# Changes in the gene expression of peripheral blood mononuclear cells during the menstrual cycle of females is associated with a gender bias in the incidence of systemic lupus erythematosus

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## Abstract

### Objective

*The incidence of systemic lupus erythematosus (SLE) is far higher in females than in males and the onset and/or disease activity is influenced by pregnancy and the menstrual cycle. Sex hormones seem to influence the pathogenesis of SLE, therefore, changes in gene expression in peripheral blood mononuclear cells (PBMC) were examined during the menstrual cycle in females, under the comparison of gene expression of patients with SLE.*

### Methods

*The detection and a quantitative analysis of the gene expression was performed by DNA microarray or real-time quantitative polymerase chain reaction (RQ-PCR) method.*

### Results

*There were thirteen known genes which showed significant quantitative changes during the menstrual cycles of females, but not in males. Among these genes, statistical quantitative differences between normal controls and SLE patients were observed in six genes.*

### Conclusion

*Based on these findings, certain genes (such as the tumor necrosis factor receptor superfamily, member 14; TNFRSF14, and signal regulatory protein, gamma; SIRPG) appear to contribute to gender difference of SLE.*

**Key words**

Systemic lupus erythematosus, menstrual cycle, sex hormone, DNA microarray, TNFRSF14, SIRPG.

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*This work was supported by grants from The Institute for Environment and Gender-specific Medicine, Juntendo University Graduate School of Medicine.*

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*Received on May 5, 2008; accepted in revised form on October 8, 2008.*



*Competing interests: none declared.*

## Introduction

SLE, which is a representative autoimmune disease, occurs far more frequently in females than in males, and there have been numerous studies concerning this issue in humans and mouse models (1-3). Sex hormones such as estrogen seem to be an important factor in this gender difference in the incidence of SLE (4). Furthermore, the onset and/or activity of SLE are known to be influenced by the menstrual cycle or pregnancy. In general, the disease activities of SLE tend to be exacerbated during the premenstruation period. A flare-up of SLE commonly occurs during early pregnancy, as well as in the puerperium. These phenomena suggest a close relationship between exacerbation of SLE and increasing concentrations of plasma estrogen or progesterone (3, 5). Based on these findings, changes in the gene expression in PBMC during the menstrual cycles of healthy females were examined using DNA microarrays. In addition, quantitative changes in the expression of specific genes which showed significant changes with menstrual cycles of normal females, but not males, and also statistical significant differences between normal persons and SLE patients were examined in active and inactive stages of the patients with SLE. These results seem to contribute to elucidation of the mechanism of gender bias of SLE.

## Materials and methods

*Patients*

Three healthy females and three males (age 26-45 years old) and SLE patients (five females and two males) were participated in this study. The patients were diagnosed as having SLE according to the 1982 revised criteria of the American College of Rheumatology (6). The disease activity was assessed using the SLE Disease Activity Index (SLEDAI) (7). The patient profiles were summarized in Table I. All patients were hospitalized for the onset or exacerbation of their SLE and treated with steroids. Their PBMC were collected before (active phase) and after (inactive phase) initiation of steroids therapy or increasing their dosages. Their disease duration, main clinical symptoms, SLEDAI score during the active and inactive phase, and dosages of steroid (prednisolone) were described in Table I. All the patients and healthy individuals gave written informed consent to the study, and this study was approved by the local ethics committee.

*Hormone assay*

Plasma concentrations of estrogen (17beta-estradiol) and progesterone were measured using the Electrochemiluminescence immunoassay (ECLIA) developed by Roche Diagnostics (Indianapolis, IN) (8).

*DNA microarray*

PBMC were separated using Ficoll-Paque Plus (GE Healthcare UK Ltd, Buckinghamshire, UK). Comprehensive gene analyses were performed using the DNA microarray method, as described previously (9, 10). Briefly, total RNA was isolated from PBMC of healthy persons and patients using an RNeasy Mini Kit (Qiagen Inc., Valencia, CA). RNA quality was checked using an Agilent 2100 Bioanalyzer and an RNA 6000 Nano LabChip Kit (Agilent Technologies, Palo Alto, CA). Thereafter, fluorescence-labeled RNA targets were synthesized using an amino-allyl RNA amplification Kit (Sigma-Aldrich Co., St. Louis, MO). RNA targets synthesized from human PBMC were coupled with Cy5. RNA targets were also synthesized from Universal Human Reference RNA (Stratagene, La Jolla, CA) and coupled with Cy3 as reference samples for the two-color microarray method. These labeled targets were competitively hybridized to an AceGene Human 30K 1 Oligo Chip Version (DNA Chip Research Inc., Kanagawa, Japan) incubated overnight at 50°C, and the hybridized images were scanned by using a ScanArrray Lite (PerkinElmer, Inc., Wellesley, MA).

*Data analysis*

The signal intensity data were quantified using ScanArray Express Software (PerkinElmer, Inc.). First, the genes showing signal intensities equal to or less than 300 were omitted from data list. Subsequently, the obtained numerical data was normalized using the

**Table I.** Profiles of the patients with SLE.

| Patients | Sex | Age | Disease Duration (year) | Main symptoms | SLEDAI score | Predni-solone therapy (mg/day) | Active phase Inactive phase Before admission | After admission |
|---|---|---|---|---|---|---|---|---|
| SLE 1 | F | 14 | 0 | Nephropathy | 21 | 4 | 0 | 40 |
| SLE 2 | F | 46 | 0 | Nephropathy | 14 | 2 | 0 | 60 |
| SLE 3 | F | 24 | 2 | Nephropathy | 17 | 6 | 10 | 50 |
| SLE 4 | F | 47 | 7 | Nephropathy | 13 | 3 | 8 | 60 |
| SLE 5 | M | 42 | 8 | Pancytopenia | 10 | 3 | 7 | 35 |
| SLE 6 | F | 42 | 4 | Nephropathy | 17 | 4 | 6 | 30 |
| SLE 7 | M | 24 | 3 | Rash, Fever | 10 | 3 | 5 | 20 |

LOWESS method. In brief, quantified signal intensities were converted by taking logarithms of base two. Using the transformed data derived from each pair of competitive hybridized images, scatter diagrams were drawn to compare sample signal intensities with those derived from controls, and executed regression analysis. The given residuals explain logarithmic gene expression ratios. Therefore, genes were selected whose average residuals were more than 1 or less than-1, *i.e*. representing a two-fold difference in expression level. Whole analyses were carried out using the Microsoft Excel (Microsoft, Redmond, WA) and Avadis software programs (Strand Life Sciences, Bangalore, India) (9).

*RQ-PCR*

Total RNA was reverse transcribed and diluted by PrimeScript® RT reagent Kit (Takara Bio Inc., Japan), then the SYBR Green method RQ-PCR was performed using SYBR® Premix Ex Taq™ (Takara Bio Inc.) and ABI Prism 7700 Sequence Detection System (Perkin Elmer Applied Biosystems, Foster City, CA). Those methods were carried out according to the manufacturer's instructions. Adequate following primers sets were employed to detect the thirteen genes described in Table II; HA038650 for TNFRSF14, HA071906 for SIRPG, HA052408 for GPBP1, HA046567 for PPWD1, HA072184 for APBA2, HA072371 for ATP11A, HA074764 for POLR3D, HA043451 for UBE2C, HA087335 for SERPINA4, HA032025 for PEX7, HA071924 for CSorf27, HA032483 for IL8, HA045485 for IL20A (Takara Bio Inc.). The β-actin gene was also detected using HA067803 (Takara Bio Inc.) as an internal loading control. The results are described as an expression index (E.I.), which was calculated as the mean amount of messenger RNA (mRNA) obtained by three separate experiments from six healthy volunteers and seven SLE patients divided by the mean amount of mRNA from all samples, respectively.

*Statistical analysis*

The statistical analysis was performed by using a Student's *t*-test. An analysis was done using the GraphPad InStat Software program (GraphPad Software Inc., San Diego, CA) and a value of $p<0.05$ was considered to indicate significance.

## Results

Figure 1 shows the plasma levels of estrogen and progesterone of healthy females and males who participated in this study at each sampling point (point 1~point 4). The sampling points of plasma and PBMC were; point 1 (p1) is the first day of a menstruation. Point 2 (p2), point 3 (p3), and point 4 (p4) indicate one week, two, and three weeks later after p1, respectively. Samples from males were also collected at four similar points (p1~p4) to females sampling points.

The gene expressions from PBMC of normal females and males were analyzed at each point using the DNA microarray method. Regarding these samples, 26,852 genes were detected using the data analysis system (see

**Table II.** Symbols and names of genes described in Figure 2.

| UniGene ID | GenBank acct. no. | Gene symbol | Gene name |
|---|---|---|---|
| Hs.512898 | NM_003820 | TNFRSF14 | Tumor Necrosis Factor Receptor Superfamily, Member 14 |
| Hs.22974 | NM_018556 | SIRPG | Single Regulatory Protein, Gamma |
| Hs.444279 | AL136844 | GPBP1 | GC-rich promoter binding protein 1 |
| Hs.121432 | XM_042024 | PPWD1 | Peptidylprolyl isomerase domain and WD repeat containing 1 |
| Hs.525718 | NM_005503 | APBA2 | Amyloid Beta (A4) Precursor Protein-binding, Family A, Member 2 (X11-like) |
| Hs.29189 | AK024264 | ATP11A | ATPase, Class VI, type 11A |
| Hs.148342 | BC002603 | POLR3D | Polymerase (RNA) III (DNA directed) Polypeptide D, 44kDa |
| Hs.93002 | NM_007019 | UBE2C | Ubiquitin-conjugating enzyme E2C |
| Hs.159628 | NM_006215 | SERPINA4 | Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 4 |
| Hs.280932 | AL365223 | PEX7 | Peroxisome targeting signal 2 receptor |
| Hs.194283 | NM_007354 | C3orf27 | Chromosome 3 open reading frame 27 |
| Hs.551925 | L23931 | IL8 | Interleukin 8 |
| Hs.445868 | NM_014432 | IL20A | Interleukin 20 receptor, alpha |

Among the nineteen genes describe in Figure 2, thirteen known genes are summarized in this Table.
The expressions of ATP11A, C3orf27, IL8, IL20A genes in p2 ~ p4 decreased, while those of the other genes in p2 ~ p4 increased in comparison to p1 in Figure 2.

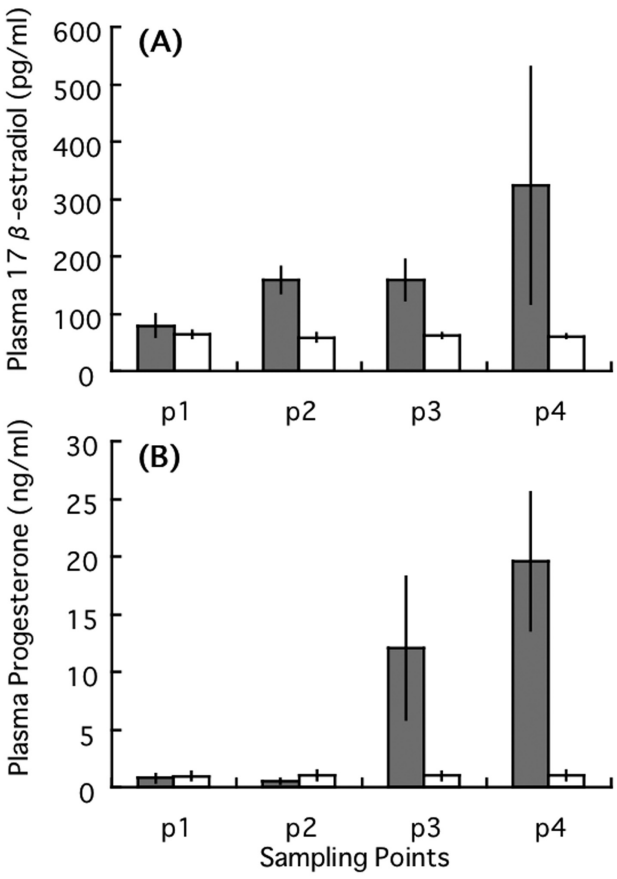


**Fig. 1.** Plasma concentration of estrogen (17beta-estradiol) (A) and progesterone (B) of healthy females (gray column) and males (white column) at each sampling point (p1 ~ p4). The bar indicates standard deviation (S.D.).

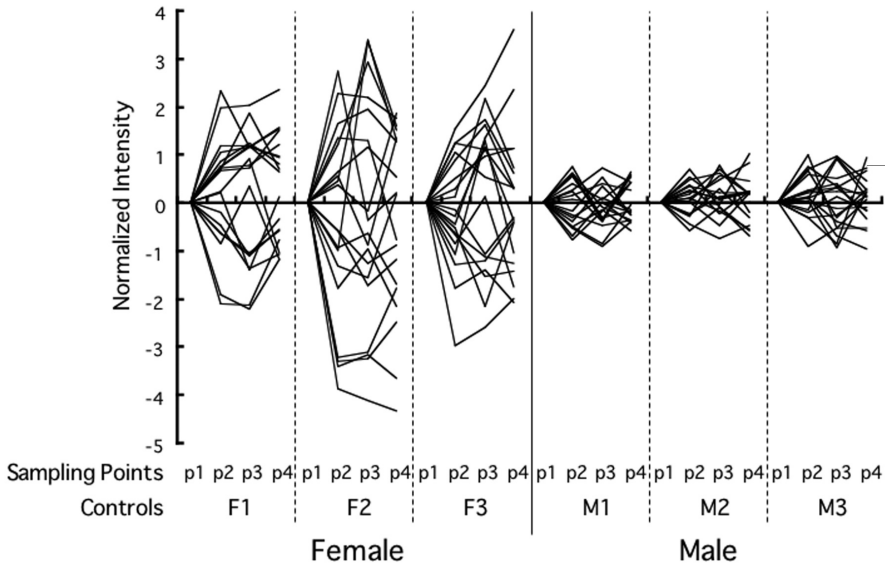


**Fig. 2.** The fluctuation of the gene expression of PBMC from three females (F1 ~ F3) and males (M1 ~ M3) at each sampling point (p1 ~ p4). Among 4,245 genes which showed strong fluctuation (more than two times when compared to p1 as a standard point) in some sampling points of females (but not males), nineteen genes described in the figure showed a similar fluctuation pattern in each sampling point of three females.

*Materials and methods*). These included 4,245 genes which showed strong changes (more than two times when compared to p1 as a standard point) in some sampling points (p2~p4) of females. Unlike females, the expression of these genes did not show any significant change at any point in males. Nineteen of those 4,245 genes showed a similar fluctuation pattern in each sampling point of all three females (Fig. 2). Thirteen genes of these were known and the others were unknown genes. The names of these thirteen known genes are listed in Table II.

Next, the quantitative expression of these thirteen genes was compared between normal controls and SLE patients using RQ-PCR. A statistical significant differences in gene expression between normal and SLE patients in the active stage were observed in six genes (Fig. 3). The precise function of some of these genes are still unclear. However, considering the pathogenesis of SLE, two genes appear to be interesting; TNFRSF14 and SIRPG. The expression of SIRPG decreased during the inactive stage of SLE in comparison to the active stage in all patients; however, the expression of TNFRSF14 was higher during inactive phase in comparison to the active phase in some patients (Fig. 4).

## Discussion

Several previous studies have suggested a potentially important role of sex hormones in the induction of SLE (3). For instance, the enhancing effect of estrogens and protective effect of androgens have been reported in SLE model mice (11). Estrogen is known to induce enhanced B cell activity, autoantibody production, and development of autoreactive T and B cells in mice (12). Exacerbation or onset of SLE after hormone replacement therapy or repeated cycles of ovulation therapy have been observed in human SLE (13). Estrogen can increase immunoglobulin production or mitogen-mediated B cell responses in human cells (14). Estrogen can also stimulate the production of T helper (Th)-2 cytokines such as interleukin (IL)-4, IL-6, and IL-10 which are dominant in the patients with SLE when compared to the Th-1 cytokines (15).

Using DNA microarray method, the change in gene expression in PBMC with menstrual cycles of females was examined in order to investigate the influence of sex hormones. Based on these results, potentially important

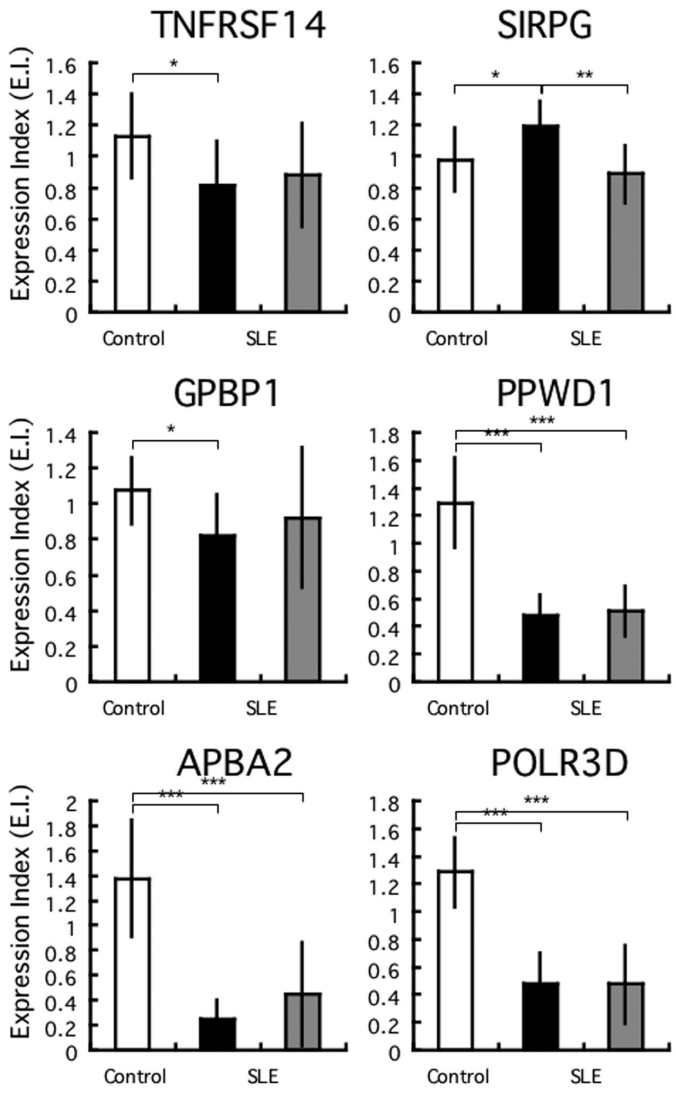


**Fig. 3.** A comparison of the gene expressions between healthy persons and SLE patients. Among the thirteen genes described in Table II, six genes showed statistical significant differences between healthy persons (F1 ~ F3, and M1 ~ M3, white column) described in Figure 2 and SLE patients (SLE 1 ~ 7, black column; active phase, gray column; inactive phase) described in Table I. Gene names of symbols refer to Table II. Data indicate the means ± S.D. of expression index (E.I.) obtained from all healthy persons and SLE patients in active or inactive phase. $^{*}p<0.05$, $^{**}p<0.001$, $^{***}p<0.0001$

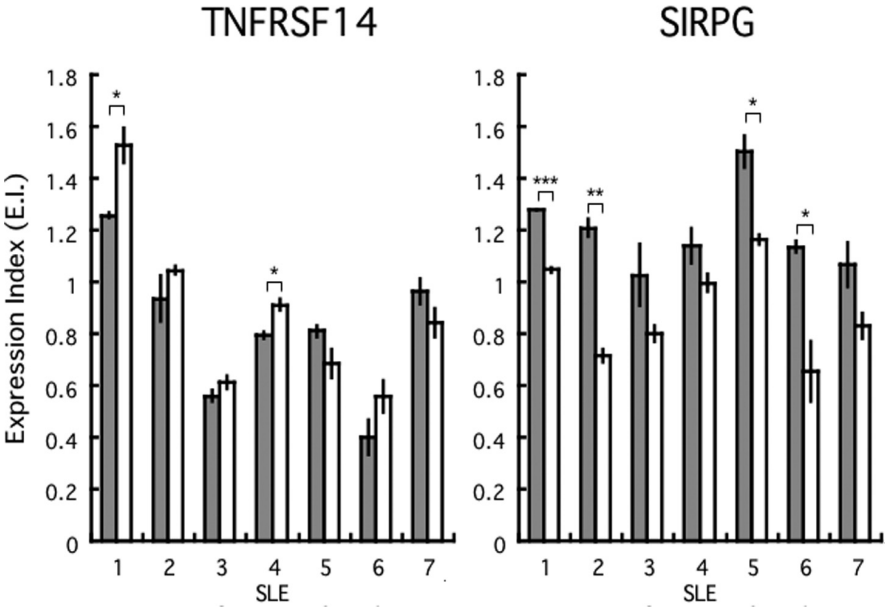


**Fig. 4.** Expression of TNFRSF14 and SIRPG in active (gray column) and inactive (white column) stages of the patients with SLE (SLE 1 ~ 7 in Table I). $^{*}p<0.05$, $^{**}p<0.005$, $^{***}p<0.001$

genes related to the gender difference of SLE were identified. Among the 26,852 genes detected by the DNA microarray method, two genes; TNFRSF14 and SIRPG were identified. The process of gene selection described in the results is summarized in Table III. However, it is possible that other genes besides TNFRSF14 and SIRPG among the final selected six genes play important roles in the gender difference of SLE.

TNFRSF14 (synonym: herpes virus entry mediator; HVEM) is a receptor for herpes simplex viruses, and ligands of LIGHT (lymphotoxin-like, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for HVEM on T cells) and BTLA (B and T lymphocyte attenuator). TNFRSF14 is located on chromosome (1p36), thus suggesting the linkage with SLE based on a genome-wide search for susceptibility genes in SLE (16). In addition, serum concentrations of the soluble form of TNFRF14 are increased in patients with SLE in comparison to normal controls (17). Furthermore, transgenic mice that express LIGHT are reported to show SLE-like symptoms (18). The interaction between TNFRSF14 and LIGHT is related to the transmission of cell information between T and B cells or T and T cells (19, 20). The interaction between them can promote the activity of nuclear factor kappa B (NF-κB), cell proliferation, and cytokine production. In contrast, it has been suggested that BTLA provides an inhibitory signal to B and T cell activations and BTLA-TNFRSF14 (HVEM) interaction is an important pathway regulating lymphocyte activation and/or homeostasis in the immune system (21). Therefore, the signals through TNFRSF14 can involve two different types of function (activation via LIGHT, and inhibition via BTLA) into cells. SIRPG is a member of the SIRP family and it is mainly expressed by T cells, and binds CD47 molecules (22). CD47 molecules are known to be involved with several autoimmune-associated cell functions such as integration, migration, phagocytosis, T-cell activation, anergy, and apoptosis (23). The interaction between CD47 and the receptor SIRP alpha (SIRPA/SHPS-1),

**Table III**. Process of gene selection.

| Category | Numbers of detected genes |
|---|---|
| Detected genes in PBMC using the DNA microarray system. | 26,852 |
| ↓ | |
| Genes showing strong change in some sampling points in only females, but not males. | 4,245 |
| ↓ | |
| Genes showing similar fluctuation pattern in each sampling point of all three females. | 19 |
| ↓ | |
| Known genes among these nineteen genes. | 13 |
| ↓ | |
| Statistical significant difference observed genes between normal persons and SLE patients in the active stage. | 6 |
| ↓ | |
| Interesting genes in their function; SIRPG, TNFRSF14. | 2 |

**Table IV.** Possible importance of TNFRSF14 and SIRPG in gender bias of SLE incidence.

| Receptor | Ligand | Effects on immune systems | Gene expression in SLE of TNFRSF14 or SIRPG | Contribution to gender bias of SLE |
|---|---|---|---|---|
| BTLA | **TNFRSF14** | Inhibition | down-regulation[1] | yes |
| LIGHT | **TNFRSF14** | Activation | down-regulation[1] | no or unclear |
| **SIRPG** | CD47 | Activation | up-regulation[2] | yes |

[1]Expression of TNFRSF14 in normal controls versus SLE patients in active stage; $p<0.05$.
[2]Expression of SIRPG in normal controls versus SLE patients in active stage; $p<0.05$.
See *Discussion*.

which was the first discovered member of SIRP, is important in the induction of autoimmune disorders such as autoimmune hemolytic anemia (AIHA) (24). SIRPG can induce a functional interaction through CD47 molecules on T cells, although the affinity to CD47 is lower than SIRPA (22, 23). Therefore, TNFRSF14 and SIRPG appear to play an interesting role in the pathogenesis of SLE.

The average levels of TNFRSF14 expression during the active stage in SLE patients were significantly lower than those in normal volunteers ($p<0.05$) (Fig. 3). However, quantitative changes of gene expressions of TNFRSF14 with SLE disease activities are not constant among SLE patients (Fig. 4). In certain patients, the levels of TNFRSF14 were decreased in the active stages in comparison to the inactive stages, and in the other patients vice versa. Male patients (SLE 5 and 7) belonged to the latter group. On the other hand, all patients in the former group (SLE 1~4 and 6) were female, and they showed nephropathy as a main clinical symptom of SLE (Table I). Therefore, decreased levels of TNFRSF14 may be associated with the pathogenesis of nephropathy in female SLE. In addition, the average levels of SIRPG expression during the active phase of SLE patients were significantly higher than those in normal volunteers ($p<0.05$) (Fig. 3). Levels of SIRPG gene expression are decreased during inactive stages in comparison to the active stages in all patients with SLE. Taken together, there is a possibility that a decrease in the TNFRSF14 level is related to failure of homeostasis of immune system via BTLA-TNFRSF14 interaction and the resultant activation of immune system and development of such symptoms in patients with SLE, especially female patients with nephropathy. Furthermore, T-cell activation through CD47 via SIRPG signaling increasing in active stages of SLE may play an important role in the induction of SLE. Such a hypothesis based on our results was summarized in Table IV. Unlikely SLE patients, the fluctuations of these genes (TNFRSF14 and SIRPG) with the menstrual cycle of normal females seems to contribute to attenuation of the enhancing effect of sex hormones (such as estrogen) to immune systems. The quantitative and/or qualitative abnormalities of these genes influenced by menstrual cycles and the associated-sex hormones fluctuations seem to contribute to the gender differences of onset and disease activity of SLE.

The effects of estrogen on the expression of genes and proteins of TNFRSF14 and SIRPG are currently being investigated *in vitro*. A recent study reported that the expression of mRNA for estrogen receptor (ER) alpha, which is mainly expressed by CD4+ T cells, is significantly increased in the PBMC from SLE patients in comparison with normal controls using RQ-PCR (25). This may be also related to the role of estrogen in the gender bias of development of SLE because functions of estrogen are displayed via its binding to ER.

Recently DNA microarray methods have been widely applied to the investigation of autoimmune diseases including SLE. The current study appears to suggest that this method is useful for the elucidation of the etiology of rheumatic disorders such as SLE which is induced by various complicated factors. Further studies are required to elucidate the precise mechanisms of quantitative and/or qualitative abnormalities in the expression of genes (such as TNFRSF14 and SIRPG) and their related proteins in the induction of SLE and the gender bias of this disease.

## Acknowledgements

The authors would like to acknowledge the useful discussion and technical assistance of Dr. F. Yoshizaki, Dr. M. Tominaga, Mr H. Nakayama, Ms J. Tomiyama, Mr C. Iwahara, and Ms M. Yoshikawa.

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