Association of FOXP3 Regulatory Gene Expression with Systemic Lupus Erythematosus Disease Activity among Egyptian Patients

Amal A Abbass, Nesrine A Mohamed, Asmaa SM Abdel-Rehim
Departments of Clinical Pathology & Immunology and Internal Medicine Allergy & Clinical Immunology, Faculty of Medicine, Ain Shams University, Cairo, Egypt.

The concept that regulatory T cells (Treg) play a key role in both development and maintenance of autoimmune response in rheumatological diseases is well accepted. In recent years, several studies analyzed Treg cell phenotype and function in systemic lupus erythematosus (SLE), the prototypical systemic autoimmune disorder in humans. The forkhead family transcription factor FOXP3 currently represents the most specific marker molecule for T cells with suppressive/regulatory capacity (Treg). Using real-time polymerase chain reaction, we quantified messenger RNA (mRNA) expression of FOXP3 in peripheral blood mononuclear cells (PBMCs) of 19 subjects with active SLE, 16 with inactive lupus and 20 healthy subjects. Relative FOXP3 gene expression was assessed by the comparative C_T method in which FOXP3 gene expression was normalized to GAPDH gene expression in each sample. Our preliminary investigations demonstrated higher FOXP3 expression in active SLE patients as compared to inactive SLE (median 8.83 and interquartile range 0.74-347.0 versus 0.426 and 0.04-3.93 respectively; P<0.05). Compared to the control group (median of 0.01 and IQR 0.011-0.07), both active and inactive SLE patients showed increased expression for FOXP3 with P value <0.01, respectively. In the active group, FOXP3 mRNA level correlated positively with disease activity as assessed with the SLEDAI index. However, this correlation did not reach statistical significance (r=0.122; P = 0.08).

Immunological tolerance is a key feature of the immune system that allows the discrimination between self from non-self, providing defense against foreign pathogens while preventing autoimmunity. This ability of the immune system is controlled by mechanisms of central and peripheral tolerance. Central tolerance involves deletion of self-reactive T cells in the thymus at an early stage of development (Viglietta et al., 2004). However, this mechanism alone is not sufficient for preventing autoimmunity, as autoreactive T cells are also detected in healthy individuals. A second mechanism involves the thymic selection of a population of regulatory T cells (Treg), which dominantly prevents both the activation and the effector function of autoreactive T cells that have eluded other mechanisms of tolerance. The depletion or inactivation of Treg leads to the development of a wide range of autoimmune and inflammatory manifestations (Barreto et al., 2009). Natural CD4^CD25^ Treg express the transcription factor FOXP3, which controls their development and function (Hori et al., 2003). Functional defects in the FOXP3 gene lead to the absence of Treg production and to immunodysregulation, polyendocrinopathy and enteropathy, X-linked Syndrome (IPEX) (Wildin et al., 2002). Systemic lupus erythematosus (SLE) is a disorder of generalized autoimmunity characterized by pathogenic autoantibodies and immune complexes that are attributed to inappropriate regulation of hyperactivated B and T cells, defective clearance of apoptotic cells and immune complexes, and loss of immune tolerance (Mok & Lau, 2003). The role of T cells in the pathogenesis of SLE is revealed by a defect of T-cell-mediated immunological tolerance and by their support of autoantibody production by autoantigen-reactive B cells (Hoffman, 2004). The etiology of SLE is complex. Multiple susceptibility genes and
environmental factors are implicated in the initiation and perpetuation of the activation of T and B lymphocytes. Because spontaneous remission or improvements in the course of the disease are common in patients with SLE, dysfunctional regulatory cells may contribute to the onset and exacerbation of SLE (Lee et al., 2006). The aim of this study was to investigate the abnormalities in FOXP3 gene expression among Egyptian SLE patients and its relationship to disease activity.

Materials and Methods

Patients

This study included 35 adult females patients aged 30.6 ± 9.22 (range 18–50 years), presenting at outpatient Rheumatology clinics of Ain-Shams University Hospitals. These patients met the American College of Rheumatology revised criteria for systemic lupus erythematosus (Hochberg, 1997).

A written informed consent was taken from all participants prior to enrolment in this study which was approved by the Ain Shams Medical Research Ethics Committee.

Patients were subjected to thorough clinical examination with focus on disease duration and disease activity in the last 6 months prior enrollment. Disease activity was assessed by measuring serum levels of anti-double stranded DNA (dsDNA) antibody titer and Disease Activity Index (SLEDAI) scores. Serum levels of anti–double-stranded DNA antibodies (anti-dsDNA) were detected by indirect immunofluorescence (Diasorin, USA). Serum creatinine, proteinuria and urea were recorded. Proteinuria was reported as urinary protein-to-creatinine ratio. According to the SLEDAI index (Lam and Petri, 2005) 19 of these patients had active disease (>4.0 points), and 16 patients were inactive (≤3.0 points) with or without immunosuppressive treatment. We excluded patients with a history of infection within 3 wk and comorbidities, such as diabetes mellitus. Twenty healthy individuals, with no history of autoimmune disease, comparable as regards age and sex were also included as controls.

Detection of FOXP3 gene expression by real-time polymerase chain reaction (PCR) analysis

- Preparation of peripheral blood mononuclear cells
  A total of 8 ml EDTA anti-coagulated venous whole blood from each donor was layered on a Ficoll-Paque density gradient and centrifuged at room temperature. The peripheral blood mononuclear cell layer was collected, washed and resuspended in 500µl phosphate buffer saline.

- RNA extraction, first-strand cDNA synthesis, and quantitative real-time polymerase chain reaction
  Total RNA was isolated from 2 × 10^6 PBMC using the RNeasy Mini Kit (QiAampminikit, Qiagen, USA). cDNA was synthesized by incubating 10µl total RNA (contain up to 1 µg RNA) in a 20 µl reaction mixture consist of RT Buffer, and RT Primer Mix. The entire reaction takes place at 42°C and is then inactivated at 95°C using QuantiTect® Reverse Transcription kit (QIAGEN, USA).

  Real-time PCR was performed on the Stratagene Mx3000P Real-Time Thermocycler (Corbett Research, Australia) using SYBR Green QuantiTect® Primer Assay PCR Master Mix (Qiagen, USA). 5 µl (up to 100 ng) of cDNA was used in each 25 µl PCR reaction containing 12.5µlSYBR Green PCR Master Mix, 2.5µl PCR primers specific for human Foxp3 {FOXP3_1_SG QuantiTect Primer Assay (QT00048286) (Qiagen, USA)} and 5µlRNase-free water. For each sample another reaction set was made for the internal control gene {human GAPDH (glyceraldehyde-3-phosphate dehydrogenase)} by using human GAPDH primer instead of human Foxp3 primer. The reactions consisted of an initial denaturation step at 95°C for 5 min followed by 35 denaturation at 95°C for 5s then combined annealing and extension at 60°C for 10 s. PCR reaction with human GAPDH primer was used as an internal control.

  Two different methods of presenting quantitative gene expression exist: absolute and relative quantification. Absolute quantification calculates the copy number of the gene usually by relating the PCR signal to a standard curve. Relative gene expression presents the data of the gene of interest relative to some calibrator or internal control gene. A widely used method to present relative gene expression is the comparative C(T) method also referred to as the 2 (-DeltaDeltaC(T)) method.

  In this study, we used the comparative C_T method to analyze our data as follow: for each unknown sample as well as for the calibrator sample

\[
\Delta C_T (\text{sample}) = C_T \text{FoxP3 gene} - C_T \text{reference gene (GAPDH test)}
\]

\[
\Delta C_T (\text{calibrator}) = C_T \text{FoxP3 gene} - C_T \text{reference gene (GAPDH cal)}.
\]

Next, the ∆ΔCT value for each sample is determined by subtracting the ∆CT value of the calibrator from the ∆CT value of the sample.
\[ \Delta \Delta CT = \Delta CT (\text{sample}) - \Delta CT (\text{calibrator}) \]

The normalized level of target gene expression is calculated by using the formula:

Normalized target gene expression level in sample = \[ 2^{-\Delta \Delta CT} \]

**Statistical Analysis**

Statistical analysis was performed utilizing statistical analysis for social science (SPSS) version 16. Demographic and clinical parameters exist in a normal distribution, and the results were reported as mean ± SD. As the results for FOXP3 gene expression did not conform to the normal distribution, the differences among groups were also assessed with a non-parametric Mann–Whitney U-test, and results were reported as the median and interquartile range. For all tests, \( P<0.05 \) was considered statistically significant.

**Results**

This study was conducted on 35 adult females patients aged 30.6 ± 9.22. Nineteen (19) of these patients had active disease while 16 patients were inactive. Table 1 shows demographic and clinical characteristics of the patients and controls.

<table>
<thead>
<tr>
<th></th>
<th>Active</th>
<th>Inactive</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case No.</td>
<td>19</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td>Age (years)(^{\dagger})</td>
<td>30.21±10.66</td>
<td>31.06±7.48</td>
<td>NS</td>
</tr>
<tr>
<td>Disease duration (years)(^{\ast})</td>
<td>4.0 (2.0-5.0)</td>
<td>3.25 (1.5-5.0)</td>
<td>NS</td>
</tr>
<tr>
<td>SLEDAI score(^{\ast})</td>
<td>10.0 (4.0-11.5)</td>
<td>2.0 (1.0-3.0)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Anti-dsDNA Titer(^{\ast})</td>
<td>408.42±68.44</td>
<td>719±3.75</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Serum creatinine(mg/dl)(^{\ast})</td>
<td>1.88±0.9</td>
<td>0.76±0.34</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>ESR (mm/hr)(^{\dagger})</td>
<td>69.11±31.1</td>
<td>6.33±1.43</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Bun(mg/dl)(^{\dagger})</td>
<td>38.59±17.7</td>
<td>17.63±5.68</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Protein-creatinine ratio(^{\ast})</td>
<td>2.14±0.77</td>
<td>0.22±0.14</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

\(^{\dagger}\) Data are presented as mean±SD., \(^{\ast}\) Data are presented as median (IQR)

SLEDAI, Systemic Lupus Erythematosus Disease Activity Index

\( P>0.05 \) is not significant (NS).

FOXP3 showed higher expression in active SLE patients compared with those within active SLE (median 8.83 and interquartile range 0.74-347.0 versus median 0.426 and 0.04-3.93 respectively; \( P<0.05 \)).

Compared to the control group which has median of 0.01 and IQR 0.011-0.07 both active and inactive SLE patients show increased expression for FOXP3 with \( P<0.01 \), respectively (Figure 1).
Association of FOXP3 Regulatory Gene Expression with SLE Disease Activity among Egyptian Patients

The FOXP3 gene expression was correlated positively with SLEDAI score, in patients with active SLE. However, this correlation did not reach statistical significance ($P = 0.08$). We related these findings to the small patients numbers. Additionally, the FOXP3 gene expression showed a positive correlation with disease duration in patients with active SLE with $P$ value $< 0.05$. There was no significant correlation was found between FOXp3 gene expression and Anti-ds DNA antibody titer, ESR, serum creatinine and proteinuria in the active group (Table 2).

There was no statistically significant correlation was found between FOXp3 gene expression and the studied parameters in the non active group (Table 3).

No significant difference was found in FOXP3 expression between patients receiving pulse steroids and patients receiving full dose steroids and patients receiving minimal steroid dose with $P$ value $> 0.05$ (Table 4 and Figure 2).

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**Table 2.** Correlation between FOXP3 gene expression and SLEDAI score, ESR, serum creatinine, Anti-dsDNA, proteinuria and disease duration in the active group.

<table>
<thead>
<tr>
<th></th>
<th>FOXP3 gene expression</th>
<th>$r$</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLEDAI score</td>
<td>0.122</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>ESR</td>
<td>-0.179</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Creatinine</td>
<td>-0.218</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Anti-dsDNA</td>
<td>0.252</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Protein-creatinine ratio</td>
<td>0.243</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Duration of disease</td>
<td>0.382</td>
<td>$&lt;0.05$</td>
<td></td>
</tr>
</tbody>
</table>

$P>0.05$ is not significant (NS).

**Table 3.** Correlation between FOXP3 gene expression and SLEDAI score, ESR, serum creatinine, Anti-dsDNA and proteinuria in the inactive group.

<table>
<thead>
<tr>
<th></th>
<th>FOXP3 gene expression</th>
<th>$r$</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLEDAI score</td>
<td>0.356</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>ESR</td>
<td>-0.100</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Creatinine</td>
<td>-0.218</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Anti-dsDNA</td>
<td>0.328</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Protein-creatinine ratio</td>
<td>0.479</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

$P>0.05$ is not significant (NS).
Table 4. Comparison of FOXP3 gene expression and SLEDAI score among patients with Systemic Lupus Erythematosus, taking different doses of steroids.

<table>
<thead>
<tr>
<th></th>
<th>Patients taking Pulse steroid (n=13)</th>
<th>Patients taking Full dose steroid (n=7)</th>
<th>Patients taking minimal dose steroid (n=15)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOXP3</td>
<td>8.8 (0.97-327.0)</td>
<td>6.96 (0.33-3141.0)</td>
<td>0.14 (0.03-5.4)</td>
<td>NS</td>
</tr>
<tr>
<td>SLEDAI score</td>
<td>6.00 (4.0-10.0)</td>
<td>10.00 (7.0-16.5)</td>
<td>2.00 (2.0-4.0)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

P>0.05 is not significant (NS).

Figure 2. Comparison of mRNA expression of FOXP3 gene expression in peripheral blood mononuclear cells among patients taking different doses of steroids. The data show the median (IQR), normalized to GAPDH (FoxP3/ GAPDH). Overall Kruskal–Wallis test, P>0.05.

Discussion

CD4+, CD25+ Treg cells are critical for maintaining self tolerance and preventing autoimmunity. In the present study, we found that FOXP3 gene expression is higher in patients with active lupus compared to those with inactive lupus. On a first glance, it may appear surprising that a higher FOXP3 gene expression, implying a higher activity of Tregs, is related to more severe SLE. While recent studies showed a reduced number or a functional defect of natural Tregs in patients with active SLE as compared to patients with quiescent lupus or normal subjects, and the diminished proportion of circulating Tregs inversely correlated with lupus disease activity (Bonelli et al., 2008). This apparent paradox is probably because CD4 T cells other than natural Tregs also express FOXP3 during activation in active SLE (Walker et al.,...
2003; Morgan et al., 2005), although it remains controversial whether this activation-induced FOXP3 gene expression indicates a functional Treg capacity (Banham et al., 2006; Bonelli et al., 2008). There is also a possibility that the immune response during active SLE includes the activation of destructive effector cells as well as protective Tregs, which therefore results in activation-induced FOXP3 gene expression (Muthukumar et al., 2005). Our result is in line with the above observations and further supported by Wan and Flavell (2007) study which noticed significant elevation in FOXP3 gene expression in asthmatic patients compared with control group. They concluded that this elevation is a defense mechanism against the inflammatory process occurring in asthma through the release of suppressive cytokines by T reg.

Moreover, Alvarado-Sanchez et al. (2006) reported no significant correlation between disease activity and the number of Tregs. Taken together, available evidence suggests that the overall expression of FOXP3 is increased in active SLE, but the suppressive function of CD4CD25Tregs may be defective.

There are other two possibilities for the inconsistency between the decreased number of Tregs and high expression of FOXP3 in this situation. Firstly, it had been reported that both normal and mutated FOXP3 alleles are equally expressed in the T cells of healthy carriers of immune dysregulation polyendocrinopathy X-linked syndrome and that increased numbers of FOXP3-positive Tregs are responsible for the control of pathogenic T cells (Tommasini et al., 2002; Fehervari & Sakaguchi, 2004). It is therefore possible that some kind of functional compensation (FOXP3 overexpression) might be present in the reduced numbers of Tregs to regain immune control in patients with active SLE. The second possibility is that increased mRNA expression of FOXP3 is associated with steroid usage. According to Christian Karagiannidis et al. (2004), FOXP3 mRNA expression is significantly increased in asthmatic patients receiving glucocorticoid. Our SLE patients with active disease received more prednisolone than the inactive SLE patients, implying that such treatment might have some impact upon the FOXP3 gene expression. However, In the present study, no significant difference was found in FOXP3 gene expression between patients receiving pulse steroids and patients receiving full dose steroids and patients receiving minimal steroid dose on the other hand, this could be attributed to the small number of the selected group of patients and the lack of a follow up study which is needed to assess the patients before and after treatment so the effect of therapy on the FOXP3 gene expression could be evaluated.

However, our finding still in partial agreement with Kagoshima et al. (2005) that reported an increased FOXP3 level in asthmatic patients on steroids more than asthmatics not on steroids although increase dose of steroid was not associated with increase in FOXP3 expression either in moderate or severe asthmatic groups.

Lee et al. (2006) found increased FOXP3 gene expression in active SLE disease while Yan et al. (2008) found no difference in FOXP3 expression at either the mRNA or protein level in any CD4+, CD25+ T cell subset from SLE patients as compared with Controls. Additionally, they found that IFN alpha producing APCs from active SLE patients were found to be responsible for the decreased suppressive capacity of Treg (Golding et al., 2010; Yan et al., 2008), this observation was supported by Alvarado-Sanchez et al. (2006) who reported a significant inverse correlation between disease activity of SLE and the suppressive function of Treg. In this current study, there was increased FOXP3 expression in patients with active SLE. Additionally, FOXP3 gene expression was
correlated positively with SLEDAI score. However, this correlation did not reach statistical significance ($P = 0.08$). These findings could be attributed to the small patients numbers.

In this study, FOXP3 gene expression was not correlated with anti ds-DNA titer, in patients with active SLE, this could be attributed to that the high dose of drug treatment may affect the levels of autoantibodies in patients with active SLE (Suen et al., 2008).

Foxp3 gene expression was positively correlated with disease duration, in patients with active SLE. In view of this data, we can hypothesize that the increase in Foxp3 gene expression could be an autoregulatory mechanism to induce T cell anergy (Prado et al., 2011).

In the present study, FOXP3 gene expression was not correlated with proteinuria. This finding could be explained that proteinuria may not necessarily indicate ongoing inflammation in the kidneys and may be contributed by pre-existing chronic lesions or recent damage in the kidneys during the course of the disease.

Subsequently, the use of FOXP3 as a Treg cell marker must be done cautiously, especially in patients with systemic inflammatory diseases or those under corticosteroid treatment.

**References**


