cary, NC) with receiver operating curve (ROC) analysis and logistic regression. $P<.05$ was considered statistically significant.

RESULTS

Baseline Characteristics

One thousand six hundred seventy-six patients became pregnant after systematic examination for recurrent miscarriages. Of this total, 54 (3.2%) had congenital uterine anomalies, 38 with partial bicornis unicolli, 10 with a septum, five with a unicornis, and one with a didelphys. None of them had hypoplasis/agenesis or diethylstilboestrol (DES) drug-related anomalies. Two patients with a septate uterus and a bicornuate uterus also had translocations in either partner. The 94 patients who had structural chromosome abnormalities, including 73 translocations, in either partner, were excluded from the analysis.

One thousand five hundred twenty-eight patients had neither congenital uterine anomalies nor an abnormal chromosome karyotype in either partner; 75 patients exhibited persistent aPL and were treated with low-dose aspirin and heparin combined therapy.

One of the two patients with bicornuate uteri underwent a Jones metroplasty, and the other underwent a Strassman metroplasty (8, 9). One patient with a septum also received a Jones metroplasty, and hysteroscopic TCR was performed for the other four patients with septate uteri.

We compared pregnancy outcomes between 42 patients with septate or bicornuate uteri not undergoing surgery and 1528 patients without uterine anomaly. We found no differences in baseline characteristics between the two groups (Table 1).

Pregnancy Outcome

Subsequent pregnancy outcomes are summarized in Table 2. Twenty-five of the 42 patients with a septate or bicornuate uterus (59.5%) treated without any kind of surgery had a successful outcome, while this was the case for 1096 (71.7%) of the 1528 without congenital uterine anomalies at the subsequent first pregnancy ($P=.084$). Four of five patients with a septate uterus and 21 of 37 patients with a bicornuate uterus gave birth to live babies. There was one case with a bicornuate uterus who suffered from uterine rupture in the first trimester because of the limited capacity.

One patient received surgery after further miscarriage. Thus, 32 (78.0%) of 41 patients and 1307 (85.5%) of 1528 patients with and without uterine anomalies could cumulatively have a live baby within the follow-up period (P = not significant). Live-birth rates of patients with congenital uterine anomalies tended to be lower both at the first pregnancy after ascertainment and cumulatively. Final live-birth rates/person are also shown in Table 2.

Furthermore, rates for an abnormal chromosome karyotype in aborted concepti in cases with and without uterine anomalies were 15.4% (two of 13) and 57.5% (134 of 233), respectively, at the first pregnancy after ascertainment of uterine anomalies, the difference being highly significant (Fisher's exact probability test, $P=.006$).

One of five patients with a unicornuate uterus succeeded in having a baby at the first pregnancy after examination, and four of five could have a baby, cumulatively. The patient with didelphys also succeeded at the first pregnancy after examination.

TABLE 1

Baseline characteristics of patients with and without congenital uterine anomalies.

	Patients with anomalies (n = 42)	Patients without anomalies (n = 1528)	*P* value
Maternal age, y			
Mean (SD)	31.5 (3.5)	31.1 (4.3)	NS
Median (interquartile range)	31 (29)	31 (28)	NS
Number of previous miscarriages			
2	17 (40.5)	765 (50.1)	
3	18 (42.9)	537 (35.1)	
4	7 (16.7)	136 (8.9)	
5 or more	0	90 (5.9)	.085
Mean (SD)	2.74 (0.77)	2.77 (1.12)	NS
Median (interquartile range)	3 (2)	2 (2)	NS
No. of previous live births			
0	37 (88.1)	1328 (86.9)	
1	4 (9.5)	186 (12.2)	
2 or more	1 (2.4)	14 (0.9)	NS
Mean (SD)	0.1	0.14 (0.37)	NS
No. of previous stillbirths			
0	40 (95.2)	1491 (97.6)	
One or more	2 (4.8)	37 (2.4)	NS

Note: Values are numbers (percentages) of patients unless otherwise specified.

Sugiura-Ogasawara. Uterine anomaly and recurrent miscarriage. Fertil Steril 2010.

Predictive Value for the D/C Ratio

Mean values (SD) for the D/C ratio in the miscarriage and live-birth groups were 0.8332 (0.3974) and 0.4776 (0.2745), respectively (P=.0057, 95% confidence interval [CI]; 0.1115–0.5998). When two miscarriage cases caused by an abnormal embryonic karyotype were excluded, the value for the D/C ratio in the miscarriage group was also significantly higher than in the live-birth group (P=.0051). Mean (SD) age and number of previous miscarriages for the 15 patients whose subsequent pregnancy ended in miscarriage and the 17 patients who experienced live births were 31.5 (3.0) versus 31.5 (3.8) and 2.76 (0.75) versus 2.72 (0.79), respectively (P = not significant). Ten patients were excluded because HSG films were not available.

The ROC curve is shown in Figure 1. From the figure, the cutoff value would be appropriate somewhere between 0.59 and 0.64, giving the sensitivity and specificity around 0.75–0.80. The area under the ROC curve, meaning the total diagnostic accuracy of the D/C ratio on live birth, was 0.808. From the logistic regression, the D/C ratio was found to be an independent risk factor on the failure of live birth after adjusting for age and previous number of miscarriages. The odds ratio for the 0.1 increment of D/C ratio was 1.42 (95% CI, 1.06–1.91).

DISCUSSION

In the present study, the live-birth rate of patients with congenital uterine anomalies tended to be lower, both at the first pregnancy after ascertainment and cumulatively, than that of patients with a normal uterus, although the differences were not significant. Congenital uterine anomalies were associated with miscarriages with a normal embryonic karyotype. Thus, congenital uterine anomalies impacted the progression of normal pregnancies.

Salim et al. earlier found no significant difference in the relative frequency of various anomalies or depth of fundal distortion as determined by three-dimensional (3D) ultrasound between women with and without a history of recurrent miscarriage, although abnormalities in uterine anatomy were more severe in women with a history of recurrent miscarriages (23). In this context, the finding in the present study that the D/C ratio is a predictor of further miscarriage in recurrent cases is clearly of interest.

However, 59.5% and 78.0% of our patients with a septate or bicornuate uterus without any kind of surgery could have a baby at the first pregnancy or cumulatively. Several studies concerning obstetric outcome after removal of a uterine septum have been reported (10–16). Lee et al. described a 77.3% uncomplicated delivery rate after hysteroscopic septum resection (14). Kormayos et al. compared pregnancy outcome after removal of septum between cases with and without a residual septum in patients with a history of two or three miscarriages and concluded that the live-birth rate in cases with no remnant was significantly higher than that in cases with a remnant (15). However, the live-birth rate for patients undergoing first hysteroscopy was 35.1% (33 of 94), and the

TABLE 2

Successful reproductive outcome after examination of uterine anomalies in patients with recurrent miscarriage.

	Success rate per pregnancy						Cumulative success rate			
	With anomalies (n = 42)	Bicornuate	Septum	Without anomalies (n = 1528)	Difference in %	*P* value	With anomalies (n = 41)[a]	Without anomalies (n = 1528)	Difference in %	*P* value
Pregnancy after the ascertainment of uterine anomaly										
First	25/42 (59.5)[b]	21/37 (56.8)	4/5 (80.0)	1096/1528 (71.7)	−12.2	.084	25 (61.0)	1096 (71.7)	−10.7	.133
Second	5/9 (55.6)	4/8 (50.0)	2/2 (100)	166/275 (60.4)[c]	−4.8	.772	30 (73.2)	1262 (82.6)	−9.4	.119
Third	2/2 (100)	2/2 (100)		38/69 (55.0)	+45.0	.207	32 (78.0)	1300 (85.1)	−7.1	.215
Fourth				4/18 (22.2)				1304 (85.3)		
Fifth				3/9 (33.3)				1307 (85.5)		
Sixth				0/6 (0)				1307 (85.5)		
Final follow up							32 (78.0)	1307 (85.5)	−7.5	

Note: Values are numbers (percentages) of couples. Success rate is defined as the live birth.

[a] One case underwent surgery between the first and second pregnancy after the ascertainment of an anomaly, thus this case was excluded from the cumulative analysis.

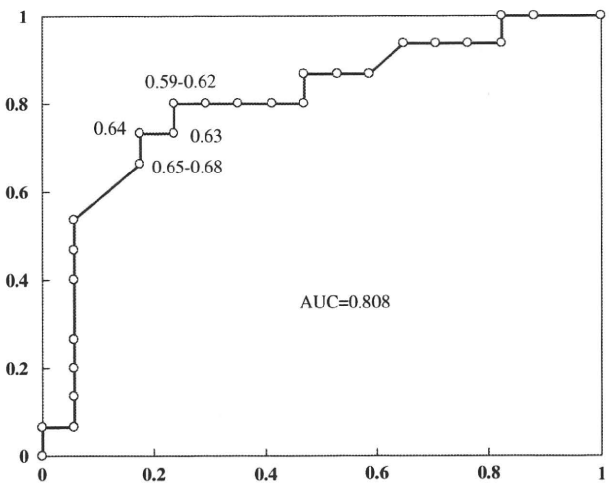
[b] Comparison was performed between patients both with anomalies and with normal uterus.

[c] Cases who could succeed in the first pregnancy were excluded from the analysis of the second and subsequent pregnancies.

Sugiura-Ogasawara. Uterine anomaly and recurrent miscarriage. Fertil Steril 2010.

FIGURE 1

ROC analysis of D/C ratio.



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cumulative live-birth rate after one or two metroplasties was 54.3% (51 of 94). Both live-birth rates were lower than that without surgery in the present study. The benefits of surgical correction (open and hysteroscopic) on pregnancy outcome have yet to be assessed in a randomized trial, but the D/C ratio might be useful in deciding who should be selected.

Limitations

In the present study, clear uterine malformations such as septate, bicornuate, or unicornuate uterus and didelphys were found in 3.2% of patients. The prevalence of clear congenital uterine anomalies in patients with a history of recurrent miscarriages has been reported to be 1.8%–20.1% with the arcuate uterus excluded (5–7) and thus higher than the 2.2% documented for fertile women (28 of 1289) (2). Minor malformations like arcuate uterus do not appear to have any impact on reproduction (2), and therefore we here excluded cases with this anomaly.

HSG is the diagnostic modality that has most often led to a tentative diagnosis of congenital anomalies (19), but when used alone it cannot distinguish between a septate and a bicornuate uterus. Thus laparoscopy has hitherto been needed for a final diagnosis. The advent of sonohysterography, MRI (20), and 3D ultrasound now allows for accurate differential diagnosis (21), although distinguishing an arcuate from a mildly subseptate or bicornuate uterus still remains difficult.

It is important to distinguish between the bicornuate uterus and the septate uterus, especially regarding the selection of surgical methods because TCR should not be performed for the former. We here ascertained the type of anomaly to study the prevalence in accordance with the American Fertility Society classification of Mullerian anomalies. Woelfer et al. proved new 3D criteria by which a bicornuate uerus can be distinguished from a septate uterus when a fundal indentation >10 mm dividing the two cornua is detectable (21). Using 3D ultrasound, it has been found that the septate uterus has the higher incidence. The criteria are useful before deciding on using TCR for the septum. It is difficult to examine the significance of the distinction between bicornuate and septate uteri because of the absence of internationally established criteria, although we have given the live-birth rate for each anomaly in Table 2. Thus we focused not on type of anomalies but rather on the D/C ratio. In addition, the sample size in the anomaly group was too small to allow any conclusion when we distinguished between the two groups.

While we examined 1676 patients who became pregnant at least one time in the present study, we failed to follow up all those who received systemic examination for causes of recurrent miscarriage at our hospital because some lived at a long distance. Some patients might become infertile after miscarriage. A prospective case-control study should therefore be conducted to compare live-birth rates between patients with and without surgery, including consideration of the infertile rate.

Conclusion

Congenital uterine anomalies have a negative impact on reproductive outcome in couples with recurrent miscarriage, being particularly associated with normal embryonic karyotype miscarriages. The height of the defect/length of the remaining uterine cavity ratio, the D/C ratio, has independent predictive value for further miscarriage in recurrent cases. Comparison of cases of anomalies with and without surgery is needed in future recurrent miscarriage studies.

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BRIEF COMMUNICATION

Japanese single women have limited knowledge of age-related reproductive time limits

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In 2007, the total fertility rate in Japan decreased to 1.34, compared with 2.10 in the USA. This decline in fertility is partly due to increased age at marriage and delay in childbearing. Increased maternal age is associated with an increased risk of infertility, miscarriage, and poor pregnancy outcome [1]. A 1986 study found that the percentage of women who did not use contraception and remained childless increased steadily according to age at marriage (6% among 20–24-year-old women compared with 64% among 40–44-year-old women) [2]. The miscarriage rate rose from 14% among patients younger than 35 years of age to 40% among those older than 40 years of age [3]. The average childbearing age in Japan has increased over the past 3 decades as more women have postponed marriage to pursue higher education and careers.

Between June 2007 and March 2008, a 2-page anonymous survey was distributed at Nagoya City University, Nagoya Women's University, and a Women's Health Week in Nagoya (sponsored by the Japanese Society for Obstetrics and Gynecology). In total, 249 single women completed the 15-item survey, which contained questions addressing attitudes toward marriage, occupation, and childbirth, in addition to knowledge about infertility, miscarriage, and age-related reproductive time limits. The Research Ethics Committee at Nagoya City University Medical School approved the study.

The mean age of participants was 25.2 ± 6.8 years. In total, 95.5% of participants expressed a desire to marry in the future, with 96.8% wanting to continue work after marriage; however, 25.7% wanted to stop working after childbirth. Overall, 91.0% of women wanted to have children, and 85.5% thought that childbirth would be important in their life. The issues of marriage, career, and childbirth had been seriously considered by 46.2%, 67.9%, and 40.6% of single women, respectively. The mean age of women considering marriage and childbirth was higher than that of women not considering these events and of women who reported that they were influenced by parental values or common social practice (Table 1).

Nearly all women (98.8%) were familiar with the term "infertility," although only 44.2% and 17.3% chose accurate rates of infertility and miscarriage, respectively. Only 10.7% chose correct answers for all aspects of reproduction. Surprisingly, 36.4% estimated their own age limit for natural pregnancy to be between 45 and 60 years. Significantly more women considering marriage and childbirth chose correct responses about the rate of infertility and about reproduction. Women considering a career chose rates of infertility and miscarriage more accurately than did women not considering this option.

Regarding questions about the source of their knowledge of the term "infertility," 85.9% of women had learned the term from the media, compared with 20.7% who had learned it from school teachers. There was no difference in overall knowledge between women who obtained their information from school teachers and those who obtained it from other sources. Women considering marriage and those with accurate knowledge of infertility rates and causes tended to want children later. Participants with an accurate knowledge of infertility causes incorrectly chose a significantly older fertility time limit. Older participant age was significantly associated with knowledge of infertility rates; however, older participants wanted children later and believed reproductive time limits to be over 40 years of age.

In the present study, there were deficits in the participants' knowledge, despite the majority reportedly knowing the term "infertility." The terms "birth control," "contraception," "induced abortion," and "sexually transmitted infections" can be found in secondary-school health and physical education, biology, and domestic science textbooks, but "infertility" is seldom seen—indicating that there is no substantial public education in Japan about this condition. Women who reported that they had gained their knowledge of reproductive health from school teachers were unaware of accurate infertility rates. This raises concern that school teachers have limited knowledge on the subject, and indicates a need for infertility content to be added to the secondary-school curriculum.

The findings from the present study imply that, without knowing their reproductive limits, many Japanese women may lose their capacity for conception; thus, increased efforts are needed to educate

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Table 1
Association between knowledge about reproductive health issues and personal aspirations[a].

	Age	Considering marriage	Considering career	Considering childbirth	School teachers listed as source of knowledge	Expected age at childbirth	Knowledge of reproductive time limit	Over 40 years of age
Age		<0.01 Yes 26.8 ± 7.4 No 23.7 ± 5.9	>0.05 Yes 25.7 ± 7.0 No 24.1 ± 6.4	<0.01 Yes 26.4 ± 7.4 No 24.4 ± 6.3				
Knowledge about infertility rate	0.01	<0.01	0.07	0.03	>0.05	0.08 (late)	>0.05	>0.05
Knowledge about male:female causes	>0.05	>0.05	0.05	>0.05	>0.05	0.07 (late)	0.01 (late)	>0.05
Knowledge about miscarriage rate	>0.05	>0.05	0.04	>0.05	>0.05	>0.05	>0.05	>0.05
Knowledge about all aspects of reproduction	0.07	0.04	>0.05	0.03	>0.05	0.09 (late)	>0.05	>0.05
Expected age at childbirth	<0.01 (late)	0.07 (late)	>0.05	>0.05	0.10 (early)			
Knowledge of reproductive time limit		>0.05	>0.05	>0.05	>0.05			
Over 40 years of age	0.06 Yes 25.4 ± 6.9 No 24.8 ± 6.5	>0.05	>0.05	>0.05	>0.05			

[a] Values are given as mean ± SD or *P* values.
[b] $P<0.05$ was considered statistically significant.

Japanese women about the influence of age on fertility. Societal pressures forcing women to choose between a career and children must change to reverse the very low fertility rate in Japan.

Conflict of interest

The authors have no conflicts of interest.

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Antiphosphatidylethanolamine antibodies might not be an independent risk factor for further miscarriage in patients suffering recurrent pregnancy loss

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ABSTRACT

The prevalence of antiphosphatidylethanolamine antibodies (aPEs) is higher in recurrent pregnancy loss patients than that in women with normal pregnancy. We conducted a cohort study to examine the predictive value of aPE for recurrent pregnancy loss and to determine its clinical significance. We examined plasma protein dependent (P+) and independent (P−) aPE IgG and IgM antibodies in 367 women with two or more unexplained consecutive pregnancy losses. We also examined conventional antiphospholipid antibodies (aPL) such as β2-glycoprotein I-dependent anticardiolipin antibodies (β2GPI-dependent aCL), lupus anticoagulant with reference to the dilute activated partial thromboplastin time (aPTT) and the diluted Russell's viper venom time (RVVT). Subsequent pregnancy outcome without medication was examined, and patients with and without aPE were compared. Totals of 37 (10.1%), 14 (3.8%), 23 (6.3%), 6 (1.6%), 9 (2.5%), 10 (2.7%) and 50 (13.6%) of the 367 patients were, respectively, positive for P+aPE IgG, P−aPE IgG, P+aPE IgM, P−aPE IgM, β2GPI-dependent aCL, lupus anticoagulant by RVVT and LA by aPTT. The patients with aPE differed from patients with β2GPI-dependent aCL or lupus anticoagulant by RVVT. No difference in live birth rate was apparent between positive and negative aPE patients with no medication. The areas under the curves for each ROC curve for the four aPEs were 0.535, 0.612, 0.546 and 0.533, respectively, so there was no significant variation in diagnostic capacity. We did not obtain any evidence that aPE elevation is an independent risk factor to predict further miscarriage in recurrent pregnancy loss patients.

1. Introduction

Established causes of recurrent pregnancy loss are abnormal chromosomes in either partner, particularly translocations, antiphospholipid antibodies (aPL) and uterine anomalies (Farquharson et al., 1984; Sugiura-Ogasawara et al., 2004, 2010). The antiphospholipid syndrome (APS) is the most important treatable etiology (Rai et al., 1997). The Sapporo criteria have been used to define APS since 1999 and preliminary classification criteria were revised more recently at a workshop in Sydney (Miyakis et al., 2006). With the new international criteria, patients can be diagnosed with APS when lupus anticoagulant and/or anticardiolipin antibodies (aCL) continue to be elevated for 12 weeks. Patients with persistent aPL should be treated with low dose aspirin and heparin combined therapy during pregnancy and about 70–80% can then experience a live birth (Rai et al., 1997).

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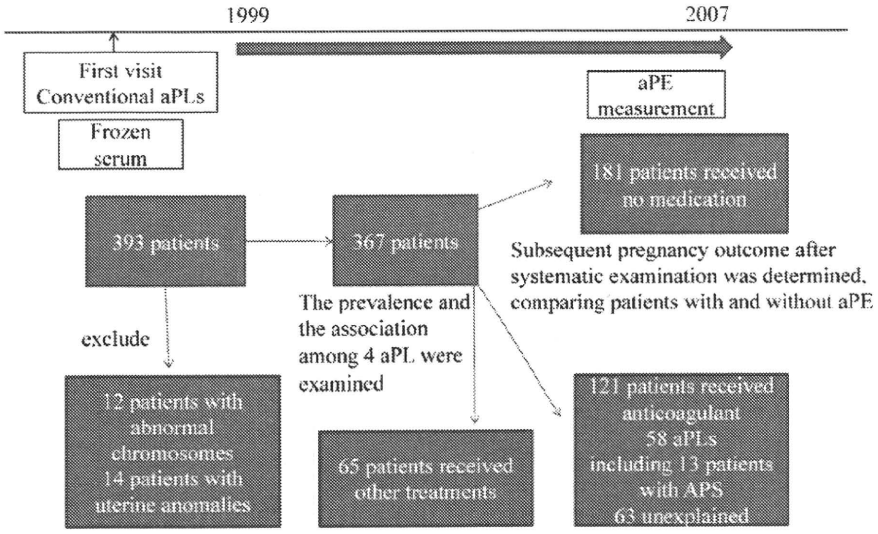

Fig. 1. Study profile.

Many kinds of aPL including antibodies against cardiolipin (CL), phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine (PE), have been measured by ELISA methods. β2-glycoprotein I (β2GPI) was found to be the true antigen of aCL in 1990 (Matsuura et al., 1990). Recently, aPL have been recognized to be autoantibodies to phospholipid-binding plasma proteins. The most common antigens are β2GPI and prothrombin (Roubey et al., 1992). β2GPI-dependent aCL, anti-β2GPI antibodies and lupus anticoagulant detected by the methods described in the International Thrombosis and Homeostasis Society are included in the International criteria for APS (Miyakis et al., 2006).

Sugi and McIntyre (1995) reported that certain antiphosphatidylethanolamine antibodies (aPEs) are not specific for PE per se but are directed to PE-binding plasma proteins, such as high molecular weight kininogen, low molecular weight kininogen, and proteins in complexes with kininogen, factor XI, or prekallikrein. The prevalence of aPE IgG and IgM were reported to be 20.1 and 12.2%, respectively, in patients suffering early pregnancy losses and significantly higher than in controls (Sugi et al., 1999). In contrast, rates for β2GPI-dependent aCL and lupus anticoagulant by dilute Russell's viper venom time (RVVT) were only 0.7 and 1.4%, respectively (Sugi et al., 1999).

However, to our knowledge, there are only limited data for any association between aPE and adverse pregnancy outcome in recurrent pregnancy loss cases (Gris et al., 2000). Thus, aPEs are not included in the international criteria for APS. We therefore here examined the predictive value of aPE and associations among β2GPI-dependent aCL and lupus anticoagulant for recurrent pregnancy loss to determine the clinical significance of aPE.

2. Materials and methods

2.1. Patients

Hysterosalpingography, chromosome analysis for both partners, determination of conventional aPLs, including both lupus anticoagulant by the 5 times diluted activated partial thromboplastin time (aPTT) method and the diluted RVVT method and β2GPI-dependent aCL, and blood tests for hyperthyroidism, diabetes mellitus and hyperprolactinemia were performed for all patients at the first visit of Nagoya City University Hospital. Serum for aPE measurement was taken at the same time when conventional aPL were measured and frozen at −70 °C. In total, we studied 367 women who had a history of two or more consecutive pregnancy losses. None of the patients had any readily identifiable causes of recurrent pregnancy loss, such as uterine or chromosomal abnormalities in either partner. None had received any medication before examination and there was no history of thrombosis. Their mean age was 31.9 ± 4.3 and the average number of previous early pregnancy losses was 2.7 ± 1.1. Twenty-two patients had a history of 26 events of intrauterine fetal death.

The patients' plasma protein dependent (P+) and independent (P−) aPE IgG and IgM were measured as aPE in 2007 using stored serum. aPE was measured once. The 367 pregnancies were recorded from August 1999 to December 2007 and subsequent pregnancy outcome was examined prospectively. A total of 58 patients were positive for at least one kind of conventional aPLs and 13 were diagnosed as APS, according to the Sapporo criteria and the Sydney revision. Sixty-three patients with unexplained causes were also treated with low dose aspirin and heparin therapy. Some 181 patients received no medication. Some 65 patients who received luteal support and a biological response modifier were excluded (Katano et al., 2000). The study profile is shown in Fig. 1.

Gestational age was calculated from basal body temperature charts. Ultrasonography was performed once or twice a week from 4 to 8 weeks' gestation. Dilation and curettage were carried out when miscarriages were diagnosed, and the karyotypes of aborted conceptuses were determined with the use of a standard G-banding technique. The present study was approved by the Research

Ethics Committee at Nagoya City University Medical School.

In the present study: (1) the prevalence and the association among 4 aPL were examined in 367 patients; (2) subsequent pregnancy outcome after systematic examination for pregnancy loss was determined in patients who received no medication or anticoagulants, comparing patients with and without aPE; (3) subsequent pregnancy outcome was examined excluding cases with abnormal karyotypes in aborted concepti; and (4) AUCs for ROC curves of aPE were calculated and receiver operating characteristic (ROC) analysis was carried out to ascertain whether aPE have predictive value for further miscarriage in 181 recurrent cases which received no medication.

2.2. Modified assays for the IgG and IgM isotypes against plasma protein binding phosphatidylethanolamine complex or phosphatidylethanolamine alone

Briefly, microtiter plates were coated with 30 μl of 50 μg/ml of PE (Aventi Polar Lipids, Birmingham, AL, USA), and each well was blocked for 1 h with 10% bovine serum albumin. To detect phospholipid-binding plasma protein dependent and independent reactivity, 50 μl aliquots of patient plasma diluted 1:100 containing either 10% adult bovine plasma or 1% bovine serum albumin were incubated for 1 h. Antibodies were detected with alkaline phosphatase labeled anti-human IgG or IgM antibodies. Nonspecific binding control wells were processed in parallel and the background values were subtracted (Sugi et al., 1999).

Cut-off levels were set at mean ± 2SD, established using sera from 122 healthy volunteers. Therefore 0.32, 0.45, 0.44 and 1.0 were considered positive for P+ aPE IgG, P − aPE IgG, P + aPE IgM and P − aPE IgM, respectively.

2.3. Assay for the lupus anticoagulant by the diluted aPTT method

Brain cephalin (Automated aPTT, Organon Teknica, Durham, NC) was employed as a phospholipid reagent for the determination of aPTT, diluted 5 times in veronal saline (Ogasawara et al., 1996a).

A: Fifty μl of non-pregnant control woman plasma, 50 μl of standard plasma, and 100 μl of diluted cephalin were mixed and incubated for exactly 3 min at 37 °C. B: At the same time 100 μl of standard plasma alone and 100 μl of diluted cephalin were mixed and incubated for 3 min at 37 °C. One hundred microlitres of 25 mM $CaCl_2$ was added and clotting time was measured with an Option 4 bioMeriux calculator, France.

Clotting times for A–B with control plasma samples from 104 healthy non-pregnant women were first examined. The mean and standard deviation values were 2.57 and 1.60 s, respectively. Lupus anticoagulant was considered positive when prolonged clotting times (>mean ± 3SD, 7.37 s) failed to correct when samples were mixed 1:1 with standard plasma.

2.4. Assay for the lupus anticoagulant with reference to the diluted Russell's viper venom time

To determine *T*1, 200 μl of healthy non-pregnant control woman plasma and 200 μl of diluted Russell viper venom and phospholipid reagents containing 25 mM $CaCl_2$ (Gradipore Ltd., Pyrmont, Australia) were mixed and clotting time was measured with an Option 4 bioMeriux calculator, France. To determine *T*2, 200 μl of the same non-pregnant control plasma and 200 μl of diluted Russell's viper venom and phospholipid-rich reagents containing 25 mM $CaCl_2$ were mixed and clotting time was measured. The mean and standard deviation values were 0.9 and 0.1 s, respectively. Lupus anticoagulant was considered positive when *T*1/*T*2 was over 1.3.

2.5. Modified assays for the IgG isotypes of β2GPI-dependent and -independent aCLs

Briefly, cardiolipin in ethanol (2.5 μg/50 μl/well) was coated onto the surfaces of polystyrene microtiter plates by evaporation under nitrogen. For the detection of β2GPI-dependent aCL, duplicate wells were incubated with 50 μl of HEPES–BSA, containing purified human β2GPI (30 μg/ml; Yamasa Corp., Choshi, Japan), for 10 min at room temperature. For the determination of β2GPI-independent aCL, duplicate wells were incubated with 50 μl of HEPES–BSA in the same manner. Fifty microlitres of test sera, diluted 1:202 in HEPES–BSA, were then introduced into the duplicate wells and incubations were performed for 30 min at room temperature. After washing with PBS–Tween, wells were exposed to 100 μl of horseradish peroxidase-labeled murine monoclonal IgG against human IgG (G-02; Yamasa Corp.) for 30 min at room temperature. After washing, a 100 μl aliquot of 0.3 mM tetramethylbendizine solution containing 0.003% of H_2O_2 was added to each well. The reaction was terminated by adding 100 μl of 2N H_2SO_4, and the optical density was measured at 450 nm. Antibody titers (units/ml) of aCL were calculated from a standard curve, obtained by running six calibration standards (Yamasa Corp., 1.3–125 units/ml) for each plate.

Test results for β2GPI-dependent and -independent aCL were considered positive when the antibody level was above the 99% confidence interval for 283 normal non-pregnant control sera. This was more than 1.9 units/ml for β2GPI-dependent and more than 6.3 units/ml for β2GPI-independent aCL. In addition, in order to avoid false positives due to nonspecific binding, a β2GPI-dependent assay had to show a higher value than the β2GPI-independent assay performed in parallel, to be considered positive (Matsuura et al., 1994; Katano et al., 1996).

2.6. Statistical analysis

Receiver operating characteristics (ROC) curves for each PE were drawn for all cut-off points. In order to examine the diagnostic values for each PE, areas under the curves (AUCs) for ROC curves were calculated. The analysis was carried out with the PROC LOGISTIC procedure in SAS sys-

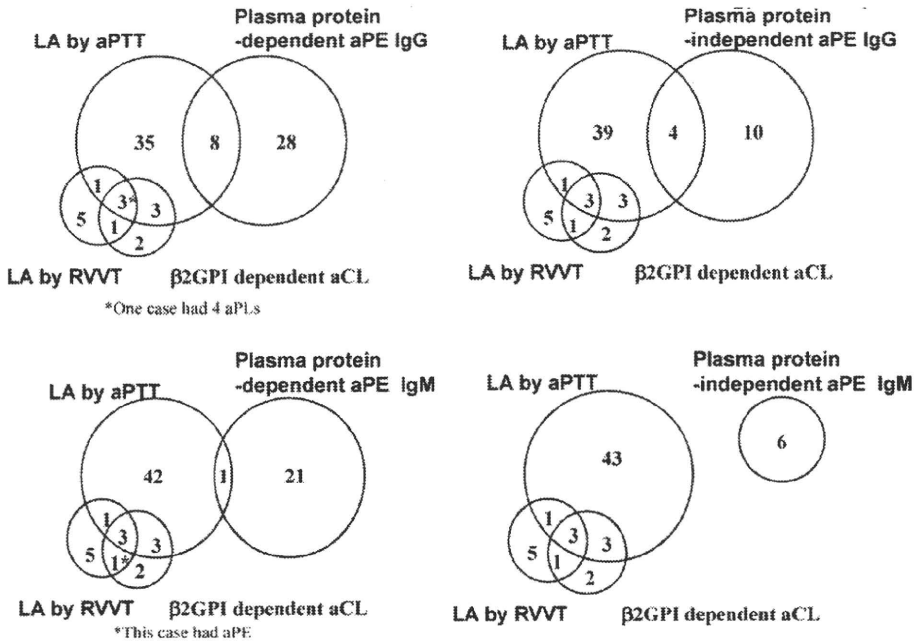

Fig. 2. Overlapping associations among the 4 aPLs for the 367 patients with a history of recurrent pregnancy loss.

tem version 9.1 (SAS Institute Inc., NC, USA) with $P < 0.05$ considered to be statistically significant.

3. Results

Totals of 37 (10.1%), 14 (3.8%), 23 (6.3%), 6 (1.6%), 9 (2.5%), 10 (2.7%) and 50 (13.6%) of the 367 patients were, respectively, positive for P+aPE IgG, P−aPE IgG, P+aPE IgM, P−aPE IgM, β2GPI-dependent aCL, lupus anticoagulant by RVVT and lupus anticoagulant by aPTT.

The relations among aPE and conventional aPL are shown in Fig. 2. Patients with P+aPE IgG were separated from those with β2GPI-dependent aCL and lupus anticoagulant by RVVT. Only one case was positive for all tests. Eight of 37 patients had both P+aPE IgG and lupus anticoagulant by aPTT. On the other hand, only one patient had P+aPE IgM and LA. Six patients with P−aPE IgM were completely separated from conventional aPL.

Eighty-eight of 367 (24.0%) patients miscarried again. Characteristics and subsequent pregnancy outcome for all 302 patients who received no medication or anticoagulant are given in Table 1. With regard to the no medication group, 10 of 14 patients (71.4%) positive for P+aPE IgG gave birth to living babies, while 127 of 167 patients (76.0%) negative for P+aPE IgG had successful pregnancies (difference not significant). A total of 4 of 7 patients (57.1%) positive for P−aPE IgG gave birth to living babies, while 133 of 174 patients (76.4%) negative for P−aPE IgG had successful pregnancies (difference not significant).

Fifty-five karyotypes of miscarried concepti could be analyzed and 31 (56.4%) were found to be abnormal. After excluding miscarriage cases caused by an abnormal embryonal karyotype, the success rate (83.3%) of patients positive did not differ from that (83.6%) of patients negative for P+aPE IgG.

AUCs for each ROC curve, as shown in Fig. 2, for P+aPE IgG, P−aPE IgG, P+aPE IgM and P−aPE IgM was 0.535, 0.612, 0.546 and 0.533, respectively. Each AUC was close to 0.5 so that there was no variation in diagnostic capacity of the test. These results thus did not suggest any significant predictive value of 4 aPE for further miscarriage (Fig. 3).

4. Discussion

In the present study, the population with aPE was found to differ substantially from those with β2GPI-dependent aCL and lupus anticoagulant by RVVT. Only 8 patients had both P+PE IgG and lupus anticoagulant by aPTT. It is well-known that purified IgG from patients with lupus anticoagulant has lupus anticoagulant activity, thus patients with lupus anticoagulant by aPTT had not aPE IgM but aPE IgG in the present study. aPTT influences the intrinsic pathway including the contact phase cascade and, in contrast, RVVT inhibits coagulation factor X directly. Lupus

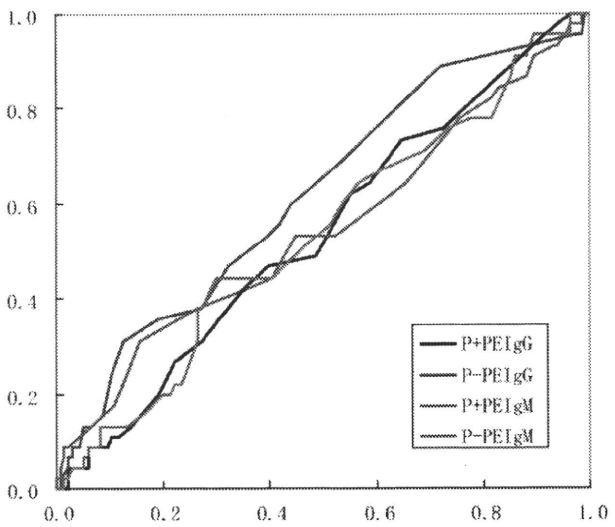

Fig. 3. ROC curves for anti-PE antibodies.

Table 1
Characteristics and pregnancy outcome of patients with antiphosphatidylethanolamine antibodies.

	No medication		Anticoagulant	
	Positive	Negative	Positive	Negative
P + aPE IgG	$n = 14$	$n = 167$	$n = 12$	$n = 109$
Mean age	31.3	31.6	34.9	32.6
Mean no. of previous losses	2.2	2.3	3.0	3.1
Failure	4 (2)	40 (25)	4 (2)	26 (17)[a]
Success	10	127	8	83
Success rate (%)	71.4 (83.3)	76.0 (83.6)	66.7 (80.0)	76.1 (83.0)[b]
P – aPE IgG	$n = 7$	$n = 174$	$n = 5$	$n = 116$
Mean age	33.0	31.5	32.0	37.8
Mean no. of previous losses	2.0	2.3	3.0	3.1
Failure	3 (1)	41 (26)	2 (2)	28 (17)
Success	4	133	3	88
Success rate (%)	57.1 (80.0)	76.4 (83.6)	60.0 (60.0)	75.9 (83.8)
P + aPE IgM	$n = 6$	$n = 175$	$n = 11$	$n = 110$
Mean age	31.7	31.5	32.2	32.5
Mean no. of previous losses	2.0	2.3	3.0	3.2
Failure	4 (3)	40 (24)	2 (1)	28 (18)
Success	2	135	9	82
Success rate (%)	33.3 (40.0)	77.1 (84.9)	81.8 (90.0)	74.5 (82.0)
P – aPE IgM	$n = 2$	$n = 179$	$n = 2$	$n = 119$
Mean age	30.5	31.4	29.5	32.7
Mean no. of previous losses	3.0	2.0	2.5	3.1
Failure	0	44 (27)	0	30 (19)
Success	2	135	2	89
Success rate (%)	100 (100)	75.4 (83.3)	100	74.8 (82.4)

[a] Miscarriages caused by an abnormal embryonal karyotype were excluded.
[b] Success rate when miscarriages caused by an abnormal embryonal karyotype were excluded.

anticoagulant comprises heterogeneous antibodies against phospholipid-binding prothrombin, factor X and/or β2GPI (Bever et al., 1991; Brandt, 1991; Roubey et al., 1992). Thus, lupus anticoagulant by aPTT included β2GPI-dependent aCL and lupus anticoagulant by RVVT. aPE can recognize kininogen–PE complexes (Sugi and McIntyre, 1995). Lupus anticoagulant acting by aPTT but not by RVVT might comprise IgG against kininogen–PE complexes.

Antigenic targets include β2GPI, prothrombin, high and low molecular weight kininogen, annexin V, protein C and protein S (Roubey et al., 1992; Roubey, 1994). In addition to approaches for conventional aPLs, new ELISA methods for aPE, anti-prothrombin and anti-annexin V antibodies are now available (Bever et al., 1991; Sugi and McIntyre, 1995; Matsubayashi et al., 2001). We have shown that β2GPI-dependent aCL is a strong predictor of intrauterine fetal death, intrauterine growth restriction and pregnancy-induced hypertension, although the frequency is low (Katano et al., 1996). These conventional aPLs are included in the international criteria for APS. However, the prevalence of β2GPI-dependent aCL and lupus anticoagulant by dRVVT are relatively low (2.5 and 2.7% in the present study) and Sugi et al. (1999) have concluded that aPEs are more strongly associated with recurrent pregnancy loss because the prevalence of PE IgG and IgM were found to be much higher (20.1 and 12.2%).

However, a high prevalence in a particular test does not necessarily imply clinical significance. With regard to antinuclear antibodies (ANA), the frequency is significantly higher than in controls, but no effects on the live birth rate were found in one study (Ogasawara et al., 1996b).

Moreover, it is unlikely that all these molecules are targeted at the same time; the whole situation rather reflects the extensive immunologic alterations that characterize the pregnant status. The exact role of even β2GPI itself in pregnancy loss remains unknown because knockout mice are fertile (Miyakis et al., 2004).

To our knowledge there have hitherto been only a few reports of the predictive value of aPE for adverse pregnancy outcome of recurrent aborters. Gris et al. (2000) described aPE IgM but not IgG to have predictive value for subsequent fetal loss from the 8th week up to and including the 22nd week of gestation, in spite of low dose aspirin treatment. Recently, Yamada et al. (2009) measured aPLs including aPE IgG during the first trimester in a consecutive series of 1155 pregnant women and found that aPE IgG was associated with developing pregnancy-induced hypertension (8.3, 2.4–29) and premature delivery (12.7, 3.1–50). However, they could not examine the association between aPE IgG and early miscarriage because the peripheral blood was obtained at 8–14 weeks' gestation.

Another issue that relates to aPE and most of the aPL is the lack of standardization and of uniform requirements for performance and interpretation of the tests. From comparing the detection methods for aPE in the present study with those in Gris's study, there are some important differences (for example, regarding overnight incubation and methanol dilutions of aPE plates, extraction of standards for OD estimation). Similar limitations and particularities exist for the measurements of most of the aPLs. Using different detection methodologies can cause different prevalence values, even though the same aPL are purportedly mea-