(Diagnostica Stago). The dilute Russell's viper venom time (dRVVT) was screened for and confirmed by use of a Gradipore LAC test (Gradipore, Sydney, New South Wales, Australia). LA was considered positive when at least one of these tests confirmed its presence.

Immunoglobulin (Ig)G and IgM aCL were measured according to a standard aCL enzyme-linked immunosorbent assay (ELISA), as described elsewhere.[35]

IgG and IgM aβ2GPI were determined by ELISA method as previously reported.[36] Purified human β2GPI was purchased from Yamasa Corp. (Tokyo, Japan). Irradiated microtiter plates, Maxisorp (Nunc, Denmark) were coated with 4 μg/ml of purified β2GPI in phosphate-buffered saline (PBS) at 4°C and washed twice with PBS. To avoid nonspecific binding of proteins, wells were blocked with 150 μl of 3% gelatin (BDH Chemicals Ltd, Poole, UK). After three washes with PBS containing 0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) (PBS-Tween), 50 μl of serum diluted with PBS containing 1% bovine serum albumin (Sigma-Aldrich) (PBS-1% BSA) in 1:50 were added in duplicate. Plates were incubated for one hour at room temperature and washed three times with PBS-Tween. Fifty microliters per well of the appropriate dilution of alkaline phosphatase-conjugated goat anti-human IgG and IgM (Sigma-Aldrich) in PBS-1% BSA was added. After one hour of incubation at room temperature and after four washes in PBS-Tween, 100 μl/well of 1 mg/ml p-nitrophenylphosphate disodium (Sigma-Aldrich) in 1 M diethanolamine buffer (pH 9.8) were added. Following color development, optical density at 405 nm was measured by a Multiskan ascent plate reader (Thermo Electron Corporation, Waltham, MA, USA). Normal ranges of IgG (> 2.2 U/ml) and IgM (> 6.0 U/ml) aβ2GPI with cut-off values of 99th percentile were previously established using nonpregnant 132 healthy controls.

IgG and IgM aPS/PT were detected by ELISA, as previously described.[37] Briefly, nonirradiated microtiter plates (Sumilon Type S; Sumitomo Bakelite, Tokyo, Japan) were coated with 30 μl of a 50 μg/ml preparation of phosphatidylserine (Sigma-Aldrich) and dried overnight at 4°C. To avoid nonspecific binding of proteins, the wells were blocked with 150 μl of Tris-buffered saline (TBS) containing 1% fatty acid-free BSA (catalog no. A6003; Sigma-Aldrich) and 5 mM $CaCl_2$ (BSA-$CaCl_2$). After three washes in TBS containing 0.05% Tween 20 (Sigma-Aldrich) and 5 mM $CaCl_2$ (TBS-Tween-$CaCl_2$), 50 μl of a 10 μg/ml preparation of human prothrombin (Diagnostica Stago) in BSA-$CaCl_2$ was added to half of the wells in the plates, and the same volume of BSA-$CaCl_2$ alone (as sample blank) was added to the other half. After one hour of incubation at 37°C, the plates were washed, and 50 μl of serum diluted 1:100 in BSA-$CaCl_2$ was added to duplicate wells. Plates were incubated for one hour at room temperature, and alkaline phosphatase-conjugated goat anti-human IgG or IgM and substrate were added. The aPS/PT antibody titer of each sample was derived from the standard curve according to dilutions of the positive control.

Genotyping

CD36 gene polymorphisms were investigated in this population using the TaqMan polymerase chain reaction (PCR) genotyping method on a 7500 Fast Real-Time PCR System® (Applied Biosystems, Foster City, CA, USA). Genomic DNA samples were extracted from peripheral blood. Related risk for having APS or SLE was approximated by odds ratio (OR).

Materials

Animal studies were reviewed and approved by Hokkaido University Institutional Animal Care and Use Committee. CD36KO mice were kindly donated by Dr Yamashita, Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, Japan.[38] FA6-152, a mouse monoclonal anti-human CD36 antibody (aCD36) with the CD36 signal blocking property, was purchased (Abcam, Cambridge, UK).[39] A mouse monoclonal aPS/PT with LA activity, 231D, was prepared as described previously.[3] Purified total IgG from APS patients either aCL- or aPS/PT-positive (Pt-aCL and Pt-aPS/PT, respectively) or healthy donors (Healthy-IgG) were prepared using MelonTM Gel IgG Purification Kit (Takara Bio, Ohtsu, Japan). Purity of IgG was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Pt-aCL and Pt-aPS/PT were confirmed to have aPL titers by ELISA as described above. Clinical profiles of the patients whose IgG was purified are shown in Table 2. All antibodies were confirmed not to be contaminated with lipopolysaccharide (LPS) using Limulus ES-II Single Test® (Wako, Osaka, Japan).

Mouse peritoneal macrophage (MPM) stimulation assay

At three days after intraperitoneal injection of 2 ml 10% proteose peptone (Becton Drive, Franklin

Table 2 Clinical profiles of patients whose IgG was purified and used in our experiments

No	*Disease*	*Age-Sex*	*Arterial thrombosis*	*Venous thrombosis*	*Pregnant morbidity*	*LA*	*aCL*	*aβ2GPI*	*aPS/PT*
Pt-aCL1	PAPS	52-F	Stroke	–	–	+	+	+	–
Pt-aCL2	PAPS	27-F	–	–	Miscarriage	+	+	+	–
Pt-aCL3	PAPS	46-F	Stroke	DVT	–	+	+	+	–
Pt-aCL4	APS-SLE	49-F	Stroke	DVT	Miscarriage	+	+	+	–
Pt-aCL5	PAPS	52-F	Stroke	–	–	+	+	+	–
Pt-aPS/PT1	PAPS	29-M	Stroke	DVT	–	+	–	–	+
Pt-aPS/PT2	APS-SLE	18-F	–	DVT	–	+	–	–	+
Pt-aPS/PT3	APS-SLE	25-F	Stroke	–	Miscarriage	+	–	–	+
Pt-aPS/PT4	APS-SLE	33-F	–	DVT	Miscarriage	+	–	–	+
Pt-aPS/PT5	APS-SLE	22-F	Stroke	PE	Miscarriage	+	–	–	+
Pt-aPS/PT6	APS-SLE	30-M	–	DVT	–	+	–	–	+
Pt-aPS/PT7	APS-SLE	45-F	Stroke	DVT		+	–	–	+

IgG: immunoglobulin G; F: female; M: male; LA: lupus anticoagulant; aCL: anticardiolipin antibodies; aβ2GPI: anti-β2 glycoprotein I antibodies; aPS/PT: phosphatidylserine-dependent antiprothrombin antibodies; PAPS: primary antiphospholipid syndrome; DVT: deep vein thrombosis; PE: pulmonary embolism.

Lakes, NJ, USA), MPM were harvested from 8- to 12-week-old female CD36KO or C57BL/6J wild-type (WT) mice. MPMs were suspended in Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA, USA) containing penicillin and streptomycin and their concentration adjusted to 1×10^6 cells/ml. MPMs were then treated with aPL (300 μg/ml Pt-aCL, 300 μg/ml Pt-aPS/PT or 10 μg/ml 231D) and incubated for four hours at 37°C 5% CO_2. Pt-aPS/PT and 231D were used in the presence of 2.5 mM $CaCl_2$ and 10 μg/ml human prothrombin (Diagnostica Stago).[3] Equal concentrations of Healthy-IgG or mouse IgG1κ isotype control (Becton Drive) was used as a negative control and 600 ng/ml LPS (Sigma-Aldrich) was used as a positive control. Data were obtained by five or more independent experiments.

Human peripheral blood mononuclear cell (PBMC) stimulation assay

Venous blood was collected in heparin from a healthy donor. Human PBMC were isolated on Ficoll-Paque plus® gradient centrifugation (Amersham Biosciences, Piscataway, NJ, USA). Human PBMCs were suspended in DMEM supplemented with 10% fetal calf serum containing penicillin and streptomycin and their concentration adjusted to 1×10^6 cells/ml. Human PBMC were then treated with aPL (200 μg/ml Pt-aCL, 200 μg/ml Pt-aPS/PT or 2 μg/ml 231D) in the presence or absence of 1 μg/ml aCD36 and incubated for four hours at 37°C 5% CO_2. Pt-aPS/PT and 231D were used in the presence of 2.5 mM $CaCl_2$ and 10 μg/ml human prothrombin. Equal concentrations of Healthy-IgG or mouse IgG1κ isotype control was used as a negative control and 1 ng/ml LPS was used as a positive control. Data were obtained by three or more independent experiments. The healthy donor was confirmed to have CD36 on both monocytes and platelets by analysis by flow cytometry before the experiments were performed (data not shown).

RNA extraction and quantitative TaqMan real-time PCR

Total RNA was isolated from MPM or human PBMC using RNeasy Mini Kit® (Qiagen, Valencia, CA, USA) and reverse-transcribed with Super ScriptTM First-Strand Synthesis System for RT-PCR (Invitrogen). Quantitative analysis of TF or IL-6 gene expression was performed by real-time PCR using 7500 Fast Real-Time PCR System® and gene-specific TaqMan Minor Groove Binder probes (Mm00438855m1, Hs01076032m1, Mm004461 90m1 and Hs00174131m1; Applied Biosystems). The level of the TF or IL-6 transcript was normalized to that of the glyceraldehyde-3-phosphate dehydrogenase. Relative quantification was performed using the comparable cycle threshold method.

Monocyte TF antigen expression by flow cytometry

Surface TF expression on human monocytes, treated with aPL as described above, was evaluated by flow cytometry with a direct double-color immunofluorescence technique. Resuspended human PBMC were incubated with phycoerythrin-conjugated mouse monoclonal anti-human CD14 (Beckman Coulter, Brea, CA, USA) and with fluorescein-conjugated mouse monoclonal anti-human

Table 3 Allele frequencies of CD36 gene polymorphisms

Group	*Minor allele frequency*	*P value*	*OR (95% CI)*
rs3765187 (C478T Pro90Ser)	(TT + TC vs CC)		
Healthy subjects ($n = 422$)	10.2% (43/422)	–	–
APS ($n = 132$)	3.8% (5/132)	0.032	0.35 (0.13 to 0.90)
SLE/non-APS ($n = 265$)	7.9% (21/265)	0.32	0.76 (0.44 to 1.31)
rs1049654 (on 5'UTR)	(C vs A)		
Healthy subjects ($n = 416$)	26.7% (222/832)	–	–
APS ($n = 123$)	26.8% (66/246)	0.96	1.01 (0.73 to 1.39)
SLE/non-APS ($n = 261$)	28.7% (150/522)	0.41	1.11 (0.87 to 1.41)

P value and OR (95% CI) for each group were obtained by comparison with healthy subjects. OR (95% CI): odds ratio (95% confidence interval); UTR: untranslated region; APS: antiphospholipid syndrome; SLE: systemic lupus erythematosus.

TF (Lifespan Biosciences, Seattle, WA, USA) for 30 minutes at 4°C. Cells were resuspended and fixed in 2% paraformaldehyde (Sigma-Aldrich). Analysis by flow cytometry was performed on an acoustic focusing cytometer (Attune; Applied Biosystems). Gating was accomplished using size, complexity and phycoerythrin gates to define the monocyte population of PBMC.

Statistical analysis

Statistical evaluation was performed by chi square test, Fisher's exact test or Student's *t* test, as appropriate. *P* values less than 0.05 were considered significant.

Results

Allele frequencies of CD36 gene polymorphisms

Allele frequencies of the two CD36 gene polymorphisms were compared among three groups: healthy subjects, APS and SLE in the absence of APS. Minor allele carrier of rs3765187 (C478T Pro90Ser), a missense mutation linked to human CD36 deficiency, was significantly less frequent in APS (3.8%) compared to healthy subjects (10.2%). In contrast, rs3765187 minor allele carrier was as frequent in SLE in the absence of APS as it was in healthy subjects. There was no significant difference in allele frequency of rs1049654, a mutation on the 5' untranslated region, among those groups (Table 3).

Expressions of TF and IL-6 on MPM induced by aPL

Expressions of TF and IL-6 were analyzed on MPM from WT or CD36KO mice cultured with each aPL and its antigen. All three aPL used in this experiment, Pt-aCL, Pt-aPS/PT and 231D, induced TF mRNA expression in MPM up to 10-fold. The aPL-induced TF mRNA expression was significantly reduced in MPM from CD36KO mice compared to MPM from WT mice (Figure 1(a)). Those three aPL induced IL-6 mRNA expression in MPM up to 60-fold. The aPL-induced IL-6 mRNA expression was significantly reduced in MPM from CD36KO mice compared to MPM from WT mice (Figure 1(b)).

TF: tissue factor; IL-6: interleukin 6; aPS/PT: phosphatidylserine-dependent antiprothrombin antibodies; IgG: immunoglobulin G; MPM: mouse peritoneal macrophage; WT: wild-type; aPL: antiphospholipid antibodies; Pt-aCL: purified total IgG from aCL-positive APS patient; Pt-aPS/PT: purified total IgG from aPS/PT-positive APS patient; LPS: lipopolysaccharide.

Expressions of TF and IL-6 on human PBMC induced by aPL

Expressions of TF and IL-6 were analyzed on human PBMC from a healthy donor cultured with each aPL and its antigen. All three aPL used in this experiment, Pt-aCL, Pt-aPS/PT and 231D, induced TF mRNA expression in human PBMC from a healthy donor up to 16-fold. The aCD36 significantly reduced aPL-induced TF mRNA expression in human PBMC. In contrast, equal concentration of mouse IgG1κ isotype control did not reduce it (Figure 2(a)). Those three aPL induced IL-6 mRNA expression in human PBMC from a healthy donor up to 40-fold. The aCD36 significantly reduced aPL-induced IL-6 mRNA expression in human PBMC. In contrast, equal concentration of the mouse IgG1κ isotype control did not reduce it (Figure 2(b)). We next performed analysis by flow cytometry to confirm the TF expression on monocytes. Those three aPL also induced surface TF expression on human

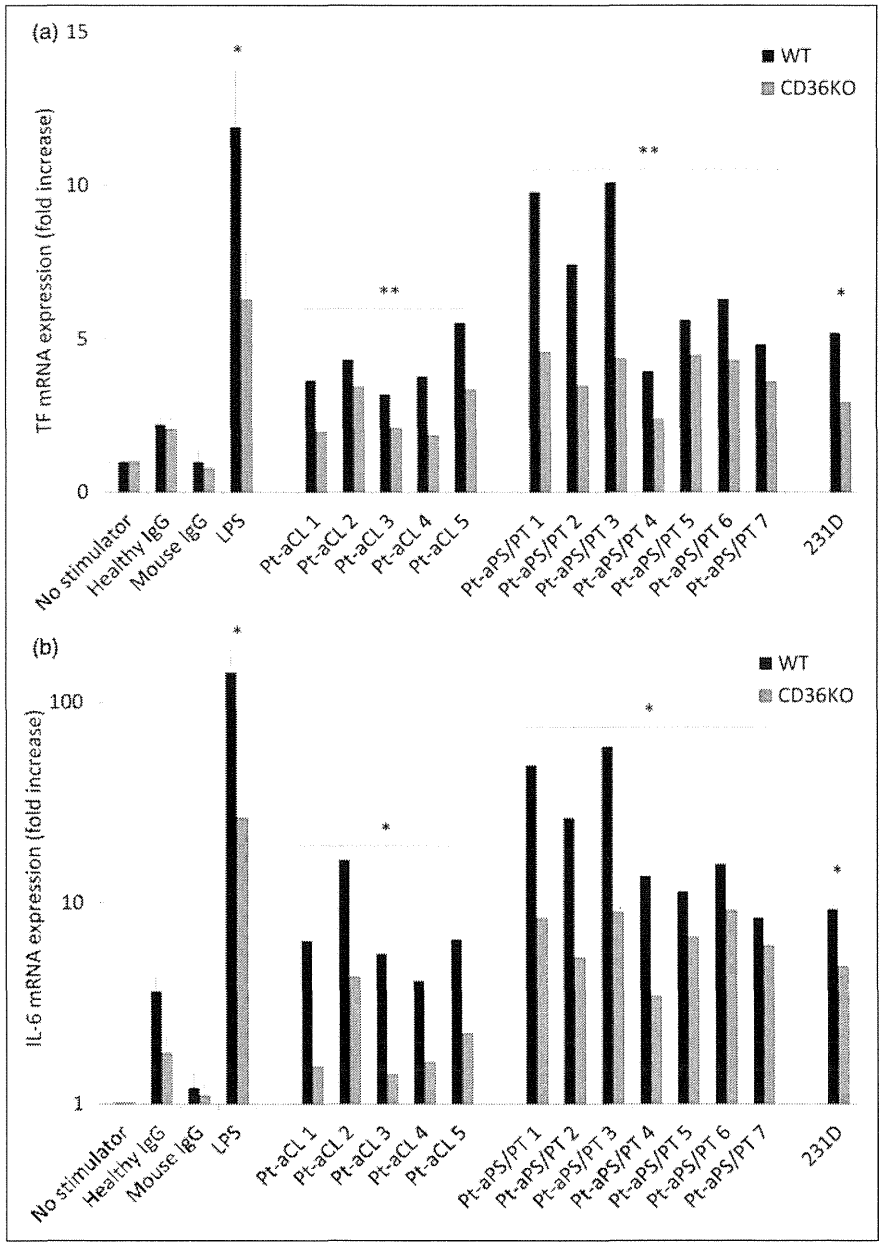

Figure 1 Evaluation of TF (a) and IL-6 (b) mRNA levels induced by aPL in mouse peritoneal macrophages. Expressions of TF and IL-6 mRNA were analyzed in MPM from WT or CD36KO mice cultured with each aPL and its antigen. Healthy-IgG represent the mean of five healthy donors. The mRNA levels induced by Pt-aCL or Pt-aPS/PT are individually indicated for each patient. Values were normalized to expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase and expressed as fold increase in the Y axis. Error bars show standard errors of the mean obtained by five or more experiments. *: $p < 0.05$ and **: $p < 0.01$. P values were obtained by comparison between WT and CD36KO using Student's t test.
TF: tissue factor; IL-6: interleukin 6; aPS/PT: phosphatidylserine-dependent antiprothrombin antibodies; IgG: immunoglobulin G; MPM: mouse peritoneal macrophage; WT: wild-type; aPL: antiphospholipid antibodies; Pt-aCL: purified total IgG from aCL-positive APS patient; Pt-aPS/PT: purified total IgG from aPS/PT-positive APS patient; LPS: lipopolysaccharide.

CD14-positive cells from a healthy donor. The aCD36 reduced aPL-induced surface TF expression on human CD14-positive cells. In contrast, equal concentration of the mouse IgG1κ isotype control did not reduce it (Figure 3).

aCD36: anti-CD36 antibody; TF: tissue factor; IL-6: interleukin 6; IgG: immunoglobulin G; aPS/PT: phosphatidylserine-dependent antiprothrombin antibodies; PBMC: human peripheral blood mononuclear cells; Pt-aCL: purified total IgG from aCL-positive APS patient; Pt-aPS/

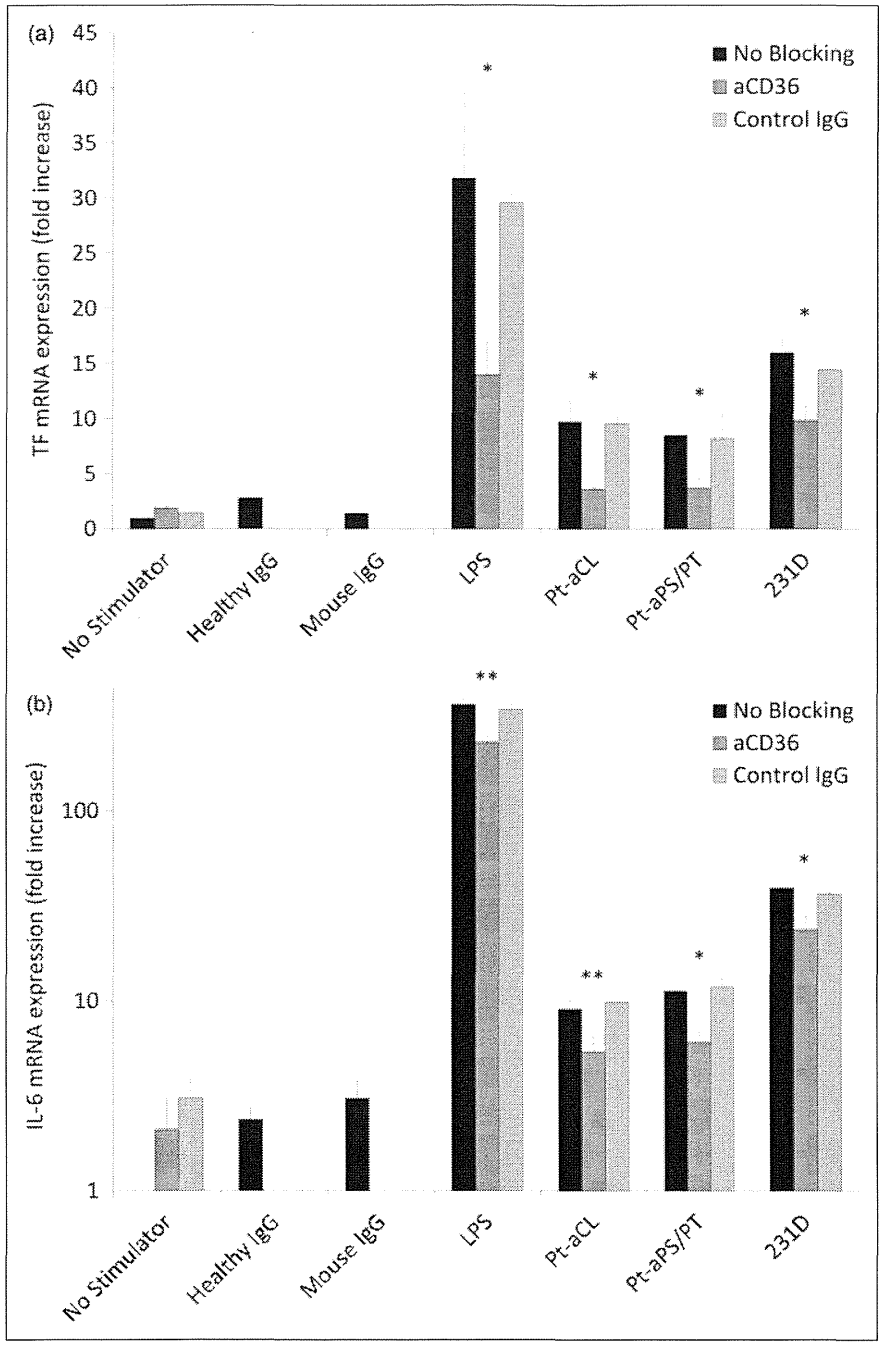

Figure 2 Evaluation of TF (a) and IL-6 (b) mRNA levels induced by aPL in human PBMC. Expressions of TF and IL-6 mRNA were analyzed in human PBMC from a healthy donor cultured with each aPL and its antigen. Healthy-IgG represent the mean of three healthy donors. Pt-aCL and Pt-aPS/PT were both from a patient with primary APS (Pt-aCL5 and Pt-aPS/PT1 shown in Table 2), which induced the highest TF expression in the response of mouse peritoneal macrophages. Values were normalized to expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase and expressed as fold increase in the Y axis. Error bars show standard errors of the mean obtained by three or more experiments. *: $p < 0.05$ and **: $p < 0.01$. P values were obtained by comparison between No blocking and aCD36 using Student's t test.
aCD36: anti-CD36 antibody; TF: tissue factor; IL-6: interleukin 6; IgG: immunoglobulin G; aPS/PT: phosphatidylserine-dependent antiprothrombin antibodies; PBMC: human peripheral blood mononuclear cells; Pt-aCL: purified total IgG from aCL-positive APS patient; Pt-aPS/PT: purified total IgG from aPS/PT-positive APS patient; aPL: antiphospholipid antibodies; APS: antiphospholipid syndrome; LPS: lipopolysaccharide.

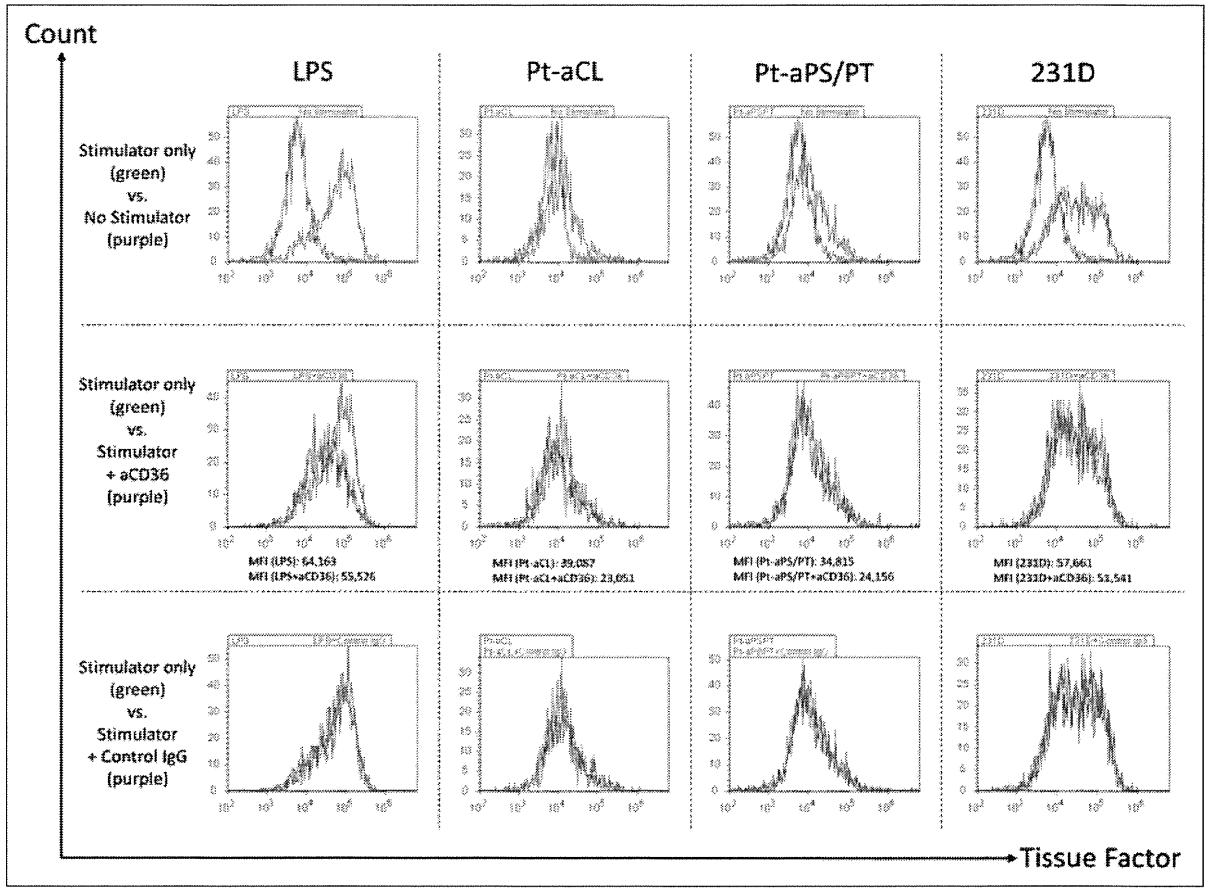

Figure 3 Evaluation of TF protein level induced by aPL on human monocytes. Expression of TF protein was analyzed on human monocytes from a healthy donor cultured with each aPL and its antigen. Histogram plots show the TF expression on CD14-positive cells. Upper, middle and lower column show the comparison of stimulator only (green line) with no stimulator, blocking with aCD36 and blocking with control IgG (purple line), respectively. Pt-aCL and Pt-aPS/PT were both from a patient with primary APS (Pt-aCL5 and Pt-aPS/PT1 shown in Table 2), which induced the highest TF expression in the response of mouse peritoneal macrophages.
aCD36: anti-CD36 antibody; MFI: mean fluorescence intensity; TF: tissue factor; IgG: immunoglobulin G; aPL: antiphospholipid antibodies; Pt-aCL: purified total IgG from aCL-positive APS patient; Pt-aPS/PT: purified total IgG from aPS/PT-positive APS patient; APS: antiphospholipid syndrome; LPS: lipopolysaccharide.

PT: purified total IgG from aPS/PT-positive APS patient; aPL: antiphospholipid antibodies; APS: antiphospholipid syndrome; LPS: lipopolysaccharide.

aCD36: anti-CD36 antibody; MFI: mean fluorescence intensity; TF: tissue factor; IgG: immunoglobulin G; aPL: antiphospholipid antibodies; Pt-aCL: purified total IgG from aCL-positive APS patient; Pt-aPS/PT: purified total IgG from aPS/PT-positive APS patient; APS: antiphospholipid syndrome; LPS: lipopolysaccharide.

Discussion

In this study, we demonstrated that the gene mutation linked to human CD36 deficiency was less frequent in patients with APS and that the deficient or suppressed CD36 function significantly reduced aPL-induced TF/IL-6 expressions in vitro. CD36 may be involved in the thrombotic pathophysiology in patients with APS. A few patients with APS, however, had the gene mutation linked to human CD36 deficiency and knocking out CD36 did not lead to complete diminuendo of aPL-induced TF expression. Taken together, CD36 may be one of the cell surface receptors involved in the pathogenesis of APS.

CD36 resides in lipid raft domains and interacts with a variety of membrane receptors, such as integrin $\alpha 3\beta 1$, $\alpha 6\beta 1$, tetraspanins and TLRs. The latest was elegantly demonstrated on macrophage in studies showing cooperation between CD36 and TLR2 or TLR6 in the recognition and response to

Table 4 Clinical profiles of patients with APS who have minor allele of rs3765187 (C478T Pro90Ser)

No	*nt478T*	*Disease*	*Age-Sex*	*Arterial thrombosis*	*Venous thrombosis*	*Pregnant morbidity*	*LA*	*aCL*	*aβ2GPI*	*aPS/PT*
1	Homo	PAPS	32-F	–	CRVO	–	–	+	–	–
2	Hetero	PAPS	42-F	–	–	Miscarriage	+	–	–	+
3	Hetero	APS-SLE	23-F	Splenic infarct	DVT	–	+	+	+	+
4	Hetero	APS-SLE	24-F	–	DVT	–	+	–	–	–
5	Hetero	APS-SLE	49-F	Stroke	DVT	Miscarriage, eclampsia	+	+	–	+

APS: antiphospholipid syndrome; LA: lupus anticoagulant; F: female; aCL: anticardiolipin antibodies; aβ2GPI: anti-β2 glycoprotein I antibodies; aPS/PT: phosphatidylserine-dependent antiprothrombin antibodies; PAPS: primary antiphospholipid syndrome; CRVO: central retinal vein occlusion; DVT: deep vein thrombosis.

bacteria cell wall components, such as *Staphylococcus*-derived lipoteichoic acid and diacylated lipoproteins.[31,40] Several CD36 functions, including microglial phagocytosis and platelet response, require integrin α3β1, α6β1 or tetraspanins.[41,42]

The mechanism of the thrombotic tendency in APS has been clarified at the molecular level by many investigations. TF upregulation on procoagulant cells is considered to be the most important procedure in the pathogenesis of APS. Elevation of plasma TF level and upregulation of TF expression on monocytes, which was accompanied by an increase in TF pathway inhibitor, were found in patients with APS.[6,43] Elevated plasma level of soluble fibrin and that of D-dimer, which reflects thrombin generation and fibrin turnover, were also found, presumably related to the "chronic" TF upregulation and activation of extrinsic coagulation pathway.[3] In in vitro studies, monocytes and endothelial cells treated with aPL demonstrated upregulation of TF and adhesion molecules.[5,6] NF-κB and p38MAPK were shown to participate in the procoagulant cell activation as intracellular signaling pathways. We[7] and others[8] showed that p38 MAPK protein was phosphorylated with NF-κB activation by aCL/β2GPI treatment and that SB203580, a specific p38 MAPK inhibitor, decreased the aCL/β2GPI-induced TF mRNA expression.

A number of candidates for the cell surface receptor involved in this pathogenesis have been reported.[9–16] Sorice et al.[44] showed the lipid raft recruitment of β2GPI and TLR-4 in human monocytes when interacting with aCL/β2GPI, suggesting that the procoagulant cell activation by aPL may involve the recruitment of cell surface receptors on lipid rafts. Given that CD36 resides in lipid raft domains and interacts with a variety of membrane receptors, our data support those findings and suggest that CD36 interacts with other β2GPI/prothrombin receptors involved in the pathogenesis of APS.

In clinical practice, treatment of APS has focused on utilizing antithrombotic medications such as warfarin, heparin or aspirin. Despite long-term antithrombotic medications, thrombosis can recur in patients with APS and antithrombotic medications can be associated with bleeding.[45] Given that thrombotic events occur only occasionally in patients with APS, aPL increase the thrombophilic threshold as the "first hit," and then clotting takes place only when a "second hit" exists, such as an infection or a surgical procedure.[46] Current antithrombotic medications in APS are directed to modulate the final event or "second hit." However, treatments that modulate the "first hit" would be more beneficial and potentially less harmful than current antithrombotic medications.

Our results suggest that inhibition or reduction of CD36 can be one of the options for the prophylaxis against thrombosis in patients with APS. Treatment targeting CD36 might be safe because heredity CD36 deficiency is not associated with serious clinical manifestations including bleeding disorders, suggesting that CD36 is a strong potential target of the treatment of patients with APS. CD36 expression is regulated by multiple agents on monocytes. It can be upregulated by adhesion, macrophage-colony stimulating factor, granulocyte/macrophage-colony stimulating factor, native and modified LDL, cellular cholesterol, IL-4 and high glucose conditions, while downregulated by corticosteroids, transforming growth factor-β1, high-density lipoprotein and LPS.[47] Statin and cilostazol, medical agents having some pleiotropic effects, were reported to downregulate CD36 expression on monocytes.[48,49] These agents might have implications for treatment of APS.

Given that CD36 deficiency may be protective for developing APS, we suspected some specific clinical features in patients with APS who have

the minor allele rs3765187. In our study, one patient with APS who carried the homozygous minor allele of rs3765187 exhibited central retinal vein occlusion as a sole APS manifestation and had aCL as a sole aPL; on the other hand, four heterozygous carriers exhibited typical APS manifestations and serological abnormalities (Table 4). Further studies will better delineate the correlation between minor allele of rs3765187 and severity of APS manifestations.

In conclusion, both genetically and biologically, our results suggest that in a susceptible background CD36 scavenger receptor function may be involved in the thrombotic pathophysiology in patients with APS.

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Conflict of interest

The authors have no conflicts of interest to declare.

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Prediction of Response to Treatment by Gene Expression Profiling of Peripheral Blood in Patients with Microscopic Polyangiitis

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Abstract

The JMAAV study was an open-labeled prospective clinical trial, which proposed severity-based treatment protocols for patients with microscopic polyangiitis (MPA). The results suggest that the proposed protocols are useful (remission rate: 89.4%), but are also indicative of relapse or patient demise regardless of the treatment (recurrence rate: 19.0%; mortality rate: 10.6%). The aim of this study is to develop the method to predict response to the treatment in patients with MPA. In the present study, transcriptome analysis was performed using peripheral blood from patients enrolled in the JMAAV study before and 1-week after the beginning of treatment. The gene expression profile before treatment was not directly related to the response to the treatment. However, when the samples from 9 patients with good response (persistent remission for 18 months) were examined, the expression of 88 genes was significantly altered by the treatment. Thirty statistically reliable genes were selected, and then the alteration of expression by the treatment was examined among 22 patients, including 17 with good response, which was defined as persistent remission for 18 months and 5 with poor response, which was defined as relapse after remission or no remission. Discrimination analysis between the alteration of expression of the 30 genes by the treatment and the response identified a combination of 16 genes as the most valuable gene set to predict the response to the treatment. This preliminary study identified IRF7, IFIT1, IFIT5, OASL, CLC, GBP-1, PSMB9, HERC5, CCR1, CD36, MS4A4A, BIRC4BP, PLSCR1, DEFA1/DEFA3, DEFA4, and COL9A2 as the important genes that can predict the response to the treatment in patients with MPA at an early point during the therapy.

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Introduction

The spectrum of anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis (AAV) includes microscopic polyangiitis (MPA), eosinophilic granulomatosis with polyangiitis (EGPA, Churg-Strauss syndrome), and granulomatosis with polyangiitis (GPA, Wegener's granulomatosis) [1]. The two major antigens of ANCA are myeloperoxidase (MPO) [2] and proteinase 3 (PR3) [3]. MPO-ANCA is often detected in the sera of patients with MPA and EGPA; while, PR3-ANCA is a useful marker for GPA. Although it remains unsolved why ANCA is produced, immunological mechanisms are considered to be involved in the development of AAV. Therefore, corticosteroids and immunosuppressive agents have been used as treatments for AAV. Based on previous clinical trials, the standard protocol of treatment for AAV was established in Western countries [4–6].

The prevalence of MPA is strikingly higher in Japanese population compared to the Caucasoid [7]. Accordingly, clinical trials to establish a guideline for the management of patients with this subtype of AAV should be held in Japan. Ozaki and colleagues instituted a Japanese study group for MPA and conducted an open-labeled prospective clinical trial, the JMAAV study (The University Hospital Medical Information Network, Clinical Trials Registry; http://www.umin.ac.jp/ctr/index-j.htm, registration number ID 000000867) [8]. In the JMAAV study, patients newly diagnosed with MPA were stratified into 3 categories based on

disease severity, including mild form, severe form, and most severe form. The mild form included patients with slight disorder of one or more organs, renal-limited type (except for rapidly progressive glomerulonephritis (RPGN)), and pulmonary-limited type (except for pulmonary hemorrhage). The severe form included patients with generalized type (MPA with involvement of more than 2 organs), pulmo-renal type (glomerulonephritis plus either limited pulmonary hemorrhage or extended interstitial pneumonia), and RPGN type. The most severe form included patients with diffuse alveolar hemorrhage, intestinal perforation, acute pancreatitis, cerebral hemorrhage, or concurrent presence of anti-glomerular basement membrane antibodies. This form also included patients with the severe form who were resistant to the severity-based treatment protocol described below.

After the establishment of diagnosis, the patients were treated according to the following protocols. 1) Mild form: Low-dose corticosteroids (0.3–0.6 mg/kg/day) were administered. Oral immunosuppressive agents (cyclophosphamide or azathioprine, 0.5–1.0 mg/kg/day or 25–75 mg/day, respectively) were optional. 2) Severe form: High-dose corticosteroids (0.6–1.0 mg/kg/day) and oral cyclophosphamide (0.5–2.0 mg/kg/day) were given. Intravenous methylprednisolone (0.5–1.0 g/day for 3 days) was considered as an alternative. Instead of oral administration, the use of intravenous cyclophosphamide (0.5–0.75 g/m^2 monthly) was also allowed. 3) Most severe form: Plasmapheresis (2.0–3.0 L/day for 3 days) was employed together with the regimen for the severe form described above.

Fifty-two patients were registered to the JMAAV study, but 4 were excluded due to the exclusive prescriptions. The remaining 48 patients were divided into the mild form (n = 23), severe form (n = 23), and most severe form (n = 2) groups. Treatment was administered according to the stated protocol. They were followed-up for 18 months. Since 1 patient in the mild form was lost to follow-up within 6 weeks, the study population for further analysis consisted of the remaining 47 patients. Remission, which was defined as the absence of clinical manifestations of active vasculitis (Birmingham Vasculitis Activity Score 2003: 0 or 1 point), was achieved in 42 out of 47 patients (remission rate: 89.4%). Among the 42 patients, 8 patients showed relapse of the disease (recurrence rate: 19.0%). Relapse was defined as the recurrence or development of at least one manifestation of vasculitis. The involvement of each organ was diagnosed as described elsewhere [8]. Ultimately, 5 of the 47 patients died (mortality rate: 10.6%).

These results suggest that the proposed severity-based protocols are applicable for patients with MPA, but the possibility of relapse is indicated and, in the worst scenario, death may occur regardless of the treatment. We considered that if the response to the treatment would be predicted prior to the beginning of treatment or at an early point during the therapy, careful follow-up or application of additional regimens to the treatment could expectedly improve the outcome.

Materials and Methods

Ethics statement

Peripheral blood was obtained from patients with written informed consent in accordance with the Declaration of Helsinki. The use of human materials was permitted by the Institutional Clinical Research Committee in Hokkaido University Hospital (No. 0903-0398).

Patient cohorts and blood samples

The list of patients registered to the JMAAV study is shown in Table 1. Peripheral blood samples (10 mL) were obtained from 39 out of the 47 patients with MPA before and 1-week after the beginning of treatment. Total RNAs were extracted using PAXgene Blood RNA System (BD, Franklin Lakes, NJ). Among the 39 pairs of blood samples, 5 pairs did not suit for the following assay because of poor quality or low amount of the extracted RNAs. The 34 patients with paired RNA samples were randomly divided into 2 cohorts, namely Cohort 1 and Cohort 2. The comparison of clinical characteristics between the 2 cohorts, including age, sex, and serum levels of creatinine and MPO-ANCA, is summarized in Table 2. Although the gender distribution seemed to be imbalance between the 2 cohorts, there was no statistically significant difference ($p = 0.0543$ in Fisher's exact test). In addition, age and serum levels of creatinine and MPO-ANCA were equivalent between the 2 cohorts ($p = 0.3077$, $p = 0.5055$, and $p = 0.7026$, respectively, in Mann-Whitney *U*-test). Since there was no significant gender difference in age and serum levels of creatinine and MPO-ANCA between male and female genders in Cohort 1 and Cohort 2 (Table S1), the imbalance of gender between the 2 cohorts, if any, was not likely to be a problem.

Cohort 1. The blood samples from this cohort were subjected to gene chip analysis in order to discover genes relevant to the response to the treatment. This cohort included 6 patients categorized into the mild form, 5 patients into the severe form, and 1 patient into the most severe form. Remission was achieved in 11 patients, but the disease relapsed in 2 of them. The time of relapse is shown in Table 1. Remission was not achieved in 1 patient. In the present study, persistent remission for 18 months was regarded as good response. On the other hand, relapse or no induction of remission was regarded as poor response. Accordingly, this cohort included 9 patients with good response and 3 patients with poor response.

Cohort 2. The blood samples from this cohort were subjected to quantitative expression analysis concerning the genes listed in Cohort 1. For this purpose, low density array technology was applied. Subsequently, data mining was performed to identify the most valuable genes to predict the response to the treatment. This cohort included 11 patients categorized into the mild form, 10 patients into the severe form, and 1 patient into the most severe form. Remission was achieved in 21 patients, but the disease relapsed in 4 of them. Remission was not achieved in 1 patient. Accordingly, this cohort included 17 patients with good response and 5 patients with poor response.

Gene chip analysis

GeneChip Human Genome Focus Array (Affymetrix, Santa Clara, CA) was used. This gene chip was equipped with 8,793 genes related to inflammation and immune response [9]. Raw data of all samples were imported into GeneChip Operating Software (Affymetrix). Each signal value was pre-normalized by MAS5.0 method, which is the manufacturer's recommended method for pre-normalization of the array data. After the pre-normalization, the array data were imported into GeneSpring GX7.3.1 Software (Agilent Technologies, Santa Clara, CA). Signal values less than 0.01 were regarded as 0.01 because minus signals are nonsense in biology. In this procedure, 0.01 was employed instead of 0 toward operation in which the corrected values would be used as denominators. For normalization per chip, each signal value was divided by the 50th percentile value in the chip. For the specific detection of each gene, the gene chip was equipped with 11 to 20 probe pairs per gene, including perfect match (PM) probes and

Table 1. Patients registered to JMAAV study.

Case No.*	Age/Sex	Disease form (Involved organs**)	Weeks for remission	Relapse	Time of relapse (months)	Response	Cohort
1	72/M	severe (L/K/N)	6	–		good	
2	75/F	severe (L/K)	6	–		good	1
3	64/M	excluded					
4	76/F	severe (E/L/K/N)	6	–		good	
5	73/F	mild (N)	6	–		good	1
6	no record	mild (M)	6	–		good	
7	no record	severe (K/S)	6	–		good	1
8	62/F	mild (M)	not achieved			poor (dead[1])	1
9	57/F	mild (L)	6	–		good	1
10	84/F	excluded					
11	72/F	mild (L/M)	6	+	9	poor	
12	73/M	mild (L/K/S)	6	–		good	2
13	77/F	mild (B/N)	24	+	9	poor	1
14	62/F	severe (K)	6	–		good	1
15	74/F	severe (L/K)	6	+	6	poor	1
16	57/M	severe (L/K/N)	6	+	6	poor	2
17	78/M	mild (K)	6	–		good	2
18	70/F	severe (L/K/N)	6	+	6	poor (dead[2])	2
19	51/F	severe (K)	6	–		good	2
20	60/F	mild (L)	6	–		good	2
21	71/F	most severe (B/L/K/I)	not achieved			poor (dead[3])	2
22	68/M	severe (K)	6	–		good	2
23	75/F	mild (K/N/J)	6	–		good	2
24	76/F	mild (K/N)	6	–		good	2
25	72/M	severe (L/K/N)	12	–		good	2
26	67/M	mild (N/S/M)	6	–		good	2
27	70/M	mild (L/M)	6	–		good	2
28	45/F	mild (K)	dropped				
29	76/M	severe (K)	6	–		good	
30	71/M	severe (K)	6	–		good (dead[4])	
31	69/F	mild (K/N)	6	–		good	2
32	72/F	severe (L/K/N)	6			dead[5]	
33	64/F	excluded					
34	62/M	severe (E/L/K)	6	–		good	2
35	58/F	mild (L/H)	6	–		good	
36	79/F	mild (L/K)	6	–		good	2
37	58/F	severe (L/K)	12	–		good	2
38	63/F	severe (K/N/M/Li)	6	–		good	2
39	71/F	severe (K)	not achieved	dropped			
40	56/M	severe (K)	not achieved			poor[6]	
41	70/M	severe (K/N/S)	12	–		good	2
42	68/M	severe (K)	12	–		good	2
43	74/M	severe (L/K/N)	12	dropped			
44	80/F	excluded					
45	75/F	mild (L/N/M)	6	+	9	poor	2
46	64/F	mild (L)	12	+	7	poor	2
47	26/F	mild (B/K/N/S/M/J)	6	–		good	1
48	62/M	severe (L/K/N/M)	6	–		good	1
49	55/F	mild (E/L/N/S)	6	–		good	1

Table 1. Cont.

Case No.*	Age/Sex	Disease form (Involved organs**)	Weeks for remission	Relapse	Time of relapse (months)	Response	Cohort
50	62/F	most severe (L/K/N)	6	−		good	1
51	58/F	mild (L/K/J)	6	+	6	poor	
52	65/M	mild (K/N)	6	−		good	

Patients died by.
[1]Interstitial pneumonia at 3 months,
[2]opportunistic infection at 11 months,
[3]cerebral bleeding at 9 days,
[4]respiratory failure without relapse at 10 months, and
[5]cerebral bleeding due to atherosclerosis at 10 weeks.
[6]Persistent hemodialysis was introduced to this patient from 1 week after diagnosis.
*The case numbers are different from the patient numbers in Takakuwa *et al.* paper (Takakuwa *et al.* Arthritis Rheum 63:3613–3624, 2011).
**Letters represent organs as follows; B: brain, E: eye/ear/nose, L: lung, K: kidney, N: peripheral nervous system, S: skin, M: muscle, I: intestine, J: joints, and Li: liver/gallbladder/pancreas.
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mismatch (MM) probes. The sequence of the paired PM and MM probes was identical, except for a change to the Watson-Crick complement in the middle of the MM probe sequence. For normalization per gene, signal values of PM probes were divided by the median value of the signal of MM probes.

Low density array analysis

The real-time RT-PCR-based TaqMan Low Density Array (Applied Biosystems, Carlsbad, CA) was applied to quantify the expression of 30 genes listed by the gene chip analysis (Table 3). The accession numbers in Table 3 belonged to GenBank repository. The low density array data were analyzed as follows. First, the expression level of the target gene was standardized by the expression level of the house-keeping β-actin gene. For this purpose, the Ct value of real-time PCR was applied. The Ct value represents the cycle number in which the PCR products reach the threshold level [10]. The expression level of the target gene was shown as ΔCt (ΔCt = Ct value of the target gene − Ct value of the β-actin gene). Next, the changed amount of expression of the target gene by the treatment was shown as ΔΔCt (ΔΔCt = ΔCt 1-week after the beginning of treatment − ΔCt before treatment). It is considered that when ΔΔCt is 1, the expression level of the target gene before treatment is 2-fold higher than 1-week after the beginning of treatment. Accordingly, when the expression level of the target gene before treatment is set as 1, the fold expression of the target gene 1-week after the beginning of treatment is shown as $2^{-\Delta\Delta Ct}$. Subsequently, the outcome was replaced by a dummy number; wherein, "good outcome (persistent remission)" was regarded as 0 and "poor outcome (relapse after remission or no remission)" as 1.

After these preparations, discrimination analysis was conducted concerning 22 patients in Cohort 2 using the fold expression of 16 genes randomly extracted from the 30 genes. In this analysis, the influence of the target gene on the prediction of the response to the treatment was calculated. Thereafter, the gene which showed the minimum influence on the prediction was replaced by another gene in the remaining 14 genes. This operation was repeated until all genes were used. Up to this, 15 combinations of genes consisting of 16 genes were generated. Subsequently, the gene with the minimum influence on the prediction was excluded one by one until the last gene remained, which resulted in the generation of another 15 combinations of genes consisting of diverse number of genes (15-1). In total, 30 combinations of genes (model #1 – model #30) were generated, and then were examined for prediction of the response to the treatment. In order to identify the most adequate model among the 30 combinations, Akaike Information Criterion (AIC) was applied. AIC is one of the suitable indices to evaluate such a model, and the algorithm showing the smallest AIC value is considered to be the most desirable [11,12].

Lastly, in order to identify the best predictors of the response to the treatment among the genes listed in Table 3, logistic analysis was conducted concerning 22 patients in Cohort 2, including 17 with good response and 5 with poor response. Discrimination analysis and logistic analysis were performed using the add-in Excel software 2012 (SSRI, Tokyo, Japan).

Table 2. Comparison of clinical characteristics between cohorts.

Clinical characteristics	Cohort 1	Cohort 2	p-value
Age	62.3±14.2	68.0±7.2	0.3077*
Sex (M/F/no record)	1/10/1	10/12/0	0.0543**
Serum creatinine (mg/dL)	1.73±1.79	1.84±1.38	0.5055*
Serum MPO-ANCA (U/mL)	334.9±291.2	293.5±211.7	0.7026*

*Mann-Whitney *U*-test.
**Fisher's exact test.
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Results

Comparison of gene expression profile before treatment and response of treatment

First, the gene expression profile before treatment was compared among patients in Cohort 1 with good response (persistent remission for 18 months, n = 9) versus with poor response (relapse after remission or no remission, n = 3). No gene showed a significant difference in expression among the patients; thereby, no correlation was indicated between the gene expression profile before treatment and the response.

Alteration of gene expression profile by treatment

When the gene expression profile obtained from 9 patients with good response in Cohort 1 was compared between before and 1-

Table 3. List of 30 genes equipped with low density array.

Alteration	p-Value	Gene symbol	Description	Accession No.*
Decrease	0.00441	CLC	Charcot-Leyden crystal protein	NM_001828
Decrease	0.00692	GBP1	Guanylate binding protein 1, interferon-inducible, 67 kDa	NM_002053
Decrease	0.00692	NGFRAP1	Nerve growth factor receptor (TNFRSF16) associated protein 1	NM_206917 NM_206915 NM_014380
Decrease	0.00692	IFIT1	Interferon-induced protein with tetratricopeptide repeats 1	NM_001548
Decrease	0.00696	PSMB9	Proteasome (prosome, macropain) subunit, beta type, 9 (large multifunctional peptidase 2)	NM_002800
Decrease	0.0106	CCR3	Chemokine (C-C motif) receptor 3	NM_001837 NM_178329
Decrease	0.0149	TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	NM_003810
Decrease	0.0149	MX1	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	NM_002462
Decrease	0.0149	HERC5	Hect domain and RLD 5	NM_016323
Decrease	0.0160	IFIT3	Interferon-induced protein with tetratricopeptide repeats 3	NM_001549 NM_001031683
Decrease	0.0200	IRF7	Interferon regulatory factor 7	NM_001572 NM_004029 NM_004031
Decrease	0.0200	OAS1	2′,5′-oligoadenylate synthetase 1, 40/46 kDa	NM_016816 NM_002534 NM_001032409
Decrease	0.0206	CCR1	Chemokine (C-C motif) receptor 1	NM_001295
Decrease	0.0234	CD36	CD36 antigen (collagen type I receptor, thrombospondin receptor)	NM_001001548 NM_001001547 NM_000072
Decrease	0.0234	MS4A4A	Membrane-spanning 4-domains, subfamily A, member 4	NM_024021 NM_148975
Decrease	0.0234	IFIH1	Interferon induced with helicase C domain 1	NM_022168
Decrease	0.0234	IFIT5	Interferon-induced protein with tetratricopeptide repeats 5	NM_012420
Decrease	0.0234	EMR1	Egf-like module containing, mucin-like, hormone receptor-like 1	NM_001974
Decrease	0.0278	OAS2	2′-5′-oligoadenylate synthetase 2, 69/71 kDa	NM_016817 NM_002535 NM_001032731
Decrease	0.0278	BIRC4BP	XIAP associated factor-1	NM_017523 NM_199139
Decrease	0.0284	OAS3	2′-5′-oligoadenylate synthetase 3, 100 kDa	NM_006187
Decrease	0.0303	MMD	Monocyte to macrophage differentiation-associated	NM_012329
Decrease	0.0324	HIST1H3H	Histone 1, H3h	NM_003536
Decrease	0.0324	PLSCR1	Phospholipid scramblase 1	NM_021105
Decrease	0.0324	MT2A	Metallothionein 2A	NM_005953
Decrease	0.0418	OASL	2′-5′-oligoadenylate synthetase-like	NM_003733 NM_198213
Increase	0.0234	COL9A2	Collagen, type IX, alpha 2	NM_001852
Increase	0.0324	DEFA4	Defensin, alpha 4, corticostatin	NM_001925
Increase	0.0324	VSIG4	V-set and immunoglobulin domain containing 4	NM_007268
Increase	0.0490	DEFA1 DEFA3	Defensin, alpha 1 Defensin, alpha 3, neutrophil-specific	NM_004084 NM_005217

*GenBank.

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week after the beginning of treatment, 88 genes showed a statistically significant alteration (Figure 1). Among the 88 genes, the expression of 66 genes was significantly decreased, while expression of the other 22 genes was significantly increased by the treatment. On the other hand, no gene in the peripheral blood showed a significant alteration when the samples from 3 patients with poor response in Cohort 1 were examined (data not shown). Although the sample number was limited, these findings suggest the possibility that the response to the treatment may be predicted based on the characteristic alteration of gene profile in the peripheral blood at an early point during the therapy.

Identification of most valuable genes to predict response to treatment

In order to identify the most valuable genes to predict the response to the treatment, 30 statistically reliable genes were selected from the 88 genes. The list of the 30 genes is shown in Table 3. These 30 genes included 26 genes significantly decreased and 4 genes significantly increased by the treatment. Next, peripheral blood samples obtained from 22 patients in Cohort 2 were subjected to discrimination analysis using the low density array. Data mining indicated that model #13 showed the minimum AIC value, which meant that the model contained the most valuable genes to predict the response to the treatment

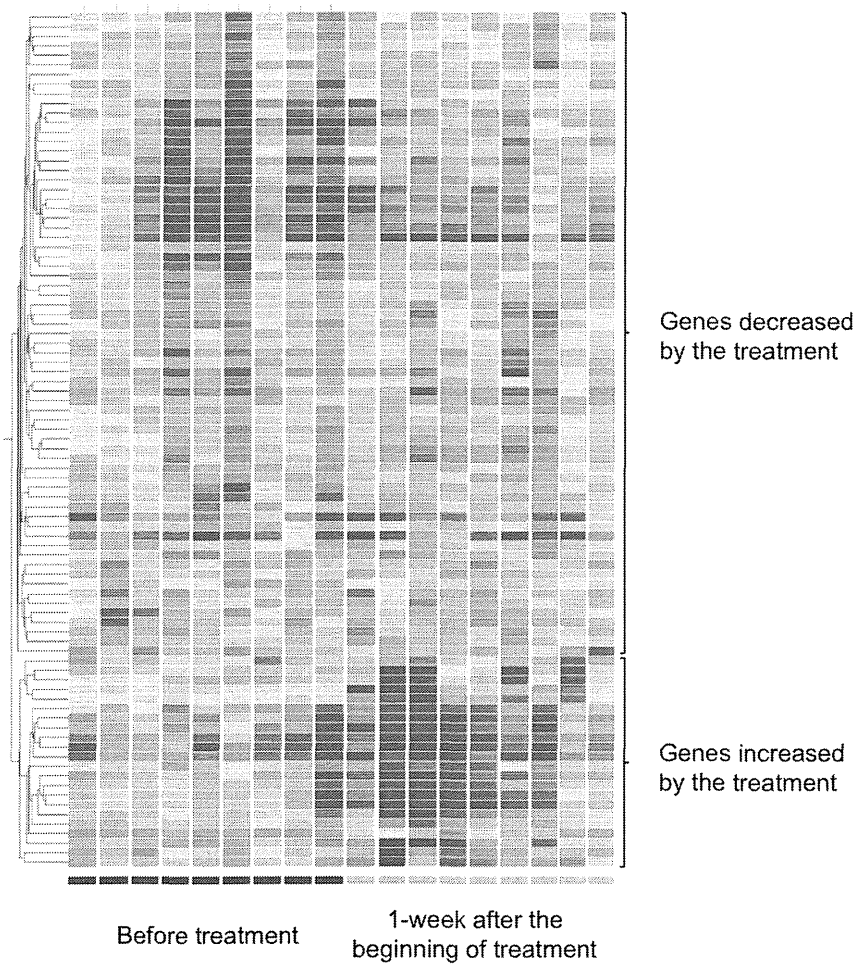

Figure 1. Gene chip analysis of peripheral blood samples obtained from MPA patients (n = 9) with good response (persistent remission) in Cohort 1. The normalized signal values of each gene were compared between before and 1-week after the beginning of treatment. First, genes that showed more than 1.5-fold change in expression level between before and 1-week after the beginning of treatment were extracted. Next, genes that exhibited a significant difference between before and 1-week after the beginning of treatment ($p<0.05$ in Student's *t*-test assisted by the Benjamin and Hochberg False Discover Rate (FDR) of 0.05) were distilled. As a result, 88 genes were nominated as indicators that reflected a characteristic alteration in expression by the treatment (fold change>1.5, $p<0.05$ in Student's *t*-test with FDR of 0.05). Hierarchical clustering analyses (Similarity measure: Person correlation, Clustering algorithm: Average linkage) were performed concerning the 88 genes and 18 samples from 9 cases.
doi:10.1371/journal.pone.0063182.g001

(Figure 2). The model #13 contained 13 genes decreased and 3 genes increased by the treatment. The 13 genes are as follows: interferon (IFN) regulatory factor 7 (IRF7); IFN-induced protein with tetratricopeptide repeats 1 (IFIT1); IFIT5, 2′-5′-oligoadenylate synthetase-like (OASL); Charcot-Leyden crystal protein (CLC); guanylate binding protein 1 (GBP-1); proteasome (prosome, macropain) subunit, beta type, 9 (PSMB9); hect domain and RLD 5 (HERC5); chemokine (C-C motif) receptor 1 (CCR1); CD36; membrane-spanning 4-domains, subfamily A, member 4 (MS4A4A); XIAP-associated factor-1 (BIRC4BP); and phospholipid scramblase 1 (PLSCR1). The 3 genes increased by the treatment included defensin α1 and α3 (DEFA1 and DEFA3), defensin α4 (DEFA4), and collagen type IX α2 (COL9A2).

Moreover, in the logistic analysis concerning the genes listed in Table 3, PLSCR1, one of the genes nominated by the discrimination analysis, was identified as the best predictor of the response to the treatment in MPA patients.

Discussion

This pilot study discovered, for the first time, a set of genes that potentially indicates the response to the treatment in patients with MPA at an early point during the therapy. The gene set consisted of 16 genes present in peripheral blood, including IRF7, IFIT1, IFIT5, OASL, CLC, GBP-1, PSMB9, HERC5, CCR1, CD36, MS4A4A, BIRC4BP, PLSCR1, DEFA1/DEFA3, DEFA4, and COL9A2.

Over the past decade, transcriptome analysis has been energetically performed to identify both diagnostic and prognostic biomarkers of diseases. This has been very successful in the field of oncology, in which gene expression signatures of neoplasm are well associated with the biological behavior and response to treatment. Transcriptome analysis also provides insight into the underlying molecular pathology of the neoplasm [13]. In immune-mediated diseases, peripheral blood samples, e.g., total leukocytes and peripheral blood mononuclear cells (PBMCs), instead of biopsy specimens from the neoplasm have been used for examining gene expression profile [14]. However, unlike oncol-

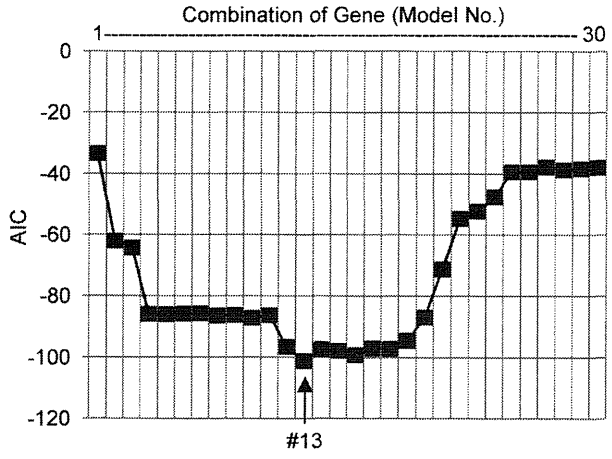

Figure 2. Identification of the most valuable genes to predict the response to the treatment in patients with MPA. Among 88 genes that showed a significant alteration in expression by the treatment, 30 statistically reliable genes were selected for further investigation using TaqMan Low Density Array. Discrimination analysis was conducted concerning 22 patients in Cohort 2, including 17 patients with good response (persistent remission) and 5 patients with poor response (relapse after remission or no remission) as described in the section of Materials and Methods. During the procedure, 30 combinations of genes (model #1 - model #30) were examined for prediction of the response to the treatment. In order to identify the most adequate model among the 30 models, AIC was applied. The model that exhibited the minimum AIC value (model #13) was regarded as the most adequate model, including the most valuable genes to predict the response to the treatment.
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ogy, results are sometimes elusive. Since there are plural subsets of cells in peripheral blood, the gene signatures, if any, might be compensated when total leukocytes or PBMCs are subjected to the analysis. In addition, the timing of blood sampling might influence the results. Recently, Lyons and colleagues reported that transcriptome analysis of leukocyte subsets, but not PBMCs, enabled the identification of gene signatures of AAV [15]. Similarly, Mckinney *et al.* reported that the $CD8^+$ T cell transcription signature could predict prognosis in autoimmune diseases, including AAV [16]. In the present study, although no correlation was determined between the gene profile of peripheral blood obtained from patients with MPA before treatment and the outcome of the treatment, a characteristic alteration of the gene profile by the treatment at an early point during the therapy was revealed. It seemed likely that the addition of an external factor, that is treatment, made the gene signatures clearer, and that the timing of blood sampling (1-week after the treatment) was appropriate for detection of the signatures.

The 16 genes identified in the present study included 13 genes significantly decreased and 3 genes significantly increased by the treatment. The interaction between the genes that showed significant alteration of expression by the treatment and the pathogenesis of MPA should be considered. It should also be considered whether the alteration of gene expression reflected the effects of the treatment. The 13 genes significantly decreased by the treatment included some IFN-related genes, such as IRF family genes, IFIT family genes, and OAS family genes. These genes are closely related to type 1 IFN and are critically implicated in the pathogenesis of systemic lupus erythematosus (SLE) [17–19]. However, it has been reported that the IFN signature of $CD4^+$ T cells is not apparent in MPA patients compared with SLE patients [15]. Therefore, the decreased expression of IFN-related molecules, such as IRF7, IFIT1, IFIT5, and OASL, in the present study is unlikely to reflect the pathogenesis of MPA. These IFN-related molecules are mainly expressed in monocytes. Although the number of monocytes was not measured in this study, the reduction of monocytes could be achieved by the treatment. Similarly, the reduction of the gene that codes for CLC protein was regarded as a result of the treatment. CLC proteins are mainly expressed in eosinophils, and the number of eosinophils is rapidly reduced by corticosteroid treatment. These issues should be confirmed in future studies by examining whether the numbers of monocytes and eosinophils in peripheral blood would be actually decreased by the treatment. However, the adequate reduction of the IFN-related molecules and CLC proteins in peripheral blood could be critical for good response to the treatment. The decrease in expression of proinflammatory genes, such as IFN-related molecules and CLC, after starting the therapy in MPA patients with good response may simply indicate the individual strength of anti-inflammatory response to the treatment employed according to the protocols. In other words, stronger immunosuppressants might be needed for patients with poor response.

The genes increased by the treatment included defensins. The induction of defensin genes is also interpreted as the effect of the treatment because circulating neutrophils that express defensins are transiently increased by corticosteroids. Interestingly, the mRNA expression of defensins was up-regulated in peripheral blood in SLE patients, but it was reduced by corticosteroid therapy [20,21]. Therefore, the contradictory response of the defensin genes to corticosteroid therapy between SLE patients and MPA patients possibly suggests the difference in the pathogenesis of these diseases.

The discrimination analysis nominated 16 genes as distinguished indicators, of which alteration of expression at an early point during the therapy was related to the response to the treatment. Since the number of genes (16) is too much for routine clinical analysis, further efforts to extract the best predictors among them are needed. As a trial for this purpose, logistic analysis was conducted independently to the discrimination analysis. The result indicated PLSCR1, one of the genes nominated by the discrimination analysis, as the sole statistically significant predictor at this time. This result, namely the extraction of a single gene, might be related to the limitation of this study with small sample size. We expect that further investigations with more samples would extract the best predictors among the 16 genes, including PLSCR1.

It remains elusive whether differences in treatment, including oral corticosteroids, intravenous methylprednisolone, immunosuppressive agents, and plasmapheresis, have an effect on the results of gene expression in this study. Further sub-analysis is not feasible due to the limited number of patients. However, it is considered that sufficient alteration of the nominated genes suggests the appropriate strength of the treatment. Thus, the relative intensity of treatment against the disease activity, but not the modality of treatment itself, could affect the results of gene expression in this study.

The remaining challenge is the validation of the results. In order to validate the practical significance of the prediction using the 16 genes nominated in this study, as well as to establish a guideline for the management of patients with MPA, larger clinical trials should be conducted.

Supporting Information

Table S1 Comparison of clinical characteristics between male and female genders in Cohort 1 and 2.
(DOC)

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Author Contributions

Conceived and designed the experiments: AI T. Yoshiki SO. Performed the experiments: AI TM T. Yamamoto. Analyzed the data: AI UT TM T. Yamamoto. Contributed reagents/materials/analysis tools: TA WY KY HY SK MSK MS HM SO. Wrote the paper: AI UT TM T. Yamamoto TA.

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Frequency of recurrent spontaneous abortion and its influence on further marital relationship and illness: The Okazaki Cohort Study in Japan

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Abstract

Aims: The aim of this study was to examine the influence of recurrent spontaneous abortion (RSA) on marital relationships, and the association between past/present illness and RSA.

Material and Methods: A total of 2733 Japanese women who underwent a medical examination responded to the questionnaire.

Results: The frequency of recurrent miscarriage and two or more consecutive RSA were 0.88% and 4.2%, respectively. Women with a history of miscarriages (hazard ratio: 1.596) and RSA (hazard ratio: 3.103) were at a higher risk of their relationships ending as compared with the women without a history of miscarriage. Existence of a relation was seen between a history of RSA and the occurrence of gastric ulcer, gastritis, fatty liver, and atopic dermatitis. Overall, 89.5% of the women with RSA experienced cumulative live births.

Conclusions: Miscarriage was found to be a severe life event with an influence on marital relationships, and to be associated with an elevated risk of gastric disease or atopic dermatitis.

Key words: atopic dermatitis, gastric ulcer, marital relationship, recurrent miscarriage, recurrent spontaneous abortion.

Introduction

Miscarriage is the most common complication during pregnancy, and a sporadic miscarriage is defined as a single or a maximum of two episodes of spontaneous pregnancy loss prior to the completion of 20 weeks' gestation. The estimated incidence is 12–16%, and mainly depends on the woman's age.[1] Recurrent miscarriage (RM) is conventionally defined as three or more miscarriages, and the estimated incidence is about 1%.[2] Some clinical researchers define two or more miscarriages as recurrent spontaneous abortion (RSA). Established causes of RSA are the presence of antiphospholipid antibodies (aPL), uterine anomalies, and abnormal chromosomes in either partner, particularly translocations;[3–5] chromosomal abnormalities in the embryo have also been reported as a causative factor of RSA.[6] However, in some cases, RSA remains unexplained.

Recently, a prospective population-based cohort study reported that women who experienced pregnancy loss are at a substantially higher risk of developing myocardial infarction.[7] The authors stated that RM and stillbirth should be considered as important factors in cardiovascular risk monitoring and in the adoption of preventive measures against cardiovascular diseases. It is speculated that recurrent pregnancy loss, which includes RM and/or stillbirth, is associated with

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an elevated risk of cardiovascular disease mediated by aPL. Diagnostic criteria of the antiphospholipid syndrome include pregnancy complications, such as RM, intrauterine fetal death, and arterial/venous thrombophilia.[8] There is limited epidemiological information concerning the association between miscarriage and the history of past/present illness, although such population-based cohort studies have the potential to clarify the causes of miscarriage.

We conducted a population-based cohort study to examine the frequency of at least one miscarriage and RSA, and their influence on marital relationships, and also the association between miscarriage and the history of past/present illness in the Japanese population.

Methods

Participants

This study was conducted in Okazaki City, Aichi Prefecture, from April 2007 to May 2010. Study candidates were selected from female health checkup examinees ranging from 35 to 79 years old at the Okazaki Public Health Center during the period specified above. Invitation letter with study protocol and questionnaire were individually mailed before the examination. On the morning of the examination day, the study staff (medical doctors or nurses) provided a one-on-one explanation of the purpose and design of the study to the subjects. A total of 2733 women who agreed to participate in the study provided written informed consent, including answering the questionnaire described below. The percentage of the population in this age range in Okazaki City in 2009 was 2.7%.

Questionnaire

The questionnaire included questions to determine the following: sex, age, marital status, height, bodyweight at present and at 20 years old, and the menstrual and pregnancy history, including the age at menarche; the menstrual history was classified on a Likert scale of 1–4 as follows: 1, regular; 2, slightly irregular; 3, irregular; 4, no menses.

The dysmenorrhea severity was classified on a Likert scale of 1–4 as follows: 1, no dysmenorrhoea; 2, have pain sometimes; 3, often have (had) pain; 4, frequently have severe pain during menstruation.

The experience of pregnancy, no or yes; total number of pregnancies, including miscarriages and stillbirths.

Experience of miscarriage, no or yes; total number of consecutive miscarriages; total number of miscarriages.

RM was defined as three or more consecutive miscarriages. RSA was defined as two or more consecutive spontaneous abortions.

Sporadic miscarriage was defined as one or two miscarriages that were not consecutive and were interrupted by live births.

No miscarriage was defined as no history of miscarriage in women with a pregnancy.

Experience of birth, no or yes; total number of births, including stillbirths; age at the time of first birth.

Experience of breast-feeding, no or yes.

Self-score from 0–100 points to describe the physical health status.

Self-score from 0–100 points to describe the feeling of happiness.

History of past/present illness: gastric ulcer, duodenal ulcer, gastritis, large intestinal polyp, hepatitis B, hepatitis C, cirrhosis, fatty liver, tuberculosis, asthma, bronchitis, diabetes, hyperlipidemia, hypertension, atopic dermatitis, urinary calculi, mastopathy, myocardial infarction, stroke.

We examined the association between the experience of at least one miscarriage and RSA and the history of past /present illness.

The study was approved by the Research Ethics Committee at Nagoya City University Medical School.

Statistics

Data were analyzed by *t*-tests and χ^2-test using SAS software. $P < 0.05$ was considered to denote statistical significance.

Results

The total number of participants in the Okazaki Cohort Study was 6086 (female: 2733). The mean age was 56.8 ± 10.3 years. The mean (SD) age of the first marriage was 23.9 years (3.4).

The mean (SD) number of pregnancies in a total of 2503 women was 2.96 (1.21). In all, 38.3% (958/ 2503) of the women with a history of pregnancy had experienced at least one miscarriage. Among these, 22 women gave a history of three or more miscarriages and 105 gave a history of two or more miscarriages; 83 women had two, 20 had three, and two had four miscarriages. Thus, the frequency of RM and RSA were 0.88% and 4.2%, respectively. The mean age of the women with sporadic miscarriage and RSA was higher than that of the women who had never suffered a miscarriage (<0.0001, 0.057, Table 1).

Table 1 Characteristics of women with recurrent spontaneous abortion, sporadic miscarriage and no miscarriage

	No miscarriage		Sporadic miscarriage		*P*-value	RSA		*P*-value
Number	1542		853			105		
Age*	56.3	(10.0)	59.1	(9.8)	<0.0001	58.2	(10.5)	0.057
BMI at 20 years of age*	20.6	(2.2)	20.8	(2.4)	0.12	20.7	(2.3)	0.82
Menstrual cycle								
Regular	1125	73.0%	612	71.7%	0.35	68	64.8%	0.028
Slightly irregular	268	17.4%	147	17.2%		20	19.0%	
Irregular	148	9.6%	93	10.9%		17	16.2%	
No menses	1	0.1%	1	0.1%		0	0.0%	
Dysmenorrhoea								
Yes (at least 'sometimes')	991	64.3%	558	65.4%	0.6	76	72.4%	0.11
Not at all	549	35.6%	295	34.6%		29	27.6%	
Marital status								
Married	1476	97.0%	804	95.3%	0.043	93	91.2%	0.0043
Divorce	46	3.0%	40	4.7%		9	8.8%	
Cumulative birth								
Yes	1506	99.0%	820	97.0%	0.0007	100	95.2%	0.0033
No	15	1.0%	25	3.0%		5	4.8%	
Breast-feeding								
Yes	1450	95.5%	767	93.8%	0.095	94	94.0%	0.067
No	69	4.5%	51	6.2%		6	6.0%	
Feeling about physical health status*	74.4	(14.2)	75.2	(14.9)	0.41	75.7	(13.3)	0.32
Feeling of happiness*	78.4	(14.8)	79.8	(14.8)	0.52	79.4	(14.6)	0.48

*Average (SD). *P*-values: vs 'No miscarriage'. BMI, body mass index.

The divorce rate in women with a history of RSA or sporadic miscarriage was significantly higher than that in the women who had no experience of a miscarriage (8.8% vs 4.7% vs 3.0%). Women with a history of miscarriages (hazard ratio: 1.596; 95% confidence interval [CI], 1.036–2.460; $P = 0.043$) and RSA (hazard ratio: 3.103; 95%CI: 1.474–6.53; $P = 0.0043$) were at a higher risk of their relationships ending, as compared with the women who had never suffered a miscarriage.

Body mass index (BMI) and obesity at 20 years of age and a history of dysmenorrhoea were not associated with miscarriage. A history of irregular menstrual cycles was not found to be associated with miscarriage, but was associated with RSA ($P = 0.028$).

There was no correlation between feeling about physical health status and the experience of miscarriage, with scores for this item in the women with RSA, sporadic miscarriage and no miscarriage, of 74.4 (14.2), 75.2 (14.9) and 75.7 (13.3), respectively. There was also no correlation between the score for a feeling of happiness and the experience of miscarriage, because the scores for this item in the women with RSA, sporadic miscarriage and no miscarriage, were 78.4 (14.8), 79.8 (14.8) and 79.4 (14.6), respectively.

A relation was seen between a history of RSA and the occurrence of gastric ulcer ($P = 0.035$), gastritis (<0.0001), fatty liver (0.031), and atopic dermatitis (0.031, Table 2). No significant relation was noted between a history of miscarriage and the risk of development of myocardial infarction (0.065).

Of the 105 women with RSA, 100 (95.2%) experienced cumulative births, and furthermore, 94 (89.5%) described a history of breast-feeding, a surrogate marker for live birth. A discrepancy of numbers occurred because some participants did not respond to some questions.

Discussion

The frequency of RM was estimated to be 0.9%, and that of RSA to be 4.2% in Japan, and 38% of all women with a history of pregnancy had experienced at least one miscarriage.

A previous study had indicated an increased risk of early miscarriage (odds ratio [OR] 1.2, 95%CI 1.01–1.46, $P = 0.04$) and RM (OR 3.5, 95%CI 1.03–12.01, $P = 0.04$) in obese women after natural conception, as compared with women within the normal weight range.[9] The problem of obesity is reported to be the greatest among non-Hispanic African-American women (48.8%), as compared with Mexican-American (38.9%) and non-Hispanic Caucasian women (31.3%).[9] There were only