PD-1 and NFATc1 as promising immunotherapy for SLE patients
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ABSTRACT

Background: Systemic lupus erythematosus (SLE) is a chronic, autoimmune disease, with a wide range of clinical symptoms. This study aims to investigate PD-1 and NFATc1 gene expression in SLE patients.

Methodology: In this study, 50 diagnosed SLE patients (25 females as early diagnosed SLE without treatment, 25 females as SLE patients under treatment with (prednisolone, hydroxychloroquine) and 25 healthy individuals as control whose ages arrange from 20 to 45 years were included. The gene expression level of PD-1 and NFATc1 were assessed by real-time polymerase chain reaction (RT-PCR).

Results: A PD-1 gene expression level (folds) results showed a significant increase in the treated group (59.74 ± 33.26 folds) compared to early diagnosed group (23.18 ± 10.17 folds) and control group (3.46 ± 1.93 folds), and early diagnosed group showed a non-significant increase compared to control group. The NFATc1 gene expression level (folds) results showed a significant increase in the treated group (0.17 ± 0.16 folds) compared to early diagnosed group (0.003 ± 0.002 folds) and control group (0.001 ± 0.0007 folds), and early diagnosed group showed a non-significant increase compared to control group.

Conclusions: Based on these results, PD-1 and NFATc1 is important in the SLE pathogenesis. These results may hold promise for developing a new SLE immunotherapy by focusing on PD-1 and NFATc1.

Keywords: Systemic lupus erythematosus, NFATc1, PD-1

INTRODUCTION

SLE is a severe autoimmune disease. Clinical symptoms of this disease can be mild, transient, or fatal [1]. It is characterized by persistent inflammation and the generation of autoantibodies, which can impact various organs in the body [2, 3]. This disease is multifactorial and is known for an imbalance of the innate and adaptive immune systems brought on by genetic susceptibility and environmental variables [4]. Before the onset of clinical symptoms, the development of autoantibodies against self-antigens may be the disease etiology [5]. Autoantibodies play a pivotal role in the immunological dysfunction observed in SLE [6]. Lack of immunological tolerance to autoantigens like nuclear antigens is a characteristic of SLE [7,8].
SLE patients often suffer from impaired antiviral immunity and are highly vulnerable to develop life-threatening opportunistic infections, which are the main cause of death [9]. SLE is estimated to affect five million people globally, 90% are reproductive age women [10]. The surface protein known as CD279, or "programmed cell death protein 1," is a member of the immunoglobulin superfamily and functions as an immunomodulatory molecule”. Inhibitory signals are delivered by PD-1 when it binds with its ligand PD-L1, which negatively regulates the immunological response following T cell activation and maintains the immune tolerance equilibrium [11]. To stop autoimmune tissue damage, the immune checkpoint molecule PD-1 down-regulates T-cell activation during immunological responses. Long-term antigen exposure leads to PD-1 expression in tumors and chronic infections, which may impair the immune system's ability to eliminate pathogens or deteriorated cells [12]. The use of checkpoint inhibitors (PD-1, PD-L1) is revolutionizing the process to managing oncological diseases [13]. In a number of experimental animal models, such as for SLE, type 1 diabetes (T1D), and multiple sclerosis (MS), rheumatoid arthritis (RA), PD-1 has been demonstrated to maintain the immune system's balance and prevent autoimmunity in different ways [14]. The PD-1 axis in SLE regulates the innate and adaptive immune system subsets [15]. Immune-regulatory molecules such as the PD-1 receptor and its ligands (PD-L1, PD-L2) are connected to the emergence of SLE [16]. The therapy of some tumors involves the use of antibodies that target PD-1 receptors and prevent their activation [17]. The clinical signs of autoimmune disease, such as SLE, may also be brought on by this antibody therapy [18,17]. Polymorphisms in the PD-1 gene have appeared in SLE. PD1.3 and PD1.5 polymorphisms are related to SLE and lupus nephritis vulnerability [16,19]. Only a few research, meanwhile, have up to this point hinted to a potential connection between SLE and PD-1 gene expression. On the other hand, there is another protein which relationship has not been studied for SLE in the literature, yet it is significant in immune response, which is NFATc1. NFATc1 is a transcription nuclear factor of activated T cells (NFAT) family [20]. NFATc1 has been connected to T-cell inducing cell cycle arrest and death [21]. In addition to having lower levels of IgG1 and IgE, mice lacking NFATc1 also produce less IL-4 and Th2 cytokines [22]. According to several studies, NFATc1 plays a role in (Th17) response and it is crucial for T-cell development. Due to the unique functions that different family members have in proliferating and activating T-cell, NFAT inhibitors have been utilized in organ transplantation to reduce transplant rejection and to manage autoimmune diseases [23]. The study aims to investigate PD-1 and NFATc1 gene expression in SLE patients.

METHODOLOGY

Subject collection
From March 2022 to August 2022, this work was performed in the department of rheumatology at Al-Yarmouk Teaching Hospital and the Medical City (Baghdad Teaching Hospital) in Baghdad. included 75 females aged between 20 to 45 years old, it includes 25 females as early diagnosed SLE (without treatment) and 25 females as SLE patients under treatment with (prednisolone, hydroxychloroquine), and 25 females healthy individuals as control. All patients were diagnosed according to clinical examination by a rheumatologist physician and selected based on the SLE revised classification criteria published by the American College of Rheumatology (ACR) [24]. Patients with other diseases (Hypertension, cardiovascular disease, diabetes types I, II) or any other chronic disease including autoimmune diseases were excluded from this study.

Blood collection
All participant patients and control had 0.25 ml of their intravenous blood (whole blood) drawn, and this was added to a 0.75 ml TRIzolTM Reagent in eppendorf tube. By repeatedly mixing up and down, the lysate was homogenized.

Real time PCR Primers
The primers were provided by (Macrogen. USA). The β-Globin primer (Housekeeping) was designed according to Mosafeeri et al. [25] and
PD-1 and NFATc1 as promising immunotherapy for SLE patients

PD-1 primer was designed according to Ghorbani et al. [26], while NFATc1 primer is specially designed for this study by using (Primer 3) program, as shown in the table(1).

**TABLE 1: Design of PD-1, NFATc1 and β-Globin primers.**

<table>
<thead>
<tr>
<th>Name of gene</th>
<th>Primer</th>
<th>Sequences (5’→3’ direction)</th>
<th>Primer size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD-1</td>
<td>Forward</td>
<td>TAGAGAAAGTTTCAGGGAGG</td>
<td>20</td>
<td>Ghorbani et al., [25]</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ATGTGTAAAGGTGGAGG</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>NFATc1</td>
<td>Forward</td>
<td>CACCAAAGTTCAGGAGATCCCA</td>
<td>22</td>
<td>(Primer3) program</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TTCTTCCTCCGGAGATGCTGCCGCTGTCT</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>β-Globin</td>
<td>Forward</td>
<td>ACACACACTGTGTTCACCA</td>
<td>20</td>
<td>Mosaferi et al. [26]</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CAACCTTCATCCACGTTCACC</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

**Extraction of RNA**

TRIzolTM Reagent's protocol (Thermo Scientific, USA) were followed to isolate RNA from the samples.

**RT-PCR and gene expression**

The One Step RT- qPCR kit, (Promega, USA made) used, contains qPCR Master Mix 5 µl, RT mix 0.25 µl MgCl2 0.25 µl, Forward primer 0.5 µl, Reverse primer 0.5 µl, Nuclease Free Water 1 µl, Final Total Volume 10 µl. Add 1 µl of Template and 9 µl of Master mix to an aliquotor for a single reaction.

Next, it was put into a Real-time PCR. The first step involved only a cycle lasting 15 min at 37 °C for c DNA and the preliminary denaturation taking place for 5 min at a temperature of 95°C. Step 2 involved 40 cycles, which included the following: A: 20 s at 95 °C to denaturation of template, B: 20 s at 60°C, 55 °C, and 65°C for PD-1, NFATc1 and β-globin respectively, for the primers for binding to template annealing, for the related primers to be lengthened, it takes 20 s at 72°C. Step 3: Melted green (three cycles for one s at 72 °C to 95 °C). By using the Livak Method, determine the gene expression levels as indicated in:-

\[
\text{Folding} = 2^{\Delta \Delta CT}
\]

\[
\Delta CT = CT \text{ gene} - CT \text{ House Keeping gene}
\]

\[
\Delta \Delta CT = \Delta CT \text{ Treated or Control} - \Delta CT \text{ Control}
\]

**Statistical Analysis**

SPSS Statistical software (IBM SPSS 26.0) was used to examine the data. employed the least significant difference (LSD) test with a lower probability than 0.05 (p <0.05).

**RESULTS**

A PD-1 gene expression level (folds) results showed a significant increase in the treated group (59.74 ± 33.26 folds ) compared to early diagnosed group (23.18 ± 10.17 folds) and control group (3.46 ± 1.93 folds). Early diagnosed group showed a non-significant increase compared to control group, as shown in the tables (2) and (3).
TABLE 2: Fold of PD-1 expression depending on 2-ΔΔCt method

<table>
<thead>
<tr>
<th>Study groups</th>
<th>Mean of PD-1 Ct</th>
<th>Mean of PD-1 ΔCt</th>
<th>Mean of ΔCt of β-globulin</th>
<th>Mean of PD-1 ΔΔCt</th>
<th>2-ΔΔCt</th>
<th>Mean of PD-1 gene folding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>21.17</td>
<td>9.31</td>
<td>11.86</td>
<td>-0.0007</td>
<td>3.46</td>
<td>3.46 ± 1.93 B</td>
</tr>
<tr>
<td>Treated patients</td>
<td>20.66</td>
<td>6.40</td>
<td>14.27</td>
<td>-2.92</td>
<td>59.74</td>
<td>59.74 ± 33.26 A</td>
</tr>
<tr>
<td>Early diagnosed patients</td>
<td>20.33</td>
<td>7.35</td>
<td>12.98</td>
<td>-1.96</td>
<td>23.18</td>
<td>23.18 ± 10.17 B</td>
</tr>
<tr>
<td>P-value (P&lt;0.05)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.146</td>
</tr>
<tr>
<td>LSD</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>37.53</td>
</tr>
</tbody>
</table>

* Different letters denote to the significant difference at P<0.05.

NFATc1 gene expression level (folds) results showed a significant increase in the treated group (0.17 ± 0.16 folds) compared to early diagnosed group (0.003 ± 0.002 folds) and control group (0.001 ± 0.0007 folds). Early diagnosed group showed a non-significant increase compared to control group, as shown in the tables (4) and (5).

TABLE 3: Fold of PD-1 gene in Ct, ΔCt and 2-ΔCt value

<table>
<thead>
<tr>
<th>Study groups</th>
<th>Mean ± SE of PD-1 Ct</th>
<th>Mean ± SE of ΔCt of β-globulin</th>
<th>Mean ± SE of PD-1 ΔCt</th>
<th>Mean ± SE of PD-1 2-ΔΔCt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>21.17 ± 0.91</td>
<td>11.86 ± 0.76</td>
<td>9.31 ± 0.60</td>
<td>0.005 ± 0.003</td>
</tr>
<tr>
<td>Treated patients</td>
<td>20.66 ± 0.86</td>
<td>14.27 ± 0.86</td>
<td>6.40 ± 0.93</td>
<td>0.09 ± 0.05</td>
</tr>
<tr>
<td>Early diagnosed patients</td>
<td>20.33 ± 0.65</td>
<td>12.98 ± 0.72</td>
<td>7.35 ± 0.78</td>
<td>0.04 ± 0.02</td>
</tr>
<tr>
<td>P-value (P&lt;0.05)</td>
<td>0.765</td>
<td>0.026</td>
<td>0.036</td>
<td>0.144</td>
</tr>
<tr>
<td>LSD</td>
<td>0.56</td>
<td>2.38</td>
<td>1.94</td>
<td>0.06</td>
</tr>
</tbody>
</table>

TABLE 4: Fold of NFATc1 expression depending on 2-ΔΔCt method

<table>
<thead>
<tr>
<th>Study groups</th>
<th>Mean of NFATc1 Ct</th>
<th>Mean of NFATc1 ΔCt</th>
<th>Mean of ΔCt of β-globulin</th>
<th>Mean of NFATc1 ΔΔCt</th>
<th>2-ΔΔCt</th>
<th>Mean of NFATc1 gene folding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23.98</td>
<td>12.12</td>
<td>11.86</td>
<td>10.84</td>
<td>0.001</td>
<td>0.001 ± 0.0007B</td>
</tr>
<tr>
<td>Treated patients</td>
<td>24.68</td>
<td>9.24</td>
<td>14.27</td>
<td>7.97</td>
<td>0.17</td>
<td>0.17 ± 0.16A</td>
</tr>
<tr>
<td>Early diagnosed patients</td>
<td>25.12</td>
<td>12.14</td>
<td>12.98</td>
<td>10.86</td>
<td>0.003</td>
<td>0.003 ± 0.002B</td>
</tr>
<tr>
<td>P-value (P&lt;0.05)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.338</td>
</tr>
<tr>
<td>LSD</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.11</td>
</tr>
</tbody>
</table>

* Different letters denote to the significant difference at P<0.05.
PD-1 and NFATc1 as promising immunotherapy for SLE patients

DISCUSSION

PD-1 gene expression

When PD-1 interacts with its ligands, the negative T cell regulator PD-1 causes negative signals to be sent to T cells, making it a possible gene in the development of SLE [27]. PDCD1 gene encodes the PD-1 molecule and functions to maintain peripheral tolerance by adverse regulation of the self-reactive T- and B-cells. There is a chance that the PDCD1 gene could be as a possible predispose gene for SLE [28]. The interaction between PD-1 and its ligands (PD-L) reduces and negatively co-stimulates the auto-reactive T-cell and B-cell by the reducing the production of cytokine[29]. Therefore, impaired PD-1:PD-L function is implicated in several autoimmune disorders, including SLE, according to mounting evidence [30]. Patients with SLE had a significantly rise in levels of PD-1 expression on some cells, such as NK cells and both CD3 T and CD19 B lymphocytes [31]. The higher PD-1 gene expression in patients with SLE is possibly illustrated by that PD-1 may play a role in SLE pathogenesis by other ways than its inhibitory function as a negative costimulatory molecule, it may also be linked to SLE severity and act as a negative feedback mechanisms, for the prevention of the potential tissue damage brought on the excessive autoimmune response in those with SLE [32]. Also, mutations that impair PD-1 regulation which have been linked to SLE in humans may occur[33]. The findings show a risen PD-1 expression in SLE patients may be related to the disease pathogenesis and may be helpful in SLE treatment. The current results supports Bassiouni et al [34] and Jiao et al., [27]. They discovered that compared to the healthy control, SLE patients' levels of PD-1 expression were considerably higher. While the present study contradicts Nishimura et al., [35] which discovered a lupus-like condition in animals lacking the PD-1 protein.

NFATc1 gene expression

Three of the four calcium-regulated NFAT proteins, including NFATc1, are expressed by T cells and are important regulators of T-cell activation, differentiation, and development [36]. NFATc1 has a high expression level in T cell [23]. The higher level of NFATc1 gene expression in those having SLE is explained through that, NFATc1 controls, among many other important genes, expression diverse proinflammatory cytokines and proteins including IFN-γ, IL-4, and IL-17, which is highly involved in the SLE pathogenesis [37,38]. According to the study's findings, NFATc1 plays a part in the pathogenesis and severity of the disease. By focusing on NFATc1, a new SLE immunotherapy may be developed.

CONCLUSION

Based on these results, PD-1 and NFATc1 is important in the SLE pathogenesis. These results may hold promise for developing a new SLE immunotherapy by focusing on PD-1 and NFATc1.

REFERENCES


