age-adjusted logistic regression. This analysis was applied to all the variables listed in the table except age.

Furthermore, multivariable logistic regression analysis was performed using pNK cell activity, age, number of previous miscarriages, and presence/absence of previous live births and bed rest as covariates. Linear multivariable logistic regression analysis was also performed using pNK cell activity, age, and number of previous miscarriages.

The analysis was carried out using SAS version 19.0 (SAS Institute), and $P<.05$ was considered to denote statistical significance.

RESULTS

In the subjected 1,127 patients, 4.4% (50) had an abnormal chromosome in either partner, 4.1% (46) of patients had a major uterine anomaly, 3.4% (38) had thyroid disease, 1.9% (21) had diabetes mellitus, and 2.9% (33) had APS (Fig. 1). In total, 180 patients were excluded from the cohort because several patients had two or three identifiable causes.

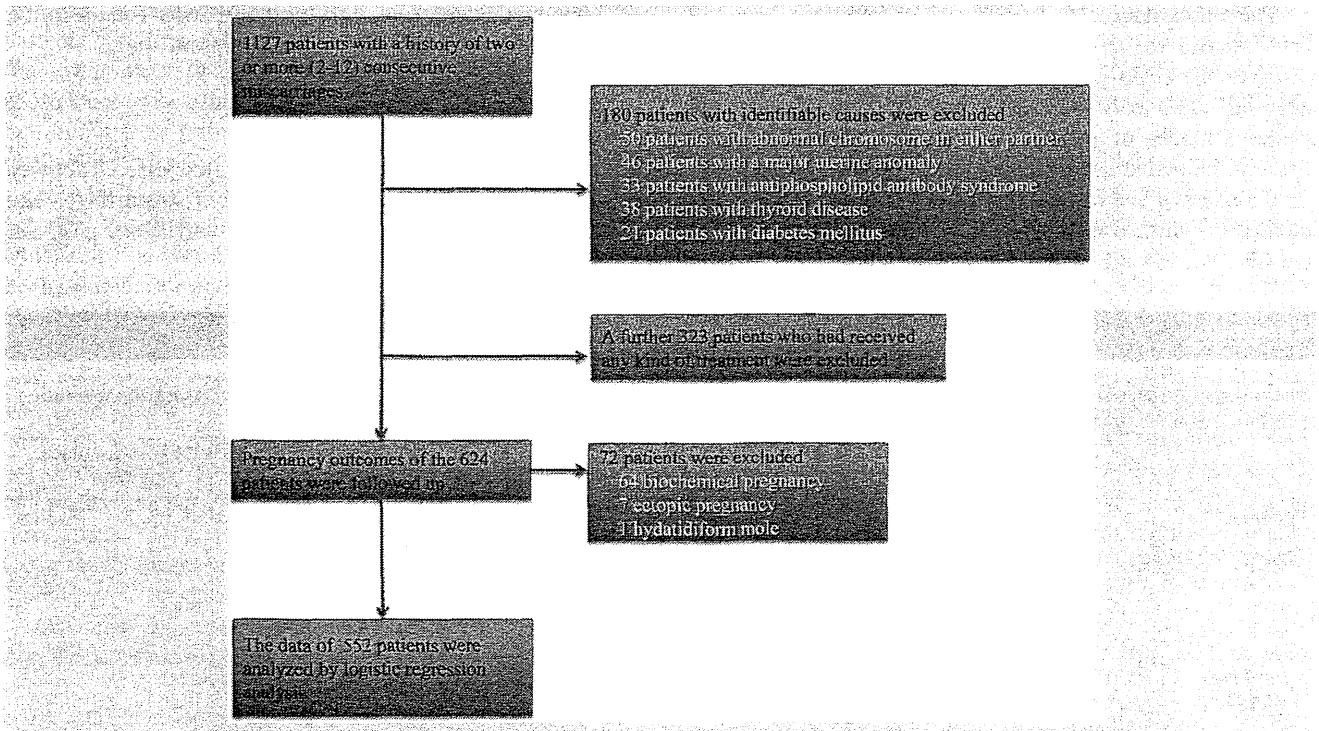
To eliminate the influence of the treatment, a further 323 patients who received any kind of treatment were excluded. A total of 72 patients—64 biochemical pregnancy, 7 ectopic pregnancy, and 1 hydatidiform mole—were excluded in the present study (Fig. 1).

The miscarriage rate of a total of 552 patients with unexplained RPL who received no medication is shown in Table 1. The subsequent miscarriage rate was 22.5% (124 of 552). The mean (SD) age and median (interquartile range) number of previous miscarriages were 31.9 (4.37) and 2 (2–3), respectively. The live birth rates of patients with previous two, three, four, five, and six miscarriages were 81.1% (309 of 381), 71.2% (99 of 139), 65.4% (17 of 26), 60.0% (3 of 5), and 0 (0 of 1), respectively.

Linear multivariable logistic regression showed that pNK was not an independent risk factor for subsequent miscarriage. However, in the crude analysis of the categorization of each variable, the miscarriage rate in the patients with 5%–24% pNK cell activity was significantly higher than that in the patients with 25%–34% pNK cell activity ($P=.046$). On the other hand, the miscarriage rate in the patients with 47%–78% pNK cell activity was similar to that in the patients with 25%–34% pNK cell activity. The plasma NK cell activity showed a weak inverse correlation with age in the 1,127 patients ($r = -0.068$).

Five variables, namely pNK cell activity, age, number of previous miscarriages, and absence of bed rest and previous live birth, were entered into the multiple logistic regression analysis for subsequent miscarriage detection in all 552 patients. The miscarriage rate in patients with 25%–34% pNK cell activity tended to be higher than that in patients with 5%–24% pNK cell activity (odds ratio [OR] 0.56, 95% confidence interval [CI] 0.31–1.00, $P=.051$). Crude, age-adjusted, and multivariable logistic regression analyses showed similar results in relation to pNK cell activity. Elevated pNK cell activity was confirmed to not be an independent risk factor for a subsequent miscarriage.

FIGURE 1



A total of 552 patients were analyzed in the present study. Of the 1,127 women initially enrolled, 180 patients with identifiable causes, 323 patients who received any kind of medication, and 72 patients whose pregnancy outcomes were biochemical or ectopic pregnancy were excluded.

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TABLE 1

Miscarriage rate according to pNK cell activity, age, and number of previous miscarriages, and age-adjusted and multivariable logistic regression analysis to identify the risk factors for subsequent miscarriage.

		Crude analysis logistic regression		Age-adjusted logistic regression[a]		Multivariable logistic regression[b]		
Parameter	Miscarriage rate, % (n)	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value	Trend *P* value
Peripheral NK cell activity (%)								.365
5–24	28.1 (41/146)	Reference		Reference		Reference		
25–34	17.9 (24/134)	0.56 (0.32–1.00)	.046	0.55 (0.31–0.98)	.042	0.56 (0.31–1.00)	.051	
35–46	22.6 (31/137)	0.75 (0.44–1.28)	.293	0.73 (0.42–1.26)	.261	0.78 (0.45–1.36)	.385	
47–74	20.7 (28/135)	0.67 (0.39–1.16)	.154	0.69 (0.39–1.20)	.186	0.73 (0.41–1.30)	.282	
Age (y)								.0002
19–29	13.2 (22/167)	Reference				Reference		
30–31	28.7 (33/115)	2.65 (1.45–4.85)	.0015			2.49 (1.35–4.59)	.0036	
32–35	20.9 (33/158)	1.74 (0.96–3.14)	.0658			1.46 (0.79–2.71)	.226	
36–45	32.1 (36/112)	3.12 (1.72–5.68)	.0002			2.54 (1.35–4.76)	.0037	
No. of previous miscarriages								.0014
2	18.9 (72/381)	Reference		Reference		Reference		
3	28.8 (40/139)	1.73 (1.11–2.71)	.012	1.57 (0.99–2.48)	.055	1.38 (0.85–2.26)	.198	
4	34.6 (9/26)	2.27 (0.97–5.30)	.052	1.84 (0.77–4.37)	.168	1.65 (0.67–1.10)	.280	
5–6	50.0 (3/6)	4.29 (0.85–21.70)	.090	3.56 (0.68–18.55)	.132	3.73 (0.69–20.10)	.126	

[a] The only covariate used was age for the age-adjusted logistic regression analysis. This analysis was applied to all the variables listed in the table except age.
[b] The covariates used for the multivariable logistic regression analysis were pNK activity, age, number of previous miscarriages, presence/absence of previous live births, and presence/absence of bed rest.

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The miscarriage rate in the patients without bed rest was significantly higher than that in the patients who were admitted for rest ($P=.016$; Table 2). However, there were differences in the mean [SD] age (30.9 [4.0] vs. 32.8 [4.5] years; $P<.0001$) and the median number of previous miscarriages (three vs. two) between the patients with and without bed rest.

The miscarriage rate in patients without previous live births tended to be lower than that in the patients with previous live births ($P=.096$). There were differences in the mean age (33.9 [3.7] vs. 31.6 [4.4] years; $P<.0001$) and the median number of previous miscarriages (three vs. two) between the patients with and without previous live births.

No effect of bed rest and previous live birth on the likelihood of live birth was observed (OR 1.28, 95% CI 0.81–2.02 and OR 0.91, 95% CI 0.52–1.59, respectively).

Age and number of previous miscarriages were determined to be risk factors for subsequent miscarriage according to both crude and linear multivariable logistic regression.

Age and numbers of previous miscarriages were confirmed to be independent risk factors. However, number of previous miscarriage, but not age, was found to be influenced by the other factors in the present study.

DISCUSSION

The results of this study suggest that elevated pNK cell activity is not a reliable predictor of subsequent miscarriage. The miscarriage rate was higher in patients with lower pNK cell activity.

TABLE 2

Miscarriage rate according to the presence/absence of previous live births and bed rest, and age-adjusted and multivariable logistic regression analysis to identify the risk factors for subsequent miscarriage.

				Crude analysis logistic regression		Age-adjusted logistic regression[a]		Multivariable logistic regression[b]	
Parameter	Miscarriage rate, % (n)	Mean (SD) age (y)	Median no. of previous miscarriage	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Absence of previous live birth									
Presence	28.4 (25/88)*	33.9 (3.7)*	3*	Reference		Reference		Reference	
Absence	21.3 (99/464)*	31.6 (4.4)*	2*	0.68 (0.41–1.14)	.096	0.68 (0.41–1.14)	.146	0.91 (0.52–1.59)	.736
Absence of bed rest									
Presence	18.2 (47/258)*	30.9 (4.0)*	3*	Reference		Reference		Reference	
Absence	26.2 (77/294)*	32.8 (4.5)*	2*	1.59 (1.06–2.40)	.016	1.59 (1.06–2)	.026	1.28 (0.81–2.02)	.288

[a] The only covariate used was age for the age-adjusted logistic regression analysis. This analysis was applied to all the variables listed in the table except age.
[b] The covariates used for the multivariable logistic regression analysis were pNK activity, age, number of previous miscarriages, presence/absence of previous live births, and presence/absence of bed rest.
* $P<.05$ was considered to denote statistical significance.

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Uterine endometrial NK (uNK) cell activity is known to be strongly involved in the maintenance of normal pregnancy. Lachapelle et al. (21) proved that the proportion of uNK cells was identical in recurrent miscarriage (RM) patients and normal controls, but the $CD56^{bright}$ and $CD16^{-}$ NK cell subset, which is predominant in normal decidua and endometrium, was significantly decreased in favor of an important contingent of $CD56^{dim}$ and $CD16^{+}$ NK cells in all patients. Quenby et al. (22) demonstrated that prednisolone therapy during the first trimester of pregnancy reduced the risk of miscarriages and improved the live birth rate in patients with idiopathic RM and increased the numbers of uNK cells in the endometrium. Measurement of pNK cell activity has been performed to determine whether it might be predictive of a successful subsequent pregnancy (23, 24). We have reported for the first time that elevated pNK cell activity might be predictive of subsequent miscarriage in patients with RM (9). Some have affirmed, whereas others have denied, the predictive value of pNK for the subsequent pregnancy outcome. However, none of these reports were based on studies of large cohorts, and there is no clear evidence yet (16, 25).

Patients with unexplained RM have been treated empirically with expensive immunoglobulin, on the basis of the conjecture that the functions of uNK cells and pNK cells are similar and that, therefore, measurement of pNK cell activity would reflect uNK cell activity. Tang et al. (16) reported a systematic review and came to the conclusion that there is no association between the subsequent pregnancy outcome and either pNK or uNK cell activity in women with RM and infertility. In the present study the correlation between the subsequent pregnancy outcome and pNK cell activity was not linear. The miscarriage rate in patients with low pNK cell activity tended to be higher than that in patients with 25%–74% pNK cell activity. Age, number of previous miscarriages, bed rest, and number of previous live births were found to exert no significant influence on pNK cell activity.

It is well known that stress and exercise increase pNK cell activity; therefore, these factors should be borne in mind while drawing blood for testing. Abnormal data pertaining to the number or activity of pNK cells may reflect transient stress reactions in daily life. It is not clear whether uNK cells may have the same significance. Peripheral blood NK and uNK cells are different types of cells, and both the models and functions of these cells are entirely different. It has been reported that measurement of pNK cell activity does not provide any information on the condition of the endometrial membrane (7, 14, 25). There is also no evidence of treatment using the data on pNK cell activity. We do not recommend measurement of pNK cell activity as part of the systematic examination in patients with RPL.

Mentally depressed patients with RPL need tender loving care (26). However, there is no evidence that subsequent miscarriage can be prevented by hospitalization. Klebanoff et al. (27) concluded that there was no difference in the miscarriage rate between women who had a heavy workload and long working hours and wives of male residents who had many kinds of jobs. Duckitt et al. (28) found no direct evidence from randomized, controlled trials regarding the influence of bed rest in women with unexplained RM. In the present study the live birth rate in patients without bed rest was significantly lower than that in the patients who were admitted for rest. However, there were significant differences in the mean age and median number of previous miscarriages between the patients with and without bed rest, because the average age of women at pregnancy is increasing year by year in Japan. Neither age-adjusted nor multivariable logistic regression analysis showed any effect of bed rest on the live birth rate. We concluded that there is no necessity to advise preventive bed rest for pregnant women, in the absence of symptoms of threatened abortion.

This study revealed that previous live birth was not predictive of a subsequent live birth, although there have been a few reports suggesting a favorable influence of a previous live birth in secondary RM patients (29, 30). Nielsen (31) reported that secondary RM is more common after the birth of a boy and that the subsequent live birth rate is reduced in secondary RM patients with a firstborn boy, owing to the pathogenic role of the aberrant maternal H-Y immune response. Both our previous study and the crude analysis in the present study indicated that the live birth rate increased as the number of previous miscarriages increased (32). However, the significant difference disappeared after adjustment for age, because age also increased with increasing number of previous miscarriages.

In more than half of the cases, the cause of RPL remains unexplained despite conventional examinations (4, 5). Recently we found that an abnormal embryonic karyotype was the most frequent cause, accounting for as much as 41% of the cases, and the percentage of truly unexplained was limited to 24.5% (33). Associations have been reported between many kinds of polymorphisms, such as those of annexin A5 and NLRP7, and RPL (34, 35). The influence of one single-nucleotide polymorphism associated with RPL might be speculated to be very small, because the OR of each gene mutation is relatively small (34). Even though it would be highly desirable, it might be difficult to identify clinically useful predictors of the outcome of a subsequent pregnancy.

We previously reported that elevated pNK cell activity may be predictive of subsequent miscarriage in patients with RPL (9). However, we wish to correct our initial conclusion, because in this study, high pNK cell activity was confirmed to not be an independent risk factor for subsequent miscarriage. Clinicians should not measure pNK activity as a systematic RPL examination, because the clinical significance or treatment method is yet to be established. Patients need not give up working, because no effect of bed rest on the likelihood of live birth was observed.

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Possible improvement of depression after systematic examination and explanation of live birth rates among women with recurrent miscarriage

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We conducted a prospective study to determine whether systematic examinations and provision of explanation regarding the successful birth rates might improve mood or anxiety disorders among childless women with recurrent miscarriages. A total of 305 first-visit patients with a history of 2–12 miscarriages completed a first questionnaire battery, including: 'K6', a new screening instrument for mood and anxiety disorders, the 'Symptom Checklist-90 Revised' (SCL-90-R) and the 'Emotional Impact' questionnaire. Of these, 170 patients who underwent routine examinations and received an explanation about successful live birth rates responded to the second questionnaire. A total of 15.4% of the patients were estimated to suffer from diagnosable depression or anxiety disorders. Patients with high scores on K6 also showed elevated scores on all the subscales of SCL-90-R, including depression and anxiety. The K6 of patients with translocation was significantly higher than that of patients with antiphospholipid antibodies. The K6 and depression scores in the 2nd questionnaire survey were significantly lower than those in the 1st survey in the 170 patients. Improvement in depression was found in patients who underwent routine examination and received an explanation.

Keywords: Depression, K6, mental distress, recurrent miscarriage, Symptom Checklist 90-Revised

Introduction

Miscarriage (spontaneous abortion) can induce depression, anxiety, denial, anger and a sense of loss and inadequacy. In one series, 10.9% of women with sporadic miscarriages experienced at least one episode of major depression (Neugebauer et al. 1997). Craig et al. (2002) reported psychiatric disorders in 33% of patients with recurrent miscarriages. However, this morbidity estimate was based on questionnaire scores and not on diagnoses. The true morbidity is therefore unknown, and there is no established treatment method for mood and anxiety disorders in patients with recurrent miscarriage.

Established causes of recurrent miscarriages include abnormal chromosomes in either partner, particularly translocations, as well as presence of antiphospholipid antibodies (aPLs) in the serum and uterine anomalies (Farquharson et al. 1984; Sugiura-Ogasawara et al. 2004; Sugiura-Ogasawara et al. 2010a). An abnormal embryonic karyotype is also a well-known cause of recurrent miscarriages and has been reported in about 25–50% of aborted conceptuses (Ogasawara et al. 2000; Carp et al. 2001; Sullivan et al. 2004). The relatively wide range may reflect differences in the maternal mean age and the mean number of previous miscarriages, since these could conceivably exert an influence.

Embryonic aneuploidy is the most important cause of miscarriage before the completion of 10 weeks' gestation, and our previous study showed that about 70% of sporadic miscarriages are caused by an abnormal embryonic karyotype (Ogasawara et al. 2000). A more recent comparative genomic hybridisation microarray analysis also indicated a rate of about 80% (Shimokawa et al. 2006). Thus, the incidence of recurrent miscarriages caused by an abnormal embryonic karyotype can be calculated as $(0.8)^n$ in n consecutive miscarriages; using this formula; that of patients with three miscarriages can be calculated as 51%. This rate is in line with the previous finding that about 50% of karyotypes were abnormal in the aborted conceptuses of a group of women with recurrent miscarriages (Ogasawara et al. 2000). An abnormal embryonic karyotype is also well-known as a predictor of subsequent successful birth (Ogasawara et al. 2000; Carp et al. 2001; Sullivan et al. 2004). In fact, about 85% of patients suffering unexplained recurrent miscarriages were found cumulatively to have successful live births (Sugiura-Ogasawara et al. 2009; 2010a; Franssen et al. 2006). We reported that the live birth rate (p) can be calculated as follows: $\text{logit}(p) = 3.964 - 0.0652 \times (\text{age}) - 0.408 \times (\text{previous number of miscarriages})$.

However, conventional examination of couples with recurrent miscarriages yields no putative cause in $> 50\%$ of cases, because the aborted conceptuses are seldom karyotyped clinically (Clifford et al. 1994). It is speculated that such patients might give up becoming pregnant because of the fear of further miscarriage.

We therefore conducted the present prospective study to examine the morbidity arising from mental disorders in childless women with recurrent miscarriages and to determine whether systematic examination and provision of an explanation concerning the expected success rates may improve the psychological distress levels in these subjects, using K6, a new screening scale for psychological distress.

Materials and methods

Patients

This subject sample consisted of 305 patients with a history of two or more (2–12) consecutive miscarriages and no child

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at the first visit, for discussion about further attempts to have a baby between January 2008 and November 2010. The mean (SD) age was 33.0 years (4.8). The first questionnaire was administered at the first visit during the patients' waiting time. Systematic examination included: hysterosalpingography; chromosome analysis for both partners; determination of serum aPL; including lupus anticoagulant and β2-glycoprotein I dependent anticardiolipin antibodies (Ogasawara et al. 1996); and blood tests for hypothyroidism, diabetes mellitus before a subsequent pregnancy.

Once all of these examinations were completed, a designated obstetrician explained the results and the expected live birth rate. We offered combined low-dose aspirin and heparin therapy for patients with the antiphospholipid syndrome and predicted a 70–80% success rate. We provided genetic counselling with regard to the mean probability of a live birth of 50% by natural pregnancy and the pre-implantation genetic diagnosis for translocation carriers (Sugiura-Ogasawara et al. 2004; Sugiura-Ogasawara et al. 2008; Kyu Lim et al. 2004), and also counselling to explain the live birth rate of 60% in the absence of surgery for congenital uterine anomalies (Sugiura-Ogasawara et al. 2010a). We provided information about the live birth rate at the first pregnancy after the examination to unexplained patients, based on the women's age and number of previous miscarriages from our database, which is 76% in patients with two miscarriages; 70% in patients with three miscarriages and 60% in patients with four miscarriages (Sugiura-Ogasawara et al. 2009). At this time, we asked the participants to reply to a second questionnaire at home 2 weeks later and provided the questionnaire and a stamped addressed envelope.

The study was conducted with the approval of the Research Ethics Committee. Each participant provided written informed consent after being provided with a full explanation about the purpose of the study and the methods to be employed.

The first questionnaire survey

The first questionnaire included K6, the Symptom Checklist-90-Revised (SCL-90-R), knowledge of miscarriage and anxiety about miscarriage before the first pregnancy and the emotional impact (EI) for each miscarriage. The frequency of depression or anxiety; the influence on depression of knowledge about miscarriage; the association between EI and the first, second, third and subsequent miscarriages or clinical, chemical or stillbirths were recorded. Knowledge regarding the frequency of miscarriage of about 15% in the first pregnancy was recorded using a four-category scale (not at all, a little bit, quite a bit, extremely).

The items in K6 covered how frequently the respondents experienced symptoms of psychological distress (e.g. feeling so sad that nothing can cheer you up) during the previous 30 days. Responses were recorded using a five-category scale (4 = all of the time; 3 = most of the time; 2 = a little of the time; 1 = none of the time), producing, therefore, a scale range 0–24 (Kessler et al. 2002). According to the Japanese validation study of K6 in the general population, the positive predictive value of K6 scores of 10 or more was 0.49 (Furukawa et al. 2008).

SCL-90-R is a self-reported questionnaire containing 90 short questions, to which the answers are classified on a five-category scale (0 = not at all, 1 = a little bit, 2 = moderately, 3 = quite a bit, 4 = extremely). It covers the whole spectrum of psychiatric symptoms, including: depression; somatisation; anxiety; obsessive-compulsive behaviour; interpersonal sensitivity; hostility (aggression and irritability); phobic anxiety; paranoid ideation (mistrust and persecutory feelings) and psychoticism.

The second questionnaire survey

The second questionnaire survey also included K6 and SCL-90-R.

Statistical analyses

Data were analysed by *t*-tests using SPSS for Windows Version 10.0. Values with $p < 0.05$ were considered to be statistically significant.

First, we examined the prevalence of mood or anxiety disorders using the cut-off of 10/9 and the associated positive predictive value discussed above with the Japanese version of K6. We then compared the psychopathological characteristics of high versus low K6 scores in terms of the SCL-90-R subscales. The association between K6 scores and the EI of miscarriages; the EI of the number of previous miscarriage; and the EI of stillbirths, early abortions and chemical abortions were also examined.

In addition, we examined the changes in the K6 scores and scores for the SCL-90-R subscales in the first to second questionnaire surveys.

Results

In the first questionnaire survey, 96 of the 305 patients (31.5%) were found to have K6 scores of ≥ 10. Given the positive predictive value of such K6 scores (Furukawa et al. 2008), it was estimated that 15.4% of the patients with recurrent miscarriages would be suffering from mood or anxiety disorders. This prevalence is significantly higher than the 1.9% reported for the Japanese general population (Kawakami et al. 2005).

The mean (SD) scores for the SCL-90-R subscales in the subjects with K6 ≥ 10 and K6 < 10 are shown in Table I. The means (SDs) for all subscales were significantly higher for K6 ≥ 10. The first K6 scores (scores in the first K6 survey) were correlated with the scores for all the subscales of the first SCL-90-R (scores in the first SCL-90-R survey), but did not differ depending on the age or number of previous miscarriages.

The K6 score was correlated with the EI of the first miscarriage (1st EI) ($r = -0.130$, $p = 0.027$) and third miscarriage ($r = -0.192$, $p = 0.03$). The EI of the second miscarriage was significantly higher than that of the first miscarriage in patients with a history of three miscarriages.

As the level of knowledge of miscarriage decreased, the negative 1st EI increased (extremely: −53.1 ± 29.3; quite a bit: −74.5 ± 33.8; a little bit: −77.7 ± 50.3; not at all: −82.4 ± 24.8, $p = 0.02$). However, the score for the subscale of depression in the SCL-90-R increased as the level of knowledge increased (extremely: 0.99 ± 0.64; quite a bit: 0.99 ± 0.66; a little bit: 0.77 ± 0.63; not at all: 0.72 ± 0.60, $p = 0.006$).

The EI (−97.86 ± 5.79) of stillbirth (more than 10 weeks' gestation) was significantly higher than of early abortion (−82.43 ± 25.02) or chemical abortion (−70.43 ± 27.23, $p = 0.002$).

Table I. The association between K6 and SCL-90-R (mean ± SD).

SCL-90-R	K6 ≥ 10 ($n = 96$)	K6 < 10 ($n = 209$)	*p* value
Depression	1.47 ± 0.64	0.55 ± 0.41	< 0.001
Somatisation	0.76 ± 0.55	0.37 ± 0.34	< 0.001
Anxiety	0.91 ± 0.66	0.26 ± 0.30	< 0.001
Obsessive-compulsive behaviour	1.10 ± 0.68	0.50 ± 0.42	< 0.001
Interpersonal sensitivity	1.16 ± 0.63	0.45 ± 0.40	< 0.001
Hostility	0.93 ± 0.68	0.31 ± 0.35	< 0.001
Phobic anxiety	0.55 ± 0.56	0.15 ± 0.28	< 0.001
Paranoid ideation	0.45 ± 0.45	0.16 ± 0.27	< 0.001
Psychoticism	0.56 ± 0.43	0.16 ± 0.22	< 0.001

A total of 170 (81.7%) of the 208 patients who had completed the systematic examinations, sent in their responses to the second questionnaire. No patients received medication to prevent miscarriages at the first or second questionnaire. No patients with translocation wished for PGD. No patients with uterine anomaly received surgery. The 2nd K6 of patients with translocation (8.0 ± 3.85) was significantly higher than that of patients with aPLs (3.44 ± 3.43, $p = 0.03$).

On average, the 2nd K6 (5.17 ± 4.28) and score for the depression subscale of SCL-90-R in the second survey (0.79 ± 0.69) were significantly lower than the 1st K6 (7.59 ± 5.22) and depression score in the first survey (0.89 ± 0.67) ($p < 0.0001$). In all, 27 of the 170 patients (15.9%) had a 2nd K6 ≥ 10, indicating that 7.8% of patients suffered from mood or anxiety disorders after undergoing routine examinations and receiving the 'explanation'. At the individual level, a significantly higher number of patients showed improved scores after the conventional examinations and the 'explanation' ($p < 0.0001$).

Discussion

Patients with K6 ≥ 10 were found to have higher scores on all the subscales of SCL-90-R, namely: depression; somatisation; anxiety; obsessive-compulsive behaviour; interpersonal sensitivity; hostility; paranoid ideation; phobic anxiety and psychoticism, than those with K6 < 10. SCL-90-R is one of the most widely used symptom questionnaires in the field of psychiatry, whose primary purpose is to depict the symptomatological profile of the respondents and not to screen for the presence/absence of certain disorders. Our results demonstrate that not anxiety but depression could improve after routine examination and provision of information concerning expected live birth rates in patients with recurrent miscarriages. K6 was found to be a useful instrument for screening for mental disorders in patients with recurrent miscarriages.

Kessler et al. (2002) developed the K6, a 6-item very short screening instrument using modern items response theory methods to select questions that maximally discriminate respondents in the 90th to 99th percentile range of the population distribution, because it is known that between 5–10% of the general population suffer from serious mental illness at any point in time. K6 was found to strongly discriminate between community cases and non-cases of the Diagnostic and Statistical Manual Disorders, 4th edn (DSM-IV). Furukawa et al. (2008) developed the Japanese version according to the standard back-translation procedure, and the screening performance was shown to be essentially equivalent to that reported for the original English version, indicating the success in producing a cross-culturally applicable screening scale. K6 was, in fact, incorporated into the World Health Organization (WHO) World Mental Health Survey undertaken in multiple countries, including Japan (Demyttenaere et al. 2004).

In a very recent epidemiological study, the morbidity associated with DSM-IV mood disorders (depression, dysthymia) or anxiety disorders (panic disorder, agoraphobia, social phobia, generalized anxiety disorder, post-traumatic stress disorder), during 1 month in the Japanese general population, was found to be 1.9% (Kawakami et al. 2005). In clear contrast, 15.4% of patients with recurrent miscarriages were speculated to suffer from mood or anxiety disorders, because theoretically, 49.0% of cases with K6 ≥ 10 can be estimated to suffer from one or more of these disorders (Furukawa et al. 2008).

In the present study, the patients undergoing systematic examinations and receiving the explanation exhibited significant improvements of the K6 scores and scores for the depression subscale of SCL-90-R. There was an interval of about 2 months between the two questionnaire surveys, and this interval itself might have alleviated the mental distress, to a certain extent. We could not conduct a case–control study because it was difficult to assess an appropriate control group. However, receiving conventional examination and information about the expected success rate might help patients' mood and improve their mental distress.

The fact that the EI of a second miscarriage was significantly higher than that of a first, in patients with a history of three miscarriages, is consistent with the results of our previous study (Aoki et al. 1998). Negative EI increased as the level of knowledge about miscarriage decreased. Japanese women have limited knowledge of reproduction (Sugiura-Ogasawara et al. 2010b). Education concerning miscarriages is needed for preventing mental disorders among women of the reproductive age.

The mental disorders in patients with translocation were found to be significantly more severe than those in patients with aPLs. Patients with aPLs received information about established treatments for a subsequent pregnancy, which could have led to desirable psychological effects.

Sugiura-Ogasawara et al. (2002) proved that depression influences the likelihood of further miscarriage in recurrent cases. It is unclear whether improvement of the K6 score might lead to improved live birth rate. Since there are no established treatment methods for preventing miscarriages in cases with unexplained recurrent miscarriages (Rai and Regan 2006), the possibility that psychosocial support might be able to prevent the patients giving up on a subsequent pregnancy, clearly warrants further attention.

We usually explain the treatment methods to patients with aPLs; provide genetic counselling for translocation carriers and provide information on the expected live birth rates for patients with unexplained miscarriages after systematic examinations. Both maternal age and reproductive history are independent predictors of further pregnancy outcomes (Sugiura-Ogasawara et al. 2009; Stephenson et al. 2002). Several couples in our experience give up trying to conceive after recurrent miscarriages, because they have the misconception that it would be impossible for them to have a live baby. Psychological tender loving care might be the most important requirement to continue trying to conceive until it results in a live birth (Liddell et al. 1991). Some patients remained depressed even after the examination, and cognitive behavioural therapy may be useful to obtain improvement in such cases.

In this study, 15.4% of patients were found to have mood or anxiety disorders. This is the first report of possible improvement of depression after conventional examinations and provision of information concerning the expected success rates in patients with recurrent miscarriages. Since our sample size was relatively small, further study with a larger sample in multiple settings would be needed to examine and confirm our conclusions.

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不育症
—治療法の変遷

杉浦 真弓

不育症の原因の変遷

不育症recurrent pregnancy lossは「妊娠はするけれど流産・死産を繰り返して児を得られない場合」と定義される。かつては習慣流産として3回以上連続する流産が検査の対象とされたが，近年，2回以上で研究対象とされるようになった。

不育症の原因には抗リン脂質抗体，子宮奇形，夫婦染色体異常がある[1,2]。実施するべき検査を表に示した。Branchら[2]は臨床家が実施すべき検査として抗リン脂質抗体，子宮奇形，夫婦染色体検査，胎児染色体検査を推奨している。

名古屋市立大学不育症1,676組を対象とした研究では，抗リン脂質抗体10.7%，子宮奇形3.2%，夫婦どちらかの染色体異常6%．糖尿病，甲状腺機能低下症，多嚢胞性卵巣症候群などの内分泌異常12%の頻度であり，約70%が原因不明であった（図B）[1]。糖尿病，甲状腺機能低下は古くから習慣流産の原因といわれてきたが，不育症に占める頻度が低いため，質の高い研究が実施しにくく，エビデンスは限られる。

胎児染色体異常が流産の原因であることは疑いようないが，反復する原因としては考えられてこなかった。我々は不育症患者の1,309妊娠について，既往流産が2～4回では50%以上に胎児染色体異常がみられ，流産回数が増加すると生児獲得率が低下することを世界で初めて報告した[3]。さらに最近，胎児染色体検査と系統的検査がすべて行われた不育症482組の原因頻度を調べたところ，41%は胎児染色体異常のみがみられ，胎児染色体正常を示す真の原因不明は25%に留まることが明らかになった（図A）[4]。また，胎児染色体検査が複数回実施されている症例では70%以上が胎児異常は異常を，胎児正常は正常の核型を反復していた。胎児染色体異常が確認された既往流産2～3回程度の患者には系統的検査をしない，という選択もある。

日本産科婦人科学会・日本産婦人科医会診療ガイドライン産科編「CQ204反復流産・習慣流産」における推奨レベルは抗リン脂質抗体A，子宮奇形A，夫婦染色体検査Bである（表）。

一方，2008～2010年厚生労働省不育症班研究が行われ，ホームページに研究成果が公開されている。この内容は班員の研究成果に基づくものであって世界のエビデンスとは異なるため，以下の問題点がある。研究班は「スクリーニング検査」を推奨している。習慣流産の病名で保険適用されている検査が多いが，スクリーニング検査は通常「自費診療」となる。一般臨床家は患者の経済的負担軽減のためにも「診療ガイドライン産科編」が示す検査を段階的に行うべきである。また，研究班はプロテインSと抗フォスファチジルエタノールアミン抗体測定を推奨しているがこれらの産科的意義は未確定である。Branchら[2]，米国胸部外科学会ガイドラインは「妊娠合併症既往女性に血栓性素因のスクリーニング検査を推奨しない」としている。

夫婦染色体均衡型転座保因者

我々は反復流産1,284組のコホート研究を行い，相互転座を持つ夫婦と染色体正常夫婦の診断後初

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表　原因精査のために必要な検査と対策

	基本的検査の実際	予防，対策	日産婦診療ガイドライン推奨レベル	Branchらの N Engl J Med総説
抗リン脂質抗体	抗カルジオリピンβ_2GPⅠ複合体抗体>1.9（230） ループスアンチコアグラント(RVVT)>1.3（290） ループスアンチコアグラント(リン脂質中和法，290)* いずれかが12週間持続したら診断する	アスピリン81 or 100 mg＋ヘパリンカルシウム(5,000 IU×2/日)自己注射	A	推奨
夫婦染色体異常	染色体G分染法(3130)**	遺伝カウンセリング 着床前診断 自然妊娠	B	推奨
子宮奇形	子宮卵管造影(512)，超音波検査(530)***	手術 非手術	A	推奨
胎児染色体	流産絨毛の染色体G分染法	薬物投与無	C	推奨
内分泌異常(糖尿病，甲状腺機能低下，多のう胞性卵巣症候群)	空腹時血糖(11)，TSH（115），FT4(140)，超音波検査，問診	糖尿病，甲状腺機能コントロール		推奨しない
血栓性疾患				推奨しない

(　)習慣流産の病名での保険点数
*リン脂質中和法はSRL社の基準値6.3よりも1.6のほうが産科的には有用である可能性があり，現在検討中である。蛇毒法と同時に保険適用されない
**染色体異常の病名で保険適用される
***習慣流産，不育症では保険適用されていない

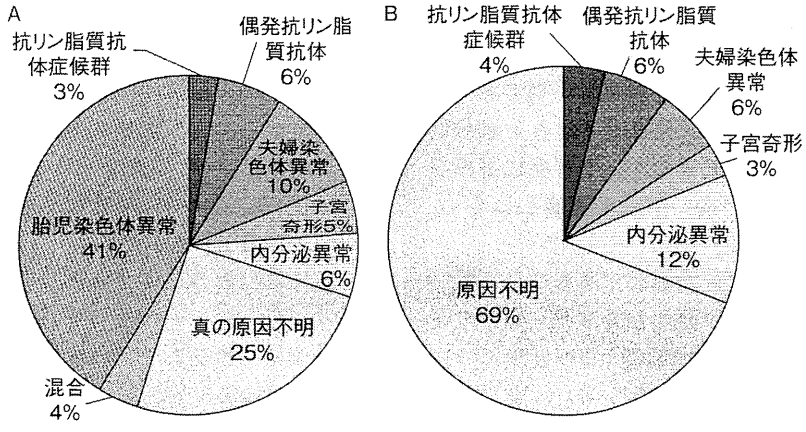

図　不育症精査を行った1,676人の異常頻度と胎児染色体検査を加えた482人の異常頻度
A：胎児染色体異常を含めた482人の不育症患者の異常頻度(Sugiura-Ogasawaraら，2012)[4]
B：1,676人の不育症患者の異常頻度(Sugiura-Ogasawaraら，2010)[1]

回妊娠での生児獲得率は31.9%（15/47）と71.7%（849/1,184）であり，均衡型相互転座保因者の流産率が有意に高いことを世界で初めて報告した[5]。累積生児獲得率は68.1%であり，オランダの研究でも83%と高い成績だった。

一方，2005年から日本でも流産予防のための着

床前診断が始まった。生児獲得率は14.3～58.6%と報告されており，自然妊娠に対する優位性は証明されていない。比較ゲノムハイブリダイゼーション法，FISH法いずれの方法においても自然妊娠を対照として着床前診断が生児獲得率を改善することを証明した報告はない。

抗リン脂質抗体症候群

プレドニゾロン・アスピリン併用療法が最初に報告されたが，流死産予防として低用量アスピリン・未分画ヘパリン療法が標準的治療法になっており，生児獲得率は70～80%とされている。国際抗リン脂質抗体学会の診断基準に準じた抗リン脂質抗体症候群APSに対しては2012年から保険適用された。診断基準には抗カルジオリピン抗体aCLもしくは抗β_2 glycoprotein I（β_2GP I）抗体（β_2GP I 依存性抗CL抗体を含む），ループスアンチコアグラント（LA，リン脂質中和法）もしくは希釈ラッセル蛇毒法（LA-RVVT）の3者が含まれている。

現在，β_2GP I，prothrombinを抗原としたELISA法が開発され多くの測定系が委託検査可能となっているが，陽性の時に抗凝固療法を行うと生児獲得率が改善できる「産科的意義」が確立されているものは少ない。

我々は5倍希釈aPTT試薬を用いて混合試験を行い（LA-aPTT），無治療では53.8%の次回流産率が抗凝固療法によって19.6%に改善できることを確認した。

リン脂質中和法は1.6（健常人の99パーセンタイル）を基準として陽性の場合，抗凝固療法によって生児獲得率が改善できることを確認した。リン脂質中和法とLA-aPTTは理論的に同じだが，試薬が違うことで異なる患者を検出した。LAに関しては試薬ごとに産科的有用性を確認する必要がある。フォスファチジルセリン依存性抗プロトロンビン抗体aPS/PT IgGも産科的意義を認めた。抗カルジオリピン抗体IgG，IgMは診断基準に含まれるが，陽性の場合に抗凝固療法を行っても生児獲得率は全く変わらなかった（論文投稿中）。Harrisの方法と記載されていても古典的Harrisの方法とは異なる点に留意する必要がある。

抗フォスファチジルエタノールアミンPE抗体IgG，IgMの陽性率はそれぞれ約10%であり，陽性率が高いために我が国では頻用されている。しかし，PE IgGはLA-aPTTと共陽性例はあるものの標準的測定法によるAPS群とかい離しており，単独陽性に関して無治療の出産率が71.4%もあり，産科的測定意義は認められなかった[6]。

現時点ではLA-リン脂質中和法，LA-RVVT法，β_2GP I 依存性抗CL抗体（基準値＞1.9）の3者を用いてAPSの診断を行うことがベストと考えられる。aPS/PT IgG測定も有用であるが保険適用はされていない。抗リン脂質抗体陰性で抗核抗体陽性例に治療の必要はない。

子宮奇形に対する手術

双角子宮に対する形成手術，中隔子宮に対する内視鏡的中隔切除術後の生児獲得率は35.1～64.9%と報告されている。しかし，これらの論文に対照はなく，長い間，手術が有用と盲目的に信じられてきた。当院の1,676例の反復流産患者の検討で単角子宮，重複子宮，双角子宮，中隔子宮の大奇形は3.2%にみられ，子宮奇形をもつ患者と正常子宮をもつ患者の非手術診断後初回妊娠成功率は59.5%（25/42），71.7%（1,096/1,528，p=0.084），累積成功率は78%，85.5%であった[1]。子宮奇形に対する手術・非手術を比較したRCTは行われていない。

胎児染色体数的異常

欧米では，原因不明習慣流産に対して胚スクリーニングが行われている。Platteauらの報告によると過去に4.46回流産歴のある原因不明習慣流産患者25人に着床前診断を行ったところ妊娠継続できたのはたったの25%だった。我々の検討では過去5回流産歴のある患者の51%が次回自然妊娠で出産できており，Platteauらも胚スクリーニングの有効性は認められないと述べている。

原因不明不育症

1981年に夫リンパ球免疫療法が報告された。当時は3回流産すると次は100%流産すると世界中が信じていた。しかし，臨床経験を積み上げた研

究者は習慣流産患者が無治療でも出産に至ることに気が付き始め，1999年には夫リンパ球と生理食塩水を比較する無作為割付け試験が報告され，夫リンパ球が無効であることが明らかになった。原因不明習慣流産に対し低用量アスピリン，アスピリン・ヘパリン療法，プラセボを無作為割付けし，これらの有効性がないことも証明された。ステロイド，イムノグロブリン，プロゲステロンなども生児獲得に関する確証は得られていない。

原因不明不育症には必ずしも薬剤投与の必要性はなく，既往流産2回流産81.1%，3回71.2%，4回65.4%，5〜6回50%の成功率が得られている。薬剤投与する場合は投与しなくても成功率に差はないことを説明し，倫理委員会の承認と患者の同意を得ることが必要である(厚生労働省臨床研究の倫理指針)。

原因不明不育症にさまざまな遺伝子変異が関与していることがわかってきた。習慣流産患者にアネキシンA5遺伝子変異が健常女性よりも高頻度にみられる危険因子であることが欧米で報告され，我々も同様の結果を得た[7]。しかし，そのオッズ比は1.6程度であり，変異有・無の間にその後の出産率の差はなかった。つまり，危険因子の影響は臨床的に大きなものではなく，不育症に関する遺伝子変異は数多く存在することで"易罹患性"を示すものと推測できる。

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A merged presentation of clinical and radiographic data using probability plots in a clinical trial, the JESMR study

In terms of the relationship between synovial inflammation and radiographic changes, including both joint damage repair and progression,[1] in rheumatoid arthritis (RA), pre-existing joint damage and persistent synovitis may promote joint destruction, while in the absence of synovitis, damaged joints may heal.[2 3] Although presentation of radiographic results using cumulative probability plots has substantially improved understanding of clinical trial data,[4] the effects of treatments on radiographic progression and improvement (regression) in individual RA patients has not yet been fully explained.

In the JESMR study,[5 6] 151 active RA patients unresponsive to treatment with methotrexate (MTX) were randomised into 1 of 2 treatment groups: etanercept (ETN) 50 mg/week with 6–8 mg/week of MTX (the E+M group), or ETN alone (the E group). Radiographs of the hands and feet before ETN (baseline) and during the first year of treatment were available from 53 (72%) and 68 (88%) patients in the E and E+M groups, respectively. Baseline characteristics of patients were comparable between those with and without available radiographic data in each treatment group (data not shown). However, most patients without data did not complete the study up to Week 52 as per protocol, chiefly due to lack of efficacy in the E group.[6] The mean baseline total Sharp-van der Heijde score (TSS)[7] was 114.5 in the E group and 113.1 in the E+M group (disease duration: 10.0 years and 8.4 years, respectively), and the smallest detectable change (SDC) in TSS over 52 weeks was 1.9.

Cumulative probability plots provided by the American College of Rheumatology (ACR)-N[8] clearly demonstrated a superior response (figure 1A,B) and a significantly greater ACR50 response rate in the E+M group at week 52 (76.5% vs 50.9%, p=0.0041, Fisher's exact test). Merged probability plots of individual radiographic change over 52 weeks (ΔTSS) suggested preferential existence of aggressive radiographic progressors among ACR50 non-responders in the E group. The relationship among treatment, clinical disease activity, and radiographic change was further addressed using time-averaged disease activity score of 28 joints (DAS28) over 52 weeks in place of ACR-N at Week 52 (figure 1C,D). Significant correlation between time-averaged DAS28 and ΔTSS was observed in the E (r^2=0.097, p=0.023) but not the E+M group (r^2=0.019, p=0.26). Aggressive radiographic progression was preferentially observed among patients with moderate or high activity on average in the E group (figure 1C), while in the E+M group, radiographic progression among these patients seemed to be balanced by radiographic regression among those in remission or with low disease activity (figures 1D–F).

The absence of radiographic regressors (>SDC) among clinical responders in the E group (figure 1A,C,E) was surprising, although 18.2% of those patients showed regression within the SDC. This may be partly explained by the limitations of the study due to the small number of patients involved. Another limitation was much lower MTX dose at study enrolment than the current global standard dosage: 7.0±1.4 (the mean±SD) and 7.4±1.1 in the E and E+M groups, respectively.

In summary, we first demonstrated the relationship between individual clinical responses and radiographic changes by merging cumulative probability plots of ACR-N or time-averaged DAS28 and ΔTSS. These presentations clearly show the relationships between two parameters as a whole, facilitating further post hoc analyses of clinical trials. Further, merged presentation of probability plots is useful in comparing a single parameter (eg, health assessment questionnaire-disability index: HAQ-DI) before and after treatments (figure 2). However, merged presentation of probability plots must be followed by statistical analyses after being classified into binary or ternary categories, as we showed here.

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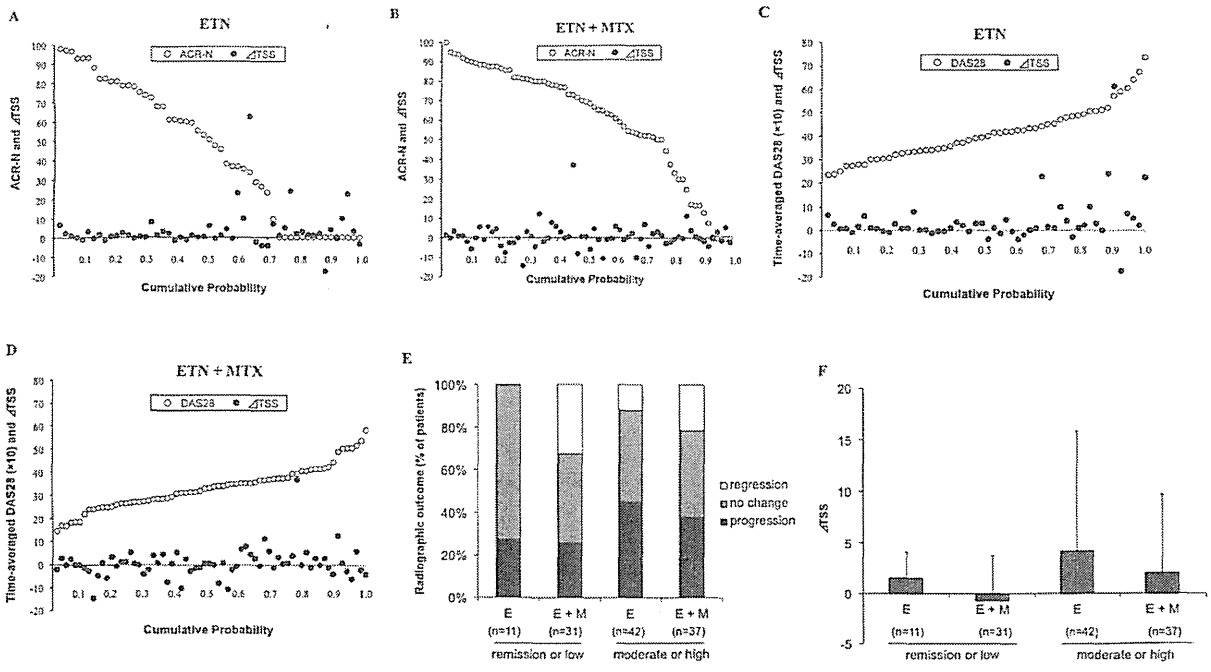

Figure 1 Cumulative probability plot analysis of ACR-N (A,B) or time-averaged DAS28 (C,D) and radiographic changes in the E (A,C) and E+M groups (B,D), merged to keep same patients on the vertical line, followed by the radiographic outcomes (E) and changes (F) stratified by the treatment and time-averaged disease activity state. Time-averaged DAS28 was calculated by the area under the curve of DAS28 at weeks 0, 2, 4, 8, 12, 24 and 52, divided by 52. No significant differences were observed between groups using Pearson's test (E) and Kruskal-Wallis test (F). ACR, American College of Rheumatology; DAS28, disease activity score of 28 joints; ETN, etanercept; MTX, methotrexate; TSS, total Sharp-van der Heijde score.

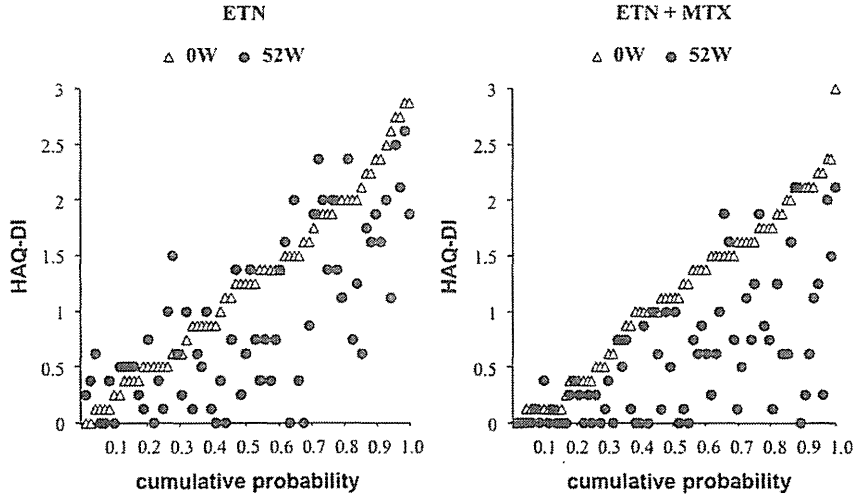

Figure 2 Merged probability plots of individual health assessment questionnaire-disability index (HAQ-DI) scores at baseline (open triangle) and Week 52 (closed circle) in the E (left) and E+M groups (right). Subsequent analyses included comparison of the rate of HAQ-DI≤0.5 at 52 weeks in patients with baseline HAQ-DI>1.5. None of 15 patients (0.0%) in the E group and 6 of 23 patients (26.1%) in the E+M group, respectively; p=0.037 by Fisher's exact test (one-sided). ETN, etanercept; MTX, methotrexate.

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Serum hepcidin level is not an independent surrogate biomarker of disease activity or of radiographic progression in rheumatoid arthritis: results from the ESPOIR cohort

Hepcidin is an interleukin-6 induced peptide hormone involved in iron metabolism and inflammation.[1] Serum hepcidin level may distinguish anaemia due to chronic inflammation and/or iron deficiency in rheumatoid arthritis (RA) patients.[2] Furthermore, some studies have suggested that serum hepcidin could reflect disease activity raising its measurement as a new surrogate biomarker of RA.[3–6] These studies have several drawbacks (unreliable pro-hormone quantification, small number of patients).[7 8] Therefore, we assessed the serum level of the mature form of hepcidin by ELISA, (Bachem, St Helens, Merseyside, UK) in 791 individuals from the French cohort of early arthritis (ESPOIR) including 632 patients with RA fulfilling the American College of Rheumatology (ACR) - European League Against Rheumatism (EULAR) criteria at inclusion and 159 with undifferentiated arthritis in order to address whether hepcidin accurately reflects RA features, disease activity or radiographic disease progression.[9 10]

Beyond expected differences between RA and undifferentiated arthritis, serum hepcidin level was higher in RA (table 1).

Baseline characteristics of 791 patients with early rheumatoid arthritis or undifferentiated arthritis

	Undifferentiated arthritis (n=159)	Early RA (n=632)	p Value
Age	47.2±13.8	48.5±12.2	0.46
Women, n (%)	117 (74)	492 (78)	0.25
First symptom (months)	6.6±7.7	6.9±8.5	0.72
DAS28 value	4.04±1.03	5.40±1.23	<0.0001
CRP level (mg/l)	17.15±29.3	21.10±33.14	0.0028
ESR (mm)	25.3±22.4	30.6±24.9	0.0014
Positive anti-CCP antibodies, n (%)	2 (1.26)	313 (49.5)	<0.0001
Positive RF, n (%)	5 (3.)	365 (57.75)	<0.0001
Swollen joint count	3.5±2.4	8.2±5.2	<0.0001
Tender joint count	3.2±2.6	9.9±7.2	<0.0001
HAQ	0.69±0.58	1.05±0.69	<0.0001
VAS fatigue	42.8±31.1	49.2±27.2	0.0118
x-ray erosion at inclusion, n (%)	0 (0)	108 (17.1)	<0.0001
Haemoglobin (g/dl)	13.0±1.21	13.0±1.3	0.9582
Ferritinemia (µg/l)	151.4±164.7	149.2±157.5	06 802
MCV (μ^3)	88.41±4.55	88.75±5.1	0.2141
Serum hepcidin level	39.6±39.9	53.0±48.5	p<0.0001

Data are mean±SD unless indicated. Baseline characteristics of RA and undifferentiated arthritis patients were compared by χ^2 or Fisher's exact tests for discrete variables and unpaired t tests, Wilcoxon signed rank tests for continuous variables.
Anti-CCP, anticyclic citrullinated protein peptide antibodies; CRP, C reactive protein; DAS28, Disease Activity Score in 28 joints; ESR, erythrocyte sedimentation rate; HAQ, Health Assessment Questionnaire; MCV, mean cell volume; RA, rheumatoid arthritis; RF, rheumatoid factor; VAS, visual analogue scale.

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ORIGINAL ARTICLE

Phospholipid scramblase 1 expression is enhanced in patients with antiphospholipid syndrome

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Abstract
Objective Thrombus formation is the key event of vascular manifestations in antiphospholipid syndrome (APS). Phosphatidylserine (PS) is normally sequestered in the inner leaflet of cell membranes. Externalization of PS occurs during cell activation and is essential for promoting blood coagulation and for the binding of antiphospholipid antibodies (aPL) to cells. One of the molecules involved in PS externalization is phospholipid scramblase 1 (PLSCR1). We evaluated PLSCR1 expression on monocytes from APS patients and analyzed the in vitro effect of monoclonal aPL on PLSCR1 expression.
Patients and methods Forty patients with APS were investigated. In vitro experiments were performed in monocyte cell lines incubated with monoclonal aPL. PLSCR1 expression was determined by quantitative real-time polymerase chain reactions. PS exposure on $CD14^+$ cell surface was analyzed by flow cytometry.
Results Levels of full-length PLSCR1 messenger RNA (mRNA) were significantly increased in APS patients compared with healthy controls (2.4 ± 1.2 vs. 1.3 ± 0.4, respectively, $p < 0.001$). In cultured monocytes, interferon alpha enhanced tissue-factor expression mediated by β2-glycoprotein-I-dependent monoclonal anticardiolipin antibody.
Conclusions Monocytes in APS patients had increased PLSCR1 mRNA expression.

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Introduction

Antiphospholipid syndrome (APS) is an autoimmune disorder characterized by the presence of antiphospholipid antibodies (aPL) in plasma of patients with thrombosis and/or pregnancy morbidity. Phospholipid-binding plasma proteins, β2-glycoprotein I (β2-GPI) and prothrombin, are the dominant antigenic targets recognized by aPL in APS [1–4].

The interaction between aPL and cells involved in hemostasis regulation is one of the most plausible mechanisms responsible for the thrombophilic state in APS. aPL react with phospholipid-binding proteins expressed on the membranes of procoagulant cells. This interaction induces a perturbation in the cells, leading to up-regulation of adhesion molecules and procoagulant substances, which results in a proinflammatory/prothrombotic response and subsequently thrombosis [5]. However, in order that aPL bind to the cell surface, the antigen–antibody complex must be present on the phosphatidylserine (PS)-exposed cell surface [6]. PS is a negatively charged phospholipid normally located in the inner leaflet of the cell membrane. PS exposure at the outer leaflet of plasma membranes occurs in activated cells and is essential for promoting blood coagulation, as PS serves as a catalytic surface for the assembly of coagulation factors [7].

Phospholipid scramblase 1 (PLSCR1), a lipid-raft-associated type II endofacial plasma protein, is involved in the regulation of PS externalization during cell activation. PLSCR1 catalyzes a rapid transbilayer movement of phospholipids between membrane leaflets [8]. We

previously reported enhanced PLSCR1 messenger RNA (mRNA) expression in monocytes in patients with systemic lupus erythematosus (SLE) [9], suggesting a role of PLSCR1 in the prothrombotic tendency in SLE. In the study reported here, we investigated the involvement of PLSCR1 in the thrombophilic state in patients with APS.

Patients and methods

Study participants

Serum and plasma samples were obtained from 40 consecutive nonselected Japanese patients with APS who visited the Rheumatic and Connective Tissue Disease Clinic. All patients—35 women and five men, mean age 50 (range 26–76) years—fulfilled the Sydney-revised Sapporo classification criteria for definite APS [10]. Fifteen patients were diagnosed as having primary APS, and 25 patients had APS in association with SLE. Patients with SLE fulfilled the American College of Rheumatology criteria [11].

The historical profiles of clinical and laboratory manifestations were verified by the authors using medical records. Twenty-two (55 %) patients experienced arterial thrombotic events such as stroke or myocardial infarction confirmed by magnetic resonance imaging, angiography, computed tomography scan, electrocardiographic changes, and increased cardiac enzymes. Deep vein thrombosis, pulmonary embolism, or retinal thrombosis were found in 16 patients (40 %) and confirmed by Doppler ultrasound, scintigraphy, phlebography, or retinal fluorescence. Four patients (10 %) had both arterial and venous thrombosis. Eight women (23 %) had pregnancy morbidity. No patient had thrombosis or pregnancy complications within 3 months before blood collection. When blood was drawn, signs of acute thrombosis were not detected in any patient: 16 patients were receiving warfarin; none were on heparin (Table 1).

Table 1 Characteristics of antiphospholipid syndrome (APS) patients

	Number	Percent
Primary APS	15	38
APS and SLE	25	62
Gender (F:M)	35:5	
Age mean (range) years	50 (26–76)	
Historical manifestations		
Thrombosis	34	85
Arterial	22	65
Venous	16	47
Arterial and venous	4	12
Pregnancy morbidity	8	23
Anticoagulant therapy/antiplatelet drugs[a]	37	93
Warfarin alone	9	24
Aspirin alone	13	35
Cilostazol alone	2	5
Combined therapy		
Warfarin + ≥1 antiplatelet drugs	7	19
Two antiplatelet drugs	6	16

APS antiphospholipid syndrome, *SLE* systemic lupus erythematosus

[a] Antiplatelet drugs: aspirin, cilostazol, clopidogrel

Blood collection

Venous blood was collected into tubes containing sodium citrate and centrifuged immediately at 4 °C. Plasma samples were depleted of platelets by filtration then stored at −80 °C until they were used in the experiments. Blood samples were also collected from 43 apparently healthy Japanese individuals who consented to join the study [25 women and 18 men, mean age 28 (range 20–38) years]. The study was performed in accordance with the Declaration of Helsinki and the Principles of Good Clinical Practice. Approval was obtained from the Local Ethics Committee, and informed consent was obtained from each study participant before enrollment.

Materials

Human monocyte cell lines THP-1, U937, KG1a (ATCC TIB-202, CRL-1593, CCL-246.1, respectively), human Burkitt's B-cell lymphoma cell line Raji (ATCC CCL-86), and murine monocyte cell line RAW 264.7 (ATCC TIB-71) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). Human interferon α (IFN-α2a) was from Santa Cruz Biotechnology Inc (CA, USA). The β2-GPI-dependent monoclonal anticardiolipin antibody (aCL), WBCAL-1, was previously established from splenocytes of a NZW × BXSB F1 male mouse, as described [12].

Methods

Isolation and preparation of PBMC or monocytes

Venous blood was collected into tubes containing heparin. Peripheral blood mononuclear cells (PBMC) were isolated on Ficoll-Paque plus® gradient centrifugation (Amersham Biosciences, Uppsala, Sweden) using standard protocols. Isolation of monocytes was performed, as reported [9]. Briefly, PBMC were pelleted by centrifugation and washed with phosphate-buffered saline (PBS) (Sigma). Contaminated red blood cells were then lysated with Red Blood Cell Lysis Buffer (eBioscience, CA, USA) and washed with PBS. Monocytes were purified using CD14 microbeads

(Miltenyi Biotec Bergisch Gladbach, Germany) as follows: PBMC pellet was suspended in 80 µl of autoMACS™ rinsing solution (Miltenyi Biotec), and 20 µl of CD14 microbeads were added. After 15 min incubation at 4 °C, cells were washed, suspended in 500 µl auto-MACS™, and separated in a magnetic separation kit (Miltenyi Biotec) according to manufacturer's instructions.

Cell culture

THP-1, U937, KG1a, and Raji cell lines were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma) and RAW 264.7 cells in Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL, Paisley, USA). Media were supplemented with 10 % fetal bovine serum (FBS) (Sigma) containing penicillin and streptomycin, and cells were maintained in a 5 % CO_2 atmosphere at 37 °C. Cultured monocyte lines were incubated in the presence and absence of several stimulators at different experimental conditions.

RNA extraction and reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA were isolated from cells using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and reverse-transcribed with the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). RT-PCR was performed as follows: 1 µl of complementary DNA (cDNA) was amplified in a total volume of 10 µl containing deoxyribonucleotide triphosphate (dNTP) (2 mM each) (Applied Biosystems, CA, USA) in a standard buffer with 1.25–1.5 mM magnesium chloride ($MgCl_2$), 0.625 U DNA Taq polymerase, and 10 pmol of each primer. The gene-specific primer sequences were as follows: for human PLSCR1, forward 5′-CAG CCT CCA TTA AAC TGT CC-3′ and reverse 5′-TCT TAG TGG TCT CTC CAGAG-3′; for mouse PLSCR1, forward 5′-CCT CCT CCA CTG AAC TGT CC-3′ and reverse 5′-CTC TCT GCC CGA GGC TGT TCT-3′. Amplification for human PLSCR1 was performed in 28 cycles: 95 °C, 45 s; 53 °C, 45 s; 72 °C, 45 s and for mouse PLSCR1 in 33 cycles: 95 °C, 45 s; 58 °C, 45 s; and 72 °C, 45 s. After all cycles were completed, a final extension step of 72 °C for 7 min was performed in all PCRs. The amplified products were resolved in 9 % polyacrylamide gel (PAGE), stained with ethidium bromide, and visualized under ultraviolet light. Bands of 273 and 148 bp were visualized for the human and mouse PLSCR1, respectively. Amplification of mRNA from the housekeeping gene β-actin was used as control of these experiments using the following primers: human β-actin forward 5′-TAC ATG GCT GGG GTG TTG AA-3′ and reverse 5′-AAG AGA GGC ATC CTC ACC CTG-3′; mouse β-actin forward 5′-ACC AAC TGG GAC GAT ATG GAG AAG A-3′ and reverse 5′-CGC ACG ATT TCC CTC TCA GC-3′.

Quantitative real-time PCR

PLSCR1 expression in monocytes from APS patients and healthy controls was evaluated by a relative quantification (RQ) of gene expression by real-time PCR, as previously reported [9]. The level of the PLSCR1 transcript was normalized to that of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). RQ was done using the comparable cycle threshold (C_T) method, as described [13].

For in vitro analysis of PLSCR1 expression, quantitative analysis of gene expression was performed by real-time PCR using the ABI PRISM 7000® Sequence Detection System (Applied Biosystems). Gene-specific sets of either Sybr® Green PCR master Mix (Applied Biosystems) and specific forward and reverse PLSCR1 and tissue factor (TF) primers, or Taq Man® Universal PCR Master Mix® and Assays-on-Demand Gene Expression Probes® (Applied Biosystems) were used. A standard curve for serial dilutions of β-actin was generated using a standard method provided by the manufacturer.

Measurement of cell-surface PS exposure

Monocytes from eight APS patients and 24 healthy individuals, who agreed to the double blood collection, were isolated, and cell-surface PS exposure was evaluated by flow cytometry using the Annexin-V-Fluos staining kit (Roche), as described [9], and FACSCalibur (Becton–Dickinson Immunocytometry Systems, San Jose, CA, USA) with the Cell Quest program. From each sample, data from 10,000 counted-gated viable cells were collected and expressed as the percentage of Annexin-V-positive cells in the total gated-cell population.

Laboratory investigations in plasma samples

D-dimer

Plasma D-dimer levels (Nanopia, D-dimer, Daiichi Kagaku, Tokyo, Japan) were measured as markers of fibrin turnover. The cutoff level was previously defined as >95 percentile of 65 healthy individuals as a routine laboratorial assay.

Antiphospholipid antibodies

Immunoglobulin G (Ig)G and M aCL were assayed according to the standard aCL enzyme-linked immunosorbent assay (ELISA) [14]. IgG and M anti-β2-GPI antibodies and IgG and M PS-dependent antiprothrombin

antibodies (aPS/PT) were determined by in-house ELISAs, as reported [15, 16]. The detection of lupus anticoagulant (LA) was based on the previous version of the guidelines recommended by the Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibody of the Scientific and Standardisation Committee of the International Society of Thrombosis and Haemostasis [17].

Statistical analysis

Statistical evaluation was performed using Student's *t* test. Spearman's rank correlation coefficient was used to analyze correlations. The significance level was set at $p < 0.05$.

Results

Expression of full-length PLSCR1 mRNA in monocytes from APS patients

Full-length PLSCR1 mRNA mean levels were evaluated by real-time PCR. Levels of PLSCR1 mRNA were significantly higher in monocytes in APS patients than in healthy controls (2.4 ± 1.2 vs. 1.3 ± 0.4, respectively, $p < 0.001$) (Fig. 1). There were no statistically significant differences in PLSCR1 mRNA levels between patients with primary APS and SLE patients or among those who had arterial thrombosis and those with venous thrombosis. Patients with pregnancy complications, without thrombotic events, have elevated PLSCR1 levels compared with those with thrombotic events (3.43 ± 1.1 vs. 2.2 ± 1.2, respectively, $p < 0.027$).

Cell-surface PS on $CD14^+$ cells

Flow-cytometric analysis showed that the amount of expressed PS on $CD14^+$ cells was increased in cells from APS patients compared with healthy controls (21.5 % ± 11.0 and 17.8 % ± 5.8, respectively). However, the difference in PS expression did not reach statistical significance. No statistically significant correlation was found between PLSCR1 mRNA levels and PS expression on monocytes in patients with APS.

Laboratory investigations

Plasma levels of D-dimer were significantly increased in patients with APS compared with those in healthy controls (1.1 ± 0.6 vs. 0.6 ± 0.2 μg/ml, $p < 0.001$). There was no difference in plasma D-dimer levels between patients with SLE and those without. aCL, anti-β2-GPI antibodies, aPS/PT, and LA were positive in 53, 58, 53, and 85 % of patients, respectively (Table 2).

There were no statistically significant correlations between the levels of PLSCR1 mRNA expression and titers of D-dimer, IgG/M aCL, IgG/M anti-β2-GPI antibodies, or IgG/IgM aPS/PT in patients with APS.

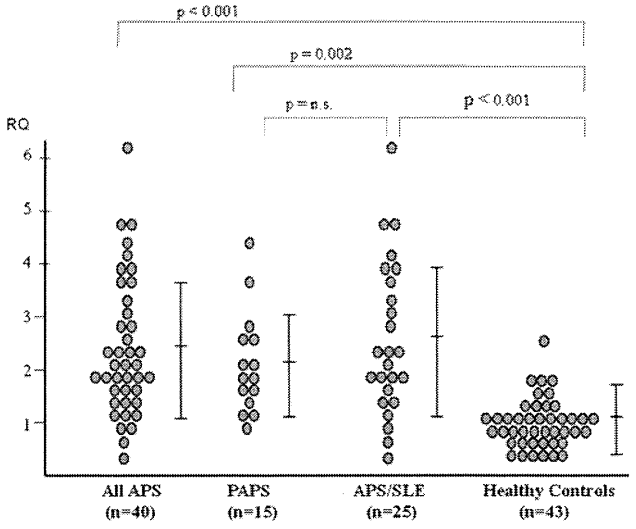

Fig. 1 Phospholipid scramblase 1 (PLSCR1) messenger RNA (mRNA) expression in monocytes from antiphospholipid syndrome (APS) patients. Gene expression of PLSCR1 in $CD14^+$ cells was evaluated in patients with antiphospholipid syndrome (APS) and in healthy individuals using real-time polymerase chain reaction (PCR). Expression values were normalized to the expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and expressed as relative quantification (RQ) in the *Y* axis. Data are shown as individual results. *Horizontal lines* show the mean ± standard deviation. PLSCR1 mRNA expression was significantly higher in patients with APS. *PAPS* primary APS; *SLE* systemic lupus erythematosus

Table 2 Laboratory investigations

	Number	Percent
D-dimer positive	14	35
Anticardiolipin antibodies	21	53
IgG	14	35
IgM	1	3
Anti-β2-GPI antibodies	23	58
IgG	17	43
IgM	4	10
aPS/PT	21	53
IgG	18	45
IgM	6	15
Lupus anticoagulant	34	85

IG immunoglobulin, *β2-GPI* β2-glycoprotein I, *aPS/PT* phosphatidylserine-dependent antiprothrombin antibodies

Effect of IFN-α on PLSCR1 mRNA expression

Cultured cell lines and PBMC from a healthy donor were incubated in the presence or absence of IFN-α2a (IFN α, 500 IU/ml) for 6 h in a 5 % CO_2 atmosphere at 37 °C. RT-PCR showed that IFN-α increased the expression of PLSCR1 mRNA (Fig. 2a). Quantitative real-time PCR demonstrated that the mean levels of PLSCR1 mRNA were significantly higher in cells treated with IFN-α (Fig. 2b).

Effect of antiphospholipid antibodies on PLSCR1 expression

RAW 264.7 cells were pretreated in the presence or absence of IFN-α2a (400 IU/ml) for 3 h, followed by treatment with β2-GPI-dependent monoclonal aCL, WBCAL-1, for an additional 5 h. IFN-α2a/WBCAL-1 combination significantly enhanced PLSCR1 mRNA expression. WBCAL-1 is a well-known inducer of TF, and TF mRNA expression was also enhanced by IFN-α2a/WBCAL-1 combination (Fig. 3).

Discussion

In our study, we showed increased expression of PLSCR1 mRNA in monocytes in patients with APS. We also demonstrated that monoclonal aPL upregulated PLSCR1 in monocytes in vitro. The association between aPL and thrombotic events is widely accepted. Numerous pathogenic

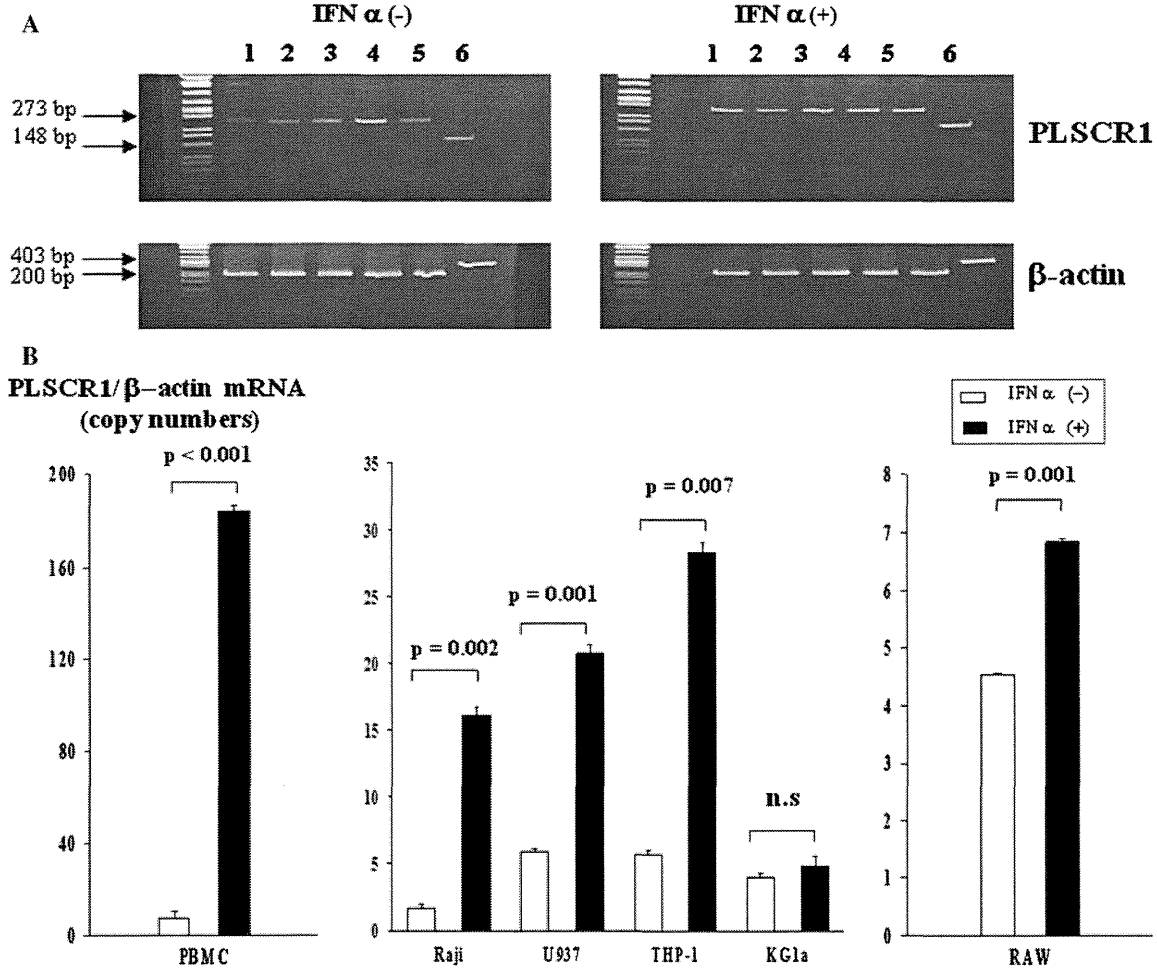

Fig. 2 Phospholipid scramblase 1 (PLSCR1) expression in cultured cells after interferon alpha (IFN-α) treatment. PLSCR1 expression was evaluated 6 h after treatment with (IFN-α^+) or without (IFN-α^-) 500 IU/ml IFN-α2a. **a** Reverse transcriptase polymerase chain reaction (RT-PCR) products are shown in 9 % polyacrylamide gel from one representative of three independent experiments. *Bands* of 273 and 148 bp correspond to human and mouse PLSCR1 products, and *bands* of 200 and 403 bp to human and mouse β-actin products, respectively. *Lanes 1–5* human cells: *lane 1* Raji, *lane 2* U937, *lane 3* THP-1, *lane 4* KG1a, *lane 5* peripheral blood mononuclear cells (PBMC); *lane 6* mouse RAW 264.7 cells. **b** Quantitative real-time analysis. Results are expressed as copy numbers of PLSCR1/β-actin messenger RNA (mRNA). *Left panel* corresponds to PLSCR1 mRNA from PBMC. *Middle panel* includes PLSCR1 mRNA from human cells lines Raji, U937, THP-1, and KG1a. *Right panel* corresponds to mouse PLSCR1 mRNA from RAW 264.7 (RAW) cell line. Data represent the mean ± standard error of triplicate samples from one representative of three independent experiments