Differential expression of Toll-like receptors 1 and 3 in patients with systemic lupus erythematosus and systemic sclerosis

Ang-Jun Liu
  China Medical University

Po-Chang Wu
  China Medical University Hospital

Jian-Ruei Ciou
  China Medical University

Pu-Wei Hou
  China Medical University Hospital

Chung-Ming Huang
  China Medical University Hospital

Po-Hao Huang
  China Medical University Hospital

Yu-Pei Chen
  Ministry of health and Welfare

Hsueh-Ting Chu
  Asia University

Hen-Hong Chang (✉ tcmchh55@gmail.com)
  China Medical University  https://orcid.org/0000-0002-7840-1504

Research article

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Abstract

**Background:** Systemic lupus erythematosus (SLE) and systemic sclerosis (SSc) are autoimmune diseases with some overlapping clinical manifestations. SLE is characterized by systemic inflammation with vasculitis and multiple organ damage, whereas SSc manifests as low-grade inflammation with vasculopathy and tissue fibrosis. Recent studies have revealed that Toll-like receptors (TLRs) play essential roles in the pathogenesis of autoimmune diseases. This study investigated the expression of TLR genes in SLE and SSc to determine their roles in the pathogenesis of these diseases.

**Methods:** Patients with SLE ($n = 15$) and SSc ($n = 9$) were recruited from a hospital in Taiwan from 2016 to 2017. RNA was extracted from peripheral blood cells of the patients for next-generation sequencing (NGS). Then, We identified differentially expressed genes and associated pathways to explore the association of TLRs with SLE and SSc pathogenesis. Several housekeeping genes, including ACTB, GAPDH, PGK1, PPIB, SDHA, and TBP, were used to normalize the expression levels of the TLR genes.

**Results:** GAPDH, PGK1, PPIB and SDHA are significantly different ($p < 0.05$) in patients of SLE and SSc. The expression levels of TLR1 ($p = 0.018$) and TLR3 ($p = 0.031$) were significantly upregulated in the patients with SSc compared with those with SLE, normalized by GAPDH.

**Conclusions:** TLR1 and TLR3 can serve as biomarkers to distinguish the gene expression between SLE and SSc, normalized with housekeeping genes such as ACTB, GAPDH and TBP.

Background

Systemic lupus erythematosus (SLE) and systemic sclerosis (SSc) are autoimmune diseases involving multiple organs. SLE is clinically characterized by systemic inflammation with vasculitis and multiple organ damage, whereas SSc is characterized by low-grade inflammation with vasculopathy and tissue fibrosis [1, 2]. Patients with SLE and SSc have immunological abnormalities due to complex interactions between immune dysfunction and genetic predisposition [2, 3].

The Toll-like receptor (TLR) family comprises 13 members, which recognize specific patterns on microbial surfaces. TLRs are involved in the innate immune system, which in turn activates antigen-specific adaptive immunity [4]. Recent studies have revealed that TLRs also play essential roles in the pathogenesis of autoimmune diseases. And recent research has suggested a connection between the TLR activation and the SLE onset [5, 6]. Thus, TLR7 and TLR9 have been shown to be involved in the pathogenesis of SLE [5, 7]. A previous study has also demonstrated the role of TLR4 in pathogenic plasma cells in the development of lupus nephritis [8]. Another research has revealed the role of TLRs in the pathogenesis of SSc via receptor activation and downstream signaling, leading to the release of profibrotic molecules [9]. The pathological role of TLRs in patients of SSc still unclear [10]. Petrackova et al. had compared 10 TLRs between SLE and SSc to Rheumatoid arthritis, finding that TLR3 is high [11]. Therefore, there could be a linkage of pathogenesis of SLE and SSc in TLRs.
The heterogeneity of SLE and SSc is not entirely understood, which limits the progress of elucidating disease pathogenesis and the development of effective treatments. Few biomarkers for SLE and SSc have been fully validated and widely accepted [9]. In this study, we tried to use the NGS technique to get precise data on gene expression by using housekeeping genes. To identify new biomarkers for surveying the progression of each disease and predicting the outcomes of the patients, we also compared the expression of TLR genes between patients with SLE and SSc.

**Methods**

**Patient recruitment**

We recruited patients diagnosed with SLE and SSc at the Rheumatology Clinic of the Chinese Medical University Hospital, Taiwan, from 2016 to 2017. The Institutional Review Board and Ethics Committee of China Medical University Hospital approved the experiments. Written informed consent was obtained from all study participants. Peripheral blood samples for next-generation RNA sequencing were collected from 9 patients with SSc and 15 patients with SLE. These patients fulfilled the 1982 American College of Rheumatology revised classification criteria for SLE [12] and 2013 European League Against Rheumatism classification criteria for SSc [13]. Patients were excluded if they had an autoimmune disease other than SLE and SSc.

**RNA sequencing**

RNA samples were processed using the TruSeq Stranded mRNA library preparation kit (Illumina, San Diego, CA, USA), and mRNA molecules were enriched using a poly(A) capture method [14]. The samples were purified and amplified using the polymerase chain reaction to create a cDNA library. The quality of the library was assessed using a 2100 Bioanalyzer system (Agilent, Santa Clara, CA, USA). An Illumina MiSeq sequencer was used to generate pair-end maximal 300-bp reads [15].

**Designing Pipeline for analyzing differentially expressed genes (DEGs)**

The raw reads in FASTQ format were aligned with the Ensemble human genome GRCh38.84 using the STAR program (version 2.5.2a) [16, 17]. The aligned reads corresponding to different genes were counted using the HTSeq program (version 0.6.1) [18]. Finally, DEGs were estimated using the DESeq2 pipeline (version 1.12.4). The results of DEG analysis were presented as log₂ fold changes and p-values [19].

**Pathway analysis**

Pathway analysis was performed using the R/Bioconductor packages, including Generally Applicable Gene-set Enrichment (GAGE) and Pathview [20, 21]. We also conducted a variety of enrichment group tests using the GAGE package with log₂ fold changes of DEGs inferred by DESeq2. The package was used to identify the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways that were significantly enriched in a set of DEGs. Changes in the KEGG pathway diagram were visualized using Pathview.

**Statistical analysis**
Statistical analyses were performed using R-Studio with different R packages, including DESeq2 and independent hypothesis weighing. DESeq2 uses shrinkage to estimate dispersion and fold changes to improve the stability and interpretability of count data for differences in analysis estimates [19]. The independent hypothesis weighing package uses a covariate that is independent of the p-value under the null hypothesis to specify the weight but provides information for the power of each test or prior probability of the null hypothesis [22]. The SPSS software (Version 22.0) was used for the Mann–Whitney U-test. Continuous variables are presented as the mean ± standard deviation, and categorical variables are presented as n (%). For all analyses, p < 0.05 was considered statistically significant.

Results

Patients’ characteristics

The demographic characteristics of the patients with SLE and SSc and their serological data are presented in Table 1. The mean age of the patients with SLE was 48 years, and that of the patients with SSc was 53 years. Among the patients with SLE, 14 (93%) were females, whereas all nine patients with SSc were females. The mean erythrocyte sedimentation rates were 18.0 ± 22.1 and 33.8 ± 29.3 mm/h in the patients with SLE and SSc, respectively. In total, 14 (93%) patients with SLE and 8 (89%) patients with SSc were positive for antinuclear antibodies. Further, six (40%) patients with SLE were anti-dsDNA positive. Three (20%) patients with SLE and four (44%) patients with SSc overexpressed the anti-Scl-70 autoantibody.
Table 1
Demographic and serological characteristics of patients with systemic lupus erythematosus and systemic sclerosis

| Characteristics | Patients with SLE (n = 15) | Patients with SSc (n = 9) |
|-----------------|-----------------------------|---------------------------|
| Age (years)     | 47.73 ± 14.35               | 53.80 ± 15.30             |
| Female sex      | 14 (93%)                    | 9 (100%)                  |
| Serological characteristics |             |                           |
| ESR (mm/h)      | 18.0 ± 22.1                 | 33.8 ± 29.3               |
| hCRP            | 0.32 ± 0.33                 | 0.23 ± 0.21               |
| IgG             | 1,112.0 ± 620.0             | 911.0 ± 1,257.2           |
| C3              | 85.67 ± 29.18               | 76.97 ± 25.22             |
| C4              | 16.62 ± 5.94                | 19.14 ± 8.99              |
| ANA positivity  | 14 (93%)                    | 8 (89%)                   |
| Coexisting autoantibodies |           |                           |
| Anti-dsDNA      | 6 (40%)                     | 0 (0%)                    |
| Anti-Scl-70     | 3 (20%)                     | 4 (44%)                   |
| Anti-SS-A       | 8 (53%)                     | 0 (0%)                    |
| Anti-SS-B       | 3 (20%)                     | 0 (0%)                    |
| Anti-Sm         | 5 (33%)                     | 0 (0%)                    |
| Anti-RNP        | 6 (40%)                     | 0 (0%)                    |

Continuous variables are expressed as the mean ± standard deviation. Categorical variables are expressed as n (%).

Abbreviations: ESR erythrocyte sedimentation rate; SLE systemic lupus erythematosus; SSc systemic sclerosis

TLR gene expression and housekeeping genes

Gene expression levels were determined in FASTQ format. The raw TLR gene expression data are presented in Table 2, which shows significant differences (p < 0.05) between the patients with SLE and SSc. Then, the expression levels of TLRs of SLE and SSc were compared. The data are presented in Fig. 1.
| Gene | Patients with SSc ($n = 9$) | Patients with SLE ($n = 15$) | $p$-Value |
|------|-----------------------------|-----------------------------|-----------|
| TLR1 | 7,096.89 ± 6,153.97         | 1,414.80 ± 821.70           | 0.0001*   |
| TLR2 | 6,550.89 ± 3,466.45         | 2,776.00 ± 1,694.09         | 0.0013*   |
| TLR3 | 48.89 ± 36.13              | 16.20 ± 12.84               | 0.0024*   |
| TLR4 | 7,429.78 ± 5,198.17         | 2,775.07 ± 1,432.09         | 0.0027*   |
| TLR5 | 822.56 ± 861.45            | 313.67 ± 174.03             | 0.0297*   |
| TLR6 | 2,522.22 ± 1,742.09         | 949.67 ± 564.37             | 0.0177*   |
| TLR7 | 434.00 ± 270.27            | 197.93 ± 142.01             | 0.0133*   |
| TLR8 | 3,900.67 ± 2,070.58         | 1,714.67 ± 805.28           | 0.0001*   |
| TLR10| 267.11 ± 139.92            | 147.73 ± 102.11             | 0.0095*   |
| ACTB | 32,554.67 ± 12,131.16       | 59,349.11 ± 70,677.27       | 0.2384    |
| GAPDH| 3,043.13 ± 1,321.43         | 5,051.33 ± 1,689.00         | 0.0021*   |
| PGK1 | 6,520.67 ± 4,210.41         | 2,592.80 ± 890.90           | 0.0001*   |
| PPIB | 866.33 ± 712.27            | 316.40 ± 94.47              | 0.0008*   |
| SDHA | 1,004.56 ± 1,027.37         | 291.07 ± 97.20              | 0.0054*   |
| TBP  | 161.00 ± 136.16            | 81.87 ± 32.73               | 0.2449    |

Data are presented as the mean (counts) ± standard deviation. * =”$p < 0.05$”

Abbreviations: SLE systemic lupus erythematosus; SSc systemic sclerosis

**Post-normalization TLR gene expression levels**

**Normalization to ACTB expression**

The TLR gene expression values normalized to those of ACTB are presented in Fig. 1a. The median relative expression levels of TLR1 and TLR3 in the patients with SSc were 12.88 (range: 9.51–35.08) and 0.13 (0.06–0.25), respectively. These levels were significantly higher than those in the patients with SLE [TLR1: 4.44 (3.08–5.28), $p = 0.0001$; TLR3: 0.040 (0.023–0.067), $p = 0.0026$]. The relative expression
levels of TLR2, TLR4, TLR5, and TLR6 were also significantly different between the patients with SSc and SLE when normalized to those of ACTB.

**Normalization to GAPDH expression**

The TLR gene expression values normalized to those of GAPDH are shown in Fig. 1b. The median relative expression levels of TLR1 and TLR3 in the patients with SSc were 69.04 (47.61–370.96) and 0.88 (0.39–1.55), respectively. These levels were significantly higher than those in the patients with SLE [TLR1: 47.42 (9.97–98.81), p = 0.018; TLR3: 0.45 (0.02–0.10), p = 0.032].

**Normalization to PGK1 expression**

The TLR gene expression values normalized to those of PGK1 are shown in Fig. 1c. The only TLR gene that showed a significant difference in the expression levels between the patients with SLE and SSc was TLR1. The median relative expression level of TLR1 in the patients with SSc was 85.66 (47.44–187.28), which was significantly higher (p = 0.0013) than that in the patients with SLE [57.48 (21.02–94.98)].

**Normalization to PPIB expression**

The expression levels of the TLR genes normalized to those of PPIB are shown in Fig. 1d. The median relative expression level of TLR1 in the patients with SSc was 66.21 (51.44–158.67), which was significantly higher (p = 0.0067) than that in the patients with SLE [46.77 (10.12–89.76)].

**Normalization to SDHA expression**

The expression levels of the TLR genes normalized to those of SDHA are shown in Fig. 1e. The median relative expression level of TLR1 in the patients with SSc was 68.05 (41.22–152.40), which was significantly higher (p = 0.021) than that in the patients with SLE [42.59 (13.92–86.24)].

**Normalization to TBP expression**

The expression levels of the TLR genes normalized to those of TBP are shown in Fig. 1f. The median relative expression levels of TLR1 and TLR3 in the patients with SSc were 40.68 (14.63–208.18) and 0.36 (0.13–0.80), respectively. These levels were significantly higher than those in the patients with SLE [TLR1: 15.06 (6.11–28.74), p = 0.001; TLR3: 0.21 (0.02–0.47), p = 0.034].

Thus, there were significant differences in the relative TLR gene expression levels between the patients with SSc and SLE. In particular, the TLR1 expression values were significantly different between the two groups when normalized to those of all of the housekeeping genes. By contrast, the TLR3 expression values were significantly different upon normalization to those of only some of the housekeeping genes. A summary of significant differences is shown in Table 3.
Table 3
Significant intergroup differences in TLR gene expression levels after normalization to those of housekeeping genes

|       | TLR1 | TLR2 | TLR3 | TLR4 | TLR5 | TLR6 | TLR7 | TLR8 | TLR10 |
|-------|------|------|------|------|------|------|------|------|-------|
| ACTB  | V    | V    | V    | V    | V    | V    | V    | V    | V     |
| GADPH | V    | V    | V    | V    | V    | V    | V    | V    | V     |
| PGK1  | V    | V    | V    | V    | V    | V    | V    | V    | V     |
| PPIB  | V    | V    | V    | V    | V    | V    | V    | V    | V     |
| SDHA  | V    | V    | V    | V    | V    | V    | V    | V    | V     |
| TBP   | V    | V    | V    | V    | V    | V    | V    | V    | V     |

"V" indicates a significant difference in the relative expression levels of TLR genes between patients with systemic lupus erythematosus and systemic sclerosis

**Discussion**

In this study, we sought to identify molecular biomarkers of SLE and SSc that might be useful for monitoring and predicting these diseases and improving the therapeutic stratification of patients. The expression of reference genes is also variable in the human body, especially to housekeeping genes [23]. Housekeeping genes are required to maintain primary cellular functions and are expressed in all the cells of an organism under normal and pathological conditions [24]. At first, the gene expression of housekeeping gene in our raw data differed greatly. Thus, we selected stably expressed housekeeping genes to reduce the bias caused by the variation in their expression. Normalized the expression of the TLR genes to that of six housekeeping genes, including ACTB, GAPDH, PGK1, PPIB, SDHA, and TBP, which have been well studied previously [23, 24].

The expression levels of TLR genes were compared between patients with SLE and SSc, and the data showed that TLR1 and TLR3 were significantly upregulated in the patients with SSc compared with those with SLE. TLR1 is involved in cytokine secretion and the inflammatory response. This receptor recognizes pathogen-associated molecular patterns present on infectious agents and mediates the production of cytokines. Inappropriate triggering of TLR pathways may initiate autoimmune reactions and lead to tissue damage [26, 27]. Patients with SSc often experience gastrointestinal tract-related symptoms, including dysphagia, nausea, regurgitation, abdominal pain, diarrhea, and constipation [13]. SSc affects the gastrointestinal tract, resulting in its progressive dysfunction. For example, small bowel disease occurs with smooth muscle atrophy, followed by the final stage of muscle fibrosis[25]. As bowel movements change, the gut microbiota also changes, which may provoke an immune response, e.g., via TLR1, in patients with SSc. This vicious cycle explains why TLR1 is highly upregulated in SSc compared with SLE. Our findings suggest that TLR1 can be used as a marker to distinguish between the two autoimmune diseases.
**TLR3** recognizes double-stranded RNA, which is a molecular pattern present in many viruses at some point in their infection cycle. **TLR3** has been detected on cell surfaces and in endosomes of fibroblasts. It is involved in the production of type I interferon, which in turn upregulates the expression of **TLR3**. It has been hypothesized that complexes of self-RNA and antimicrobial peptides can activate **TLR3**[28]. Previous research has revealed that interferon-α2 increases the **TLR3**-induced production of interleukin-6 in dermal fibroblasts and overexpression of **TLR3** in human skin, particularly in dermal fibroblasts present in the connective tissue of the dermis [25]. Our findings can be used as a reference to further investigate the association between **TLR3** and skin fibrosis.

**TLRs** significantly contribute to the pathogenesis of SLE and lupus nephritis. **TLR3** present in mesangial cells and podocytes influences the glomerular filtration barrier. **TLR3** also plays a pivotal role in liver fibrosis. The gut microbiota can stimulate liver immunity, effectively helping in the immune response to a hepatitis virus [29]. Studies have indicated a relationship between microbial composition and chronic liver disease or cirrhosis [30]. The intestinal microbiota plays an important role in the pathogenesis of the liver disease. If gut microbiota overgrows, it can exacerbate conditions such as liver injury and liver fibrosis [31].

A typical feature of SSc is that vascular injury causes Raynaud's phenomenon and results in the release of endogenous TLR ligands during inflammation and local tissue damage[32]. Induction of intracellular signaling pathways may be one of the mechanisms that initiate fibrosis [25]. Studies have shown the importance of **TLRs** in the pathogenesis of SSc, with both **TLRs** and their ligands showing increased expression [24, 25].

Overall, we found a strong correlation between **TLR1** and **TLR3** expression in SSc and SLE. Currently, few antifibrosis medications are available, and only anti-inflammation therapeutics are commonly used in the clinic. Therefore, a microRNA inhibiting the **TLR1** and **TLR3** pathways may potentially be useful for curing fibrosis.

This is the first study to analyze the expression levels of nine **TLR** genes to determine their relationship to the pathogenesis of SSc and SLE. Nevertheless, this study had several limitations. We did not evaluate the course and severity of SSc and SLE because our sample size was small. Additionally, patients were recruited from only one medical center. The patient population was heterogeneous, and the subgroups were not large enough to represent statistically significant cohorts.

**Conclusions**

Our results showing that the expression levels of **TLR1** and **TLR3** were significantly upregulated in the patients with SSc compared with those with SLE, therefore they can be used as biomarkers to distinguish these two diseases. Although SLE and SSc have some overlapping clinical manifestations, our findings provide further insight into the pathogenesis of SSc, that **TLR1** and **TLR3** may play essential roles in recognizing intestinal microbiota and inducing fibrosis pathways.
Abbreviations

DEG: differentially expressed gene; GAGE: Generally Applicable Gene-set Enrichment; KEGG: Kyoto Encyclopedia of Genes and Genomes; SLE: Systemic lupus erythematosus; SSc: systemic sclerosis; TLR: Toll-like receptor; NGS: next-generation sequencing

Declarations

Ethics approval and consent to participate

This study was authorized by the Institutional Review Board and Ethics Committee of the Chinese Medical University Hospital. Written informed consent was obtained from all patients who participated in this study.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The code is available at Github (https://github.com/htchu/ssc-tlr).

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

HTC designed the method and modified the Bioconductor software in R. AJL, HTC, and HHC wrote the manuscript. HHC led the project. All authors read, discussed, and approved the final manuscript.

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**Figures**
Expression levels of TLR genes normalized to those of housekeeping genes: a. ACTB; b. GADPH; c. PGK1; d. PPIB; e. SDHA; and f. TBP. *p < 0.05 between patients with systemic lupus erythematosus and systemic sclerosis (Mann–Whitney U-test)

Figure 1