Expression of T-bet, a type 1 T-helper cell transcription factor, in the urinary sediment of lupus patients predicts disease flare

R. W.-Y. Chan, F. M.-M. Lai¹, E. K.-M. Li, L.-S. Tam, K.-M. Chow, P. K.-T. Li and C.-C. Szeto

Background. Systemic lupus erythematosus (SLE) is characterized by the aberrant activation of T-lymphocytes. Since T-bet is the principal transcription factor for the differentiation of type-1 helper T-lymphocyte, we study the impact of urinary T-bet mRNA expression in clinically quiescent SLE patients on the risk of subsequent disease flare.

Methods. We studied 60 quiescent SLE patients. Urinary mRNA expression of T-bet was studied by the real-time quantitative polymerase chain reaction. Patients were followed for 4 yrs for disease flare.

Results. We studied 60 patients; 57 were female. The mean age was 38.8 ± 11.2 yrs. Their baseline SLE disease activity index score was 1.63 ± 1.64. During the follow-up, 28 patients (46.6%) developed lupus flare, of which 17 (28.3%) had severe flare. Receiver operating characteristic curves showed that urinary T-bet expression three times above the average level of healthy control had 64.3% sensitivity and 84.4% specificity of predicting all lupus flare. Using this cut-off, patients with a high urinary T-bet expression had a significantly higher risk of all lupus flare and severe flare than the patients with a low T-bet expression (log-rank test, \(P < 0.001\) for both). With multivariate Cox proportional hazard model to adjust for potential confounding variables, urinary T-bet expression and patient’s sex were the only independent predictors of all lupus flare and severe flare.

It could be estimated that 1-fold increase in urinary T-bet expression would result in 8.4% excess risk of all lupus flare (95% confidence interval (CI), 4.1–13.0%, \(P < 0.001\)) and 12.9% excess risk of severe flare (95% CI 7.4–18.7%, \(P < 0.001\)).

Conclusions. A high urinary T-bet expression was an independent predictor of lupus flare. Measurement of urinary T-bet may provide valuable information for the risk stratification of SLE patients.

Key words: SLE, Lupus nephritis, T-bet, GATA-3.

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by aberrant cytokines milieu and multiple organ involvement [1, 2]. Imbalance in the cytokines produced by the two subsets of T-helper cells, Th1 and Th2, probably plays an important role in the pathogenesis of SLE [3]. Although the control of the Th1/Th2 imbalance has been unclear, there is growing evidence to suggest that two transcription factors, T-bet and GATA-3, are the determining factors of T-helper cell differentiation [4–7]. T-bet promotes Th1 lineage commitment [8, 9] and forms an autoregulatory positive feedback loop with gamma interferon to maintain Th1-mediated immune response [10]. On the other hand, GATA-3 promotes Th2 differentiation [11] and induces Th2 cytokines production in an analogous way as T-bet [12]. The relative expression of T-bet and GATA-3, resulting in a swing in the Th1/Th2 pendulum, has been implicated in a number of immunological diseases [13–18].

Extensive studies focus on cytokines and T-helper cells in the peripheral blood of SLE patients in vitro and in vivo. However, reports on the Th1/Th2 imbalance in SLE have been inconsistent [19–22]. By serological measurements, SLE is often considered to be a Th2-mediated disease at the early stage [20], but the Th1 commitment may replace the Th2 pathway and takeover the progression of SLE to active nephritis [23]. Nevertheless, it should be noted that T-lymphocytes are activated at the site of disease involvement, and the study of peripheral blood mononuclear cells may therefore give misleading results.

We have previously shown that patients with active lupus nephritis have increased T-bet and depressed GATA-3 expression in the urinary sediment and kidney tissue [24], indicating a predominant Th1-type of T-lymphocyte activation. In the present study, we examine the impact of urinary mRNA expression of T-bet and GATA-3 in clinically quiescent SLE patients on the risk of subsequent disease flare.

Patients and methods

Patient selection

We recruited 60 SLE patients: 30 patients with a previous history of lupus nephritis but with no disease activity after treatment (the Remission Group), and another 30 without systemic disease activity and with no history of renal disease (the Quiescent Group). All patients fulfilled the American College of Rheumatology criteria for SLE [25]. Among them, 57 were females and 3 were males. Their mean age was 38.8 ± 11.2 yrs. Their baseline SLE disease activity index score was 1.63 ± 1.64. During the follow-up, 28 patients (46.6%) developed lupus flare, of which 17 (28.3%) had severe flare. Receiver operating characteristic curves showed that urinary T-bet expression three times above the average level of healthy control had 64.3% sensitivity and 84.4% specificity of predicting all lupus flare. Using this cut-off, patients with a high urinary T-bet expression had a significantly higher risk of all lupus flare and severe flare than the patients with a low T-bet expression (log-rank test, \(P < 0.001\) for both). With multivariate Cox proportional hazard model to adjust for potential confounding variables, urinary T-bet expression and patient’s sex were the only independent predictors of all lupus flare and severe flare.

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Rheumatology diagnostic criteria of SLE [25]. Active lupus was defined as an SLE Disease Activity Index (SLEDAI) score of 6 or more [26, 27]. Previous lupus nephritis was defined as a history of biopsy-proved World Health Organization (WHO) class III, IV or V nephritis. We also recruited 10 healthy volunteers as control (the Healthy Group). The design of the study was approved by the Clinical Research Ethical Committee of the Chinese University of Hong Kong. All SLE patients were also participants in our previous cross-sectional study on the T-bet and GATA-3 gene expression in urinary sediment [24]. The present study is a follow-up study of the SLE patients with clinically quiescent disease in our previous report [24].

Study of urinary mRNA expression

The mRNA expression of T-bet and GATA-3 in the urinary sediment was studied by reverse transcription and then real-time quantitative polymerase chain reaction (RT-QPCR). A whole-stream early morning urine specimen was collected after informed consent. The methods of mRNA extraction from urinary sediment have been described by Li et al. [28]. Briefly, the freshly collected urine sample with preservative was immediately centrifuged at 3000 g for 30 min at 4 °C. Total RNA was extracted from the sediment by the RNeasy Mini Kit (Qiagen Inc., Canada), following the manufacturer’s instructions. All specimens were pre-treated with Deoxyribonuclease I (Invitrogen™, Life Technologies, USA) and then stored at −70°C. The integrity and purity of RNA was confirmed by the 18s to 28s rRNA ratio.

For each RT-PCR reaction, ~0.5 μg of RNA was reverse transcribed to complementary DNA (cDNA) with the Superscript II RNase H− Reverse Transcriptase (Invitrogen™, Life Technologies, USA). The RT-PCR was performed by the ABI Prism 7700 Sequence Detector System (Applied Biosystems, Foster City, CA). The primer and probe sequences of human T-bet and GATA-3 were designed and synthesized by Applied Biosystems (Foster City, USA) and has been described in our previous report [24]. The level of mRNA expression of each target was normalized to a housekeeping gene (18s rRNA). RT-QPCR amplifications were performed in 20 μl volume at 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was run in triplicate.

The results of RT-QPCR were analysed by the Sequence Detection Software version 1.9 (Applied Biosystems, Foster City, CA), with the difference-in-threshold-cycle (ΔΔCt) procedure according to the manufacturer’s instructions. Briefly, the relative standard curve method was applied in the quantification of the mRNA expression of T-bet and GATA-3. Relative standard curve of each transcription factor to 18s RNA was prepared in each PCR run for comparing the relative expression across batches of reactions. cDNA derived from phorbol 12-myristate 13-acetate (PMA)-stimulated human leucocyte was used for the generation of relative standard curves. For each sample, the relative T-bet and GATA-3 expression was calculated using linear regression analysis of the respective standard curves, and their values divided by the corresponding 18s rRNA value. The expression level of T-bet and GATA-3 were expressed as dimensionless numbers relative to the corresponding average expression level of the Healthy Group.

Assessment of clinical activity

On the day of urine collection, the disease activity of SLE was assessed clinically by an independent physician with the SLEDAI score [26]. For specific assessment of the renal activity of the SLE, renal score of the SLEDAI that consisted of proteinuria, urinary casts, haematuria and pyuria of the original SLEDAI score was computed and analysed separately. The overall SLEDAI score ranged from 0 to 32, and the renal score ranged from 0 to 16.

Clinical follow-up

After the initial assessment, patients were followed every 4–12 weeks, as decided by individual clinician, for 4 yrs. During each follow-up, serum electrolytes, urea, creatinine, albumin, liver enzymes, complements and anti-double strand DNA antibody titres were measured. Lupus disease activity was assessed by the SLEDAI score. Lupus flares were defined as one or more of the following [29]: (a) >3-point increase in SLEDAI score; (b) new or worsening discoid, photosensitive or other rashes attributable to lupus (including lupus profundus, cutaneous vasculitis or bullous lupus); nasopharyngeal ulcers, pleuritis, pericarditis, arthritis or fever not attributable to infection; (c) increase in prednisolone dosage and (d) initiation of therapy with either hydroxychloroquine or non-steroidal anti-inflammatory drugs, without an increase in prednisolone dosage. Severe lupus flares were defined as one or more of the following [29]: (a) SLEDAI instrument score >12; (b) new or worsening central nervous system involvement, vasculitis, glomerulonephritis, myositis, thrombocytopenia (platelet count below 60 × 10⁹ cells/l) or haemolytic anaemia (haemoglobin level below 7.0 g/dL or decrease in haemoglobin level above 3 g/dL over a 2-week period), each requiring doubling of prednisolone dosage to a final dosage >0.5 mg/kg/day or acute hospitalization; (c) any manifestation requiring an increase in dosage of prednisolone to >0.5 mg/kg/day, or initiation of therapy with cyclophosphamide, azathioprine, mycophenolate mofetil or methotrexate and (d) hospitalization for lupus activity. All clinicians were blinded from the result of urinary mRNA measurement.

Statistical analysis

Statistical analysis was performed by Statistical Package for Social Sciences version 10.0 software (SPSS Inc., Chicago, IL). Because the data were highly skewed, the mRNA expression levels were compared between groups by Mann–Whitney U-test. A P-value of < 0.05 was considered significant. All probabilities were two-tailed.

To determine the prognostic cut-off of urinary T-bet expression, conventional receiver-operating characteristic (ROC) curves were constructed for all lupus flares and severe flares, the area under the curve (AUC) was computed. We then performed time-to-event analyses to study the effect of urinary T-bet expression on the risk of lupus flare. In this part of the analysis, lupus flare was considered as events. The urinary T-bet expression three times above the average expression of healthy control was taken as the cut-off (see subsequently), and patients were categorized into high or low T-bet expression. Univariate time-to-event analysis was performed by the simple log-rank test. To adjust for the effect of potential confounding variables, a Cox proportional hazards model was then constructed for multivariate analysis, with urinary T-bet expression, age, sex, duration of lupus, history of lupus nephritis and baseline SLEDAI score as covariates, and independent predictors were selected by the backward stepwise approach. Because the number of event was small, we used the baseline SLEDAI score as a summarizing variable, instead of individual clinical and serological marker, for the construction of the Cox model to avoid over-fitting. The result is expressed as adjusted hazard ratio and 95% CI.

Results

We studied 60 patients; 57 were female. The mean age was 38.8 ± 11.2 yrs. Their baseline SLEDAI score was 1.63 ± 1.64. The baseline serum creatinine was 117.8 ± 105.3 μmol/l and proteinuria 0.69 ± 1.25 g/day. The average duration of lupus was 10.2 ± 6.1 months. At the time of recruitment, the dosage of maintenance oral prednisolone was 2.4 ± 2.9 mg/day; 14 patients (23.3%) received azathioprine as maintenance immunosuppressant.
The baseline urinary expressions of T-bet and GATA-3 were 4.83 ± 6.72 and 1.39 ± 2.37-fold when compared with healthy controls.

Over the 4 yrs of follow-up, 28 patients (46.6%) developed flare of SLE, of which 17 (28.3%) were severe flares. Six patients with severe flares had kidney biopsy, which showed WHO class III (three cases), class IV (two cases) and pure class V (one case) lupus nephritis. Five patients (8.3%) progressed to end-stage renal failure during the follow-up.

Urinary gene expression and risk of disease flare

Patients who developed lupus flare during the 4 yrs of follow-up had higher baseline urinary T-bet (7.64 ± 8.34 vs 2.37 ± 2.15, \( P = 0.003 \)), but not GATA-3 expression (1.45 ± 1.87 vs 1.34 ± 2.76, \( P = 0.8 \)), than those who did not flare. Similarly, patients who developed severe flares had higher baseline urinary T-bet expression than those without renal flare (10.28 ± 9.76 vs 2.68 ± 2.23, \( P < 0.001 \)), but the GATA-3 expression was similar (1.72 ± 2.27 vs 1.26 ± 2.42, \( P = 0.5 \)).

We then constructed the ROC curves for the prediction of all lupus flares and severe flares in 4 yrs by urinary T-bet expression (Fig. 1). When a urinary T-bet expression three times above the average expression of healthy control was defined as the cut-off, the sensitivity, specificity, PPV and NPV of predicting severe lupus flare in 4 yrs were 64.3, 84.4, 78.3 and 73.0%, respectively. At this level of urinary T-bet expression, the sensitivity, specificity, PPV and NPV of predicting all lupus flare in 4 yrs were 76.5, 76.7, 56.5 and 73.0%, respectively. The baseline urinary expressions of T-bet and GATA-3 were 4.83 ± 6.72 and 1.39 ± 2.37-fold when compared with healthy controls.

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The effect of urinary T-bet expression on the risk of lupus flare was further analysed by multivariate Cox proportional hazard model to adjust for potential confounding variables. The results of the multivariate Cox models for the time to all lupus flare and severe flare are summarized in Table 1. In this model, urinary T-bet expression and patient’s sex were the only independent predictors of all lupus flare and severe flare; a history of lupus nephritis was not an independent predictor of future flare. It could be estimated that 1-fold increase in urinary T-bet expression would result in 8.4% excess risk of all lupus flare (95% CI, 4.1–13.0%, \( P < 0.001 \)) and 12.9% excess risk of severe flare (95% CI, 7.4–18.7%, \( P < 0.001 \)).

Discussion

Our previous study also showed that urinary T-bet expression increased, while GATA-3 expression decreased, in patients with active lupus nephritis [24]. In the present study, we found that some SLE patients without clinical disease activity had a high urinary T-bet expression, indicating a skew towards Th1 pathway of T-helper cell activation. More importantly, a high urinary T-bet expression was an independent predictor of subsequent disease flare even after adjusting for patient age, sex, duration of SLE, history of previous renal involvement and baseline SLEDAI score.

A major concern of our result is the validity of measuring urinary T-bet expression at a single time point. To the best of our knowledge, there is no published data on the intra-individual variability of urinary T-bet expression, and it remains unproved that the expression is stable for years in quiescent lupus patients. However, our recent data showed that the variability of urinary T-bet expression is relatively small over a few months, at least in lupus patients with maintenance immunosuppressive therapy [29].

Nevertheless, it remains to be clarified as to whether the urinary T-bet levels rise in lupus patients immediately before the flare or whether the expression remains static. Theoretically, there are two possible explanations for the relation between high urinary T-bet expression and lupus flare. First, there may be, possibly genetic, predisposition to the activation of Th1 pathway in some patients, resulting in their intrinsic tendency to flare at all time. Second, a high urinary T-bet expression may be an early marker of immune system activation that heralds a flare. Since almost 50% of the patients with a high T-bet expression developed disease flare within 12 months (Fig. 1), a high urinary T-bet expression is at least partly a short-term indicator of immune systemic activation.

![Fig. 1](https://academic.oup.com/rheumatology/article-abstract/46/1/44/2255969) ROC curves for the prediction of (A) all lupus flares; and (B) severe flares by urinary T-bet expression. The AUC were 0.735 and 0.790, respectively.
Severe flares have a higher risk of systemic as well as renal flare than those with a low T-bet expression. A urinary T-bet expression three times above the normal level is significantly higher than those with proliferative lupus glomerulonephritis (either class III or IV). The urinary T-bet expression is a systemic immunologic marker and therefore reflected systemic immunologic derangement in serum creatinine and proteinuria (i.e. parameters that we used to define renal flare in this study) are late markers of renal injury. Given that T-lymphocyte is the common cell type that expresses T-bet, the infiltrating CD3+ T-lymphocytes may be the major source of T-bet mRNA in urinary sediment.

It follows that serial measurement of the urinary expression of T-bet may be a valuable test for the monitoring of SLE patients. Unfortunately, we do not have the data of urinary T-bet expression of our patients at the time of disease flare, and the hypothesis needs to be tested by another prospective study. Since early treatment of isolated serologic activity in SLE patients has been advocated [31], it would be interesting to study whether empirical steroid treatment of quiescent SLE patients with high urinary T-bet expression could prevent lupus flare, or whether these patients need a higher maintenance dose of immunosuppressive agents.

We found that urinary T-bet expression is predictive of all disease flares and severe flares. In fact, our result would be almost identical if only severe flares with renal involvement were analysed (details not shown). It is, however, possible that many systemic flares may actually have occult renal involvement. In fact, derangement in serum creatinine and proteinuria (i.e. parameters that we used to define renal flare in this study) are late markers of renal disease. On the other hand, the elevated urinary T-bet expression might have come from passenger lymphocyte from systemic circulation and therefore reflected systemic immunologic activation. Unfortunately, we did not study T-bet expression in peripheral blood for comparison. In our previous report, patients with proliferative lupus glomerulonephritis (either class III or IV) had significantly higher urinary expression of T-bet than those with type V lupus nephritis [24]. Since the number of patients with renal biopsy during the follow-up period was small and only one had membranous nephritis during follow-up, we are also unable to perform further sub-group analysis for individual class of lupus nephritis.

As described in our previous reports [24, 32], the major cellular compositions in the urinary sediments were red blood cells, lymphocytes and epithelial cells. Both T and B lymphocytes, as well as macrophages, could be found in the urinary sediment. In addition, CD3+ and CD20+ lymphocytes were both markedly increased in the urine of the SLE patients with current or previous renal injury [32]. Given that T-lymphocyte is the common cell type that expresses T-bet [33, 34], the infiltrating CD3+ T-lymphocyte is probably the major source of T-bet mRNA in urinary sediment. However, our previous study also showed that T-bet and GATA-3 were expressed, albeit at a low level, by renal tubular cells in kidney biopsy of SLE patients [24]. Since immunohistochemistry is a semi-quantitative method, further experiment with laser micro-dissection of kidney biopsy tissue [35, 36] may be necessary to investigate the relative contribution of glomerular and tubulointerstitial inflammation in detail.

Theoretically, it is the degree of Th1/Th2 imbalance that predicts the risk of lupus flare [19–22]. Since the two transcription factors T-bet and GATA-3 promote Th1 and Th2 pathways, respectively, the ratio of T-bet to GATA-3 expression may be the preferred parameter to represent the degree of Th1/Th2 imbalance. In fact, re-analysis of our result by using urinary T-bet to GATA-3 expression ratio gives a similar, but not better, result. We believe that the assay of urinary T-bet expression would be a convenient option for clinical application.

In the present study, the gene expressions of T-bet and GATA-3 were measured by their relative level to a house-keeping gene. As a result, we cannot define a cut-off level of T-bet expression for clinical use. Nevertheless, our results provide the proof-of-principle evidence that measurement of gene expression in urinary sediment has potential clinical and research applications in SLE and possibly other renal diseases. Further studies are needed to standardize the batch-to-batch comparison before the test is applicable for routine clinical practice.

### Table 1. Multivariate Cox model for the time to lupus flare

<table>
<thead>
<tr>
<th>Factor</th>
<th>Adjusted hazard ratio</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All lupus flares</td>
<td>1.08</td>
<td>1.04–1.13</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sex*</td>
<td>5.76</td>
<td>1.66–20.06</td>
<td>&lt;0.006</td>
</tr>
<tr>
<td>T-bet expression</td>
<td>1.13</td>
<td>1.07–1.19</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sex*</td>
<td>15.04</td>
<td>3.76–60.27</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Male vs female.
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The authors have declared no conflicts of interest.

References


