The effect of immunosuppressive therapy on the messenger RNA expression of target genes in the urinary sediment of patients with active lupus nephritis

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Abstract

Background. Previous studies have shown that messenger RNA (mRNA) expression of target genes is increased in the urinary sediment of patients with active lupus. We study the effect of immunosuppressive therapy on the urinary gene expression profile in patients with active lupus nephritis.

Method. We recruited nine patients with active systemic lupus erythematosus (SLE) and renal disease, and required corticosteroid, with or without cytotoxic treatment. They were followed for 6 months, urine samples were collected at 0, 4, 12 and 24 weeks and gene expression profile was determined by polymerase chain reactions. The pattern of gene expression was compared to clinical parameters of therapeutic response.

Results. Amongst the target genes studied, there was a progressive decline in the urinary expression of T-bet, interleukin (IL)-10, transforming growth factor-beta (TGF-β), monocyte chemoattractant protein-1 (MCP-1), and interferon-gamma (IFN-γ) after immunosuppressive treatment, although the change of IFN-γ was not statistically significant. The time course of their urinary expression was parallel to the systemic activity as reflected by the systemic lupus erythematosus disease activity index (SLEDAI). Throughout the study period, the SLEDAI score correlated significantly with the expressions of IFN-γ (r = 0.43, P = 0.009), T-bet (r = 0.40, P = 0.016), TGF-β (r = 0.51, P = 0.002) and MCP-1 (r = 0.38, P = 0.022). The anti-double strand(anti-ds)DNA antibody titer correlated significantly with the expressions of IFN-γ (r = 0.45, P = 0.009), T-bet (r = 0.37, P = 0.034), IL-10 (r = 0.59, P < 0.001), TGF-β (r = 0.44, P = 0.010) and MCP-1 (r = 0.49, P = 0.004). On the other hand, the expression level of IL-2, IL-4, IL-12, IL-18 and GATA-3 remained static throughout the study period.

Conclusions. The mRNA expression of T-bet, IL-10, TGF-β, MCP-1, and probably IFN-γ in the urinary sediment of patients with active lupus nephritis improves with successful immunosuppressive therapy, and the change in gene expression profile is in phase with the clinical disease activity. Measurement of urinary mRNA expression of target genes may be a potential non-invasive tool for the monitoring of lupus disease activity.

Keywords: IL-10; lupus nephritis; SLE; T-bet

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease with a variety of clinical manifestations. The intricate involvement of inflammatory mediators including cytokines seems fundamental in the aberrant immune system of SLE [1,2]. Evidence indicates the crucial contributory role of the dysregulated expression of cytokines in the immunopathogenesis of SLE [3–8].

The clinical monitoring of lupus disease activity and treatment response is a difficult subject. Although systemic lupus erythematosus disease activity index (SLEDAI) score, serum anti-double strand (anti-ds) DNA level and serum complement levels are commonly used [9,10], none is entirely satisfactory. Recently, quantification of messenger RNA (mRNA) expression in urinary sediment has become possible. Extraction of mRNA from urinary sediment is an established technique [11], and mRNA expression can be examined by the reverse transcription and real-time quantitative polymerase chain reaction (RT-QPCR). Our previous
study demonstrated a predominance of T-helper cell type 1 (Th1) pattern of cytokine mRNA expression in the urinary sediment of patients with active lupus nephritis [12]. Recently, we found that the mRNA expressions of T-bet, a Th1 transcription factor, transforming growth factor-beta (TGF-β), and monocyte chemoattractant protein-1 (MCP-1) were significantly elevated in the urine sediment of active lupus patients and highly correlated with the SLEDAI score, as well as the biochemical and histological indexes of lupus activity [13,14]. We hypothesize that quantification of target gene mRNA expression in urinary sediment may be a useful and non-invasive approach to monitor the disease activity and treatment response of lupus patients.

**Patients and methods**

**Patient selection**

We recruited nine active lupus patients. All of them fulfilled the American College of Rheumatology diagnostic criteria [15]. Active lupus was defined as the SLEDAI score ≥6 [16,17] and the clinical condition, as decided by the individual clinician, required an increase in the dosage of corticosteroid. Seven patients required kidney biopsy, which was not possible in the other two patients because of concomitant thrombocytopenia. Exclusion criteria of the study included the presence of life-threatening complications other than lupus nephritis (for example, cerebral lupus, severe systemic infection), recent increase in dosage of corticosteroid prior to recruitment, a history of poor drug compliance, or pregnancy.

**Clinical follow-up**

After baseline assessment, all patients were treated with oral prednisolone 0.5 mg/kg/day, with oral cyclophosphamide 2 mg/kg/day added for the six patients with active type III or type IV nephritis according to the International Society of Nephrology classification [18]. The treatment regimen for each individual patients was decided by the responsible clinician and not affected by the study. All patients were followed at 0, 4, 12 and 24 weeks and any time point in between if clinically needed. During each follow-up, serum electrolytes, urea, creatinine, albumin, liver enzymes, complements and anti-dsDNA antibody titres were measured. Lupus disease activity was assessed by the SLEDAI score. All clinicians were blinded from the result of urinary mRNA measurement.

**Serial measurement of urinary mRNA expression**

Whole stream early morning urine specimen was collected at 0, 4, 12 and 24 weeks. RNA was extracted from urinary sediment as described in our previous studies [12,13]. Briefly, the urine specimen was centrifuged at 3000 g for 30 min at 4°C. Total RNA was extracted from the centrifuged sediment by the RNaseasy Mini Kit (Qiagen Inc., Canada), following the manufacturer’s instruction. All specimens were pre-treated with deoxyribonuclease I (Invitrogen™, Life Technologies, Carlsbad, CA) and then stored at −70°C until use.

We used 0.5 µg of RNA for reverse transcription with the Superscript II RNase H Reverse Transcriptase (Invitrogen™, Life Technologies). Target gene mRNA expressions were quantified by the RT-QPCR with the use of ABI Prism 7700 Sequence Detector System (Applied Biosystems, Foster City, CA). Based on our previous studies [12–14], the targets we studied included interleukin (IL)-2, IL-4, IL-10, IL-12, IL-18, interferon gamma (IFN-γ), TGF-β, MCP-1, T-bet and GATA-3. The RT-QPCR of IL-2, IL-4, IL-10, IL-12, IL-18, IFN-γ, TGF-β and MCP-1 was performed by commercial kits (all from Applied Biosystems) following the manufacturer’s instruction. The primer and probe sequence of T-bet and GATA-3 are summarized in Table 1. The level of mRNA expression of each target was normalized to 18s rRNA, which was the house-keeping gene (Applied Biosystems). RT-QPCR amplifications were performed in a 20 µl volume at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. All tests were performed in triplicate in the present study. Results were analysed with Sequence Detection Software version 1.9 (Applied Biosystems).

Gene expression of each target was calculated by using the difference-in-threshold-cycle (ΔΔCt) procedure, according to manufacturer’s instruction. For 18s rRNA and each target, the relative efficiency of amplification over various starting template concentrations was determined. Approximately equal efficiencies for other targets with 18s rRNA amplifications were verified by an absolute value of <0.1 for the slope of log input cDNA amount vs ΔΔCt, which was obtained by subtraction of Ct value of 18s rRNA from that of the target. Therefore, it was possible to detect 18s rRNA in the same tube with other targets. The relative quantification of using multiplex reaction with a comparative method was determined by the formula 2−(ΔΔCt), where the ΔΔCt was calculated by the subtraction of ΔCt of calibrator from ΔCt of sample [19]. We studied 10 healthy volunteers as control. The expression level of target was a ratio relative to that of the healthy volunteers.

**Statistical analysis**

Statistical analysis was performed by Statistical Package for Social Sciences version 10.0 software (SPSS Inc., Chicago, IL). The urinary mRNA expressions at the post-treatment time points were compared with the pre-treatment expression. Correlation between the expression level of target genes and clinical parameters, including the SLEDAI score and laboratory result, was explored. Because the data were highly skewed, Wilcoxon signed-rank test was used to compare pre- and post-treatment level of gene expression. Correlations were determined by Spearman’s rank correlation coefficient. A P-value <0.05 was considered as significant. All probabilities were two-tailed.

**Results**

We studied nine female SLE patients. The average age was 37.1 ± 9.2 years. Their clinical data during the study period are summarized in Table 2. The change of disease activity, as reflected by the SLEDAI score, is summarized in Figure 1. Kidney biopsy was performed in seven patients; the renal diagnoses were class III
The mean histological activity and chronicity indices of the seven patients were 5.9±3.8 and 2.8±1.6, respectively.

**Change in expression of target genes**

The change in urinary mRNA expression of target genes during the study period is summarized in Table 3 and Figure 2. We found a significant reduction of the urinary IL-10 expression after immunosuppressive treatment (Figure 2A). As compared to the pre-treatment level, the urinary expression of IL-10 was significantly reduced at 12 weeks ($P=0.050$) and dropped further at 24 weeks ($P=0.021$). The urinary expression of IFN-γ at 4, 12 and 24 weeks were lower than the pre-treatment level, but the change did not reach statistical significance (Figure 2B). The expression level of IL-2, IL-4, IL-12 and IL-18 did not have significant change throughout the study period.

We found a drastic reduction in both TGF-β and MCP-1 expressions in the urinary sediment after immunosuppressive treatment. Similar to the pattern of change we observed in IL-10, the expression of TGF-β significantly dropped at 12 weeks ($P=0.028$) and further reduced at 24 weeks ($P=0.021$) (Figure 2C). The expression of MCP-1 was reduced significantly at 12 weeks ($P=0.015$) as well as 24 weeks ($P=0.038$) as compared to the pre-treatment level (Figure 2D).

We observed a gradual decline of urinary mRNA expression of T-bet, the key Th1 transcription factor, after immunosuppressive treatment. The expression of T-bet was significantly reduced at 12 weeks ($P=0.038$) and 24 weeks ($P=0.021$) as compared to pre-treatment level. In contrast, the urinary expressions of GATA-3, the principal Th2 transcription factor, remained static throughout the study period. The pattern of cytokine profile change was similar between the six patients with cyclophosphamide therapy and those without (details not shown).

**Table 1.** Primer and probe sequences for the RT-QPCR

| Target | Forward primer | Reverse primer | Probe
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>T-bet</td>
<td>5'-GAT GTT TGT GGA CGT GGT CTT G-3'</td>
<td>5'-CTT TCC ACA CTG CAC CCA CT-3'</td>
<td>5'-6FAM-CCA GCA CCA CTG GCC GTA CCA G-TAMRA-3'</td>
</tr>
<tr>
<td>GATA-3</td>
<td>5'-CGC CTG CGG GCT CTA TC-3'</td>
<td>5'-CTT TCG CTG GGG CTT AAT GA-3'</td>
<td>5'-6FAM-CTG TCC GTT CAT TTT-TAMRA-3'</td>
</tr>
</tbody>
</table>

The probes for T-bet and GATA-3 were labeled with 6-carboxyfluorescein (6FAM) at the 5' end and N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA) at the 3' end.

**Table 2.** Demographic and clinical information of the patients

<table>
<thead>
<tr>
<th>SLEDAI</th>
<th>0 week</th>
<th>4 week</th>
<th>12 week</th>
<th>24 week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total score</td>
<td>14.4±8.3</td>
<td>11.9±7.3</td>
<td>4.4±1.9</td>
<td>2.3±1.2</td>
</tr>
<tr>
<td>Renal score</td>
<td>8.0±4.5</td>
<td>7.6±4.2</td>
<td>2.2±2.1</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Damage Index</td>
<td>0.6±0.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-dsDNA (RU/ml)</td>
<td>749.4±393.9</td>
<td>354.8±352.9</td>
<td>147.6±245.4</td>
<td>118.2±154.2</td>
</tr>
<tr>
<td>C3 (g/l)</td>
<td>0.49±0.16</td>
<td>0.57±0.12</td>
<td>0.65±0.12</td>
<td>0.64±0.11</td>
</tr>
<tr>
<td>C4 (g/l)</td>
<td>0.089±0.047</td>
<td>0.13±0.07</td>
<td>0.16±0.06</td>
<td>0.16±0.05</td>
</tr>
<tr>
<td>Serum creatinine (µmol/l)</td>
<td>124.0±104.9</td>
<td>106.0±51.1</td>
<td>100.0±64.1</td>
<td>95.8±47.1</td>
</tr>
<tr>
<td>Serum albumin (g/l)</td>
<td>29.1±5.4</td>
<td>33.0±5.5</td>
<td>36.2±5.3</td>
<td>37.4±6.4</td>
</tr>
<tr>
<td>Proteinuria (g/day)</td>
<td>2.9±1.4</td>
<td>1.8±1.5</td>
<td>1.5±2.2</td>
<td>1.1±1.4</td>
</tr>
<tr>
<td>GFR (mL/min)</td>
<td>67.7±34.7</td>
<td>65.1±25.9</td>
<td>75.5±32.8</td>
<td>73.9±30.2</td>
</tr>
<tr>
<td>Prednisolone (mg/day)</td>
<td>28.3±11.5</td>
<td>24.4±10.8</td>
<td>15.0±6.1</td>
<td>11.1±6.6</td>
</tr>
</tbody>
</table>

SLEDAI, systemic lupus erythematosus disease activity index; C, complement level; GFR, glomerular filtration rate.

**Fig. 1.** Change in SLEDAI of the nine SLE patients with time. The overall SLEDAI score of the nine SLE patients decreased with time. The bold line (—) represents a patient with transient worsening of disease activity 4 weeks after treatment.

(5 cases); class IV (1 case), and pure class V (1 case) lupus nephritis. The mean histological activity and chronicity indices of the seven patients were 5.9±3.8 and 2.8±1.6, respectively.

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We further explore the association between the urinary mRNA expression of target genes and SLE disease activity. The overall SLEDAI score significantly correlated with the expressions of IFN-γ (r = 0.43, P = 0.009), T-bet (r = 0.40, P = 0.016), TGF-β (r = 0.51, P = 0.002) and MCP-1 (r = 0.38, P = 0.022). The renal score in SLEDAI significantly correlated with the expressions of IFN-γ (r = 0.38, P = 0.023), T-bet (r = 0.38, P = 0.021), TGF-β (r = 0.47, P = 0.004) and MCP-1 (r = 0.35, P = 0.035). In addition, the anti-dsDNA antibody titer of the patients significantly correlated with the expressions of IFN-γ (r = 0.45, P = 0.009), T-bet (r = 0.37, P = 0.034), IL-10 (r = 0.59, P < 0.001), TGF-β (r = 0.44, P = 0.010) and MCP-1 (r = 0.49, P = 0.004). The degree of proteinuria significantly correlated with the expressions of IL-10 (r = 0.55, P = 0.002), TGF-β (r = 0.36, P = 0.05) and MCP-1 (r = 0.59, P = 0.001). The serum albumin level also significantly correlated with the expressions of
IL-10 (r = −0.36, P = 0.035) and MCP-1 (r = −0.38, 
P = 0.024). We did not observe any correlation between renal function, as represented by the estimated GFR, and the expression of any target gene.

**Discussion**

In the present report, we studied the change in target gene mRNA expression in the urinary sediment of nine patients with active SLE who received immunosuppressive treatment. The result of our present study is in line with our previous reports [12–14]. In our previous study [14], urinary T-bet expression significantly correlated with the SLEDAI score as well as other laboratory findings. In the present study, we found that the elevated T-bet expression in patients with active lupus nephritis decreased in parallel with the reduction in lupus activity. Although it was previously shown that GATA-3, the principal Th2 transcription factor [14], was suppressed in the patients with active lupus nephritis, the expressions of GATA-3 in the urinary sediment remained static following treatment. Taken together, our result is consistent with the idea that active lupus nephritis is related to a reversible skew towards the Th1 pathway of T lymphocytes activation [20,21].

Following the trend of SLEDAI and T-bet, the elevated IFN-γ expression in the patients with active lupus nephritis were reduced with treatment, although the change was not statistically significant. The result is consistent with a previous report that IFN-γ expression was closely related to the expression of T-bet because of their mutual interaction [22] and further supports the involvement of the Th1 pathway in the pathogenesis of SLE flare [20,21]. On the other hand, although our previous study demonstrated an up-regulation of IL-2, in another study of Th1 cytokine, in the urinary sediment of active lupus, its expression remained static after immunosuppressive treatment. Interestingly, no change in the expression of IL-4, IL-12 and IL-18 was observed. Since IL-12 and IL-18 are two of the most commonly recognized factors of Th1 differentiation [23], our results suggest that the upregulation of T-bet and IFN-γ during lupus flare, and their downregulation following treatment, are governed by factors other than IL-12 and IL-18. Further studies are needed to elucidate the cause of Th-1/Th-2 imbalance in lupus patients.

We have previously demonstrated the upregulation of TGF-β and MCP-1 mRNA expression in the urinary sediment of patients with active lupus nephritis, and the expression closely correlated with the systemic disease activity as well as the histological findings of kidney biopsy [13]. In fact, the role of TGF-β and MCP-1 in the pathogenesis of lupus nephritis has been extensively studied [24]. In this study, we found that the elevated TGF-β and MCP-1 expression in patients with active lupus nephritis decreased in parallel with the reduction in lupus activity.

Similarly, we found that the elevated IL-10 expression in patients with active lupus nephritis decreased following the trend of lupus activity. The roles of IL-10 immunoregulation [25–28] and SLE [29,30] have been studied extensively. Our results suggest an important role of IL-10 in the development of lupus nephritis. On the other hand, it is clear that IL-10 inhibits the production of Th1 cytokines, in particular to IL-12 [31,32]. The change in IL-10 expression we observed may represent the alteration of the body's own immunoregulatory and anti-inflammatory mechanisms [33,34].

It is, however, important to note that the entire panel of target genes mentioned above responded to treatment simultaneously with the clinical disease activity. Although the change in gene expression profile in the urinary sediment may shed light onto the pathogenesis of lupus nephritis, monitoring of the expression profile, of the target genes examined in the present study, does not provide additional clinical information for the monitoring of lupus disease activity. In fact, we observed no correlation between the expression levels of most of the target genes and the parameters of kidney function, suggesting that these genes may not be useful for monitoring the activity of lupus nephritis. Because of the small sample size, we did not have a collection of non-responders for the analysis of the change in gene expression levels. Nevertheless, one patient had worsening of lupus activity during the first 4 weeks of treatment (the bold line in Figure 1) together with a concomitant increase in urinary expression of some target genes, supporting a relationship between impairment in clinical disease activity and urinary gene expression profile. Our current study provides a proof-of-concept result that monitoring of urinary cytokine gene expression is technically feasible, but further studies are needed to identify target genes that respond before, and therefore can predict, clinical improvement.

In this study, we could not affirm the cellular compositions of urinary sediment. Nevertheless, urine microscopic examination was performed in all cases, and urinary sediments were mainly composed of erythrocytes, mononuclear leukocytes and tubular epithelial cells. We believe the increased expression of most of the target genes came from mononuclear leukocytes as well as tubular epithelial cells. Furthermore, our present study only involved active SLE patients with renal complications. Since the clinical features of SLE are extremely diverse and disease flare may occur with various organ involvements, further studies with recruitment of patients with active lupus and without renal involvement are needed. Due to the heterogeneous pathology of lupus nephritis, further investigation on urinary cytokine profile in different types of lupus nephritis would also be necessary.
Treatment response of urinary RNA in lupus

In conclusion, we found that the mRNA expression of T-bet, IL-10, TGF-β, MCP-1, and probably IFN-γ in the urinary sediment of patients with active lupus nephritis improves with successful immunosuppressive therapy, and the change in gene expression profile is in phase with the clinical disease activity. Measurement of urinary mRNA expression of target genes may be a potential non-invasive tool for the monitoring of lupus disease activity.

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Conflict of interest statement. The authors declare no conflict of interest.

(See related article by Colucci et al. NDT. doi:10.1093/ndt/gfl223.)

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