Original Research Paper

Gene Expression Profiling of Toll-Like Receptor 4 and 5 in Peripheral Blood Mononuclear Cells of Patients with Systemic Sclerosis

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Abstract: The Toll-Like Receptor (TLR) family is appeared to be expressed in many cell types in the immune system and plays a role in the pathogenesis of various autoimmune diseases. The expression profile and role of TLRs in Systemic Sclerosis (SSc) have been partly explained. It is aimed through this investigation to evaluate the expression pattern of TLR 4 and 5 in Peripheral Blood Mononuclear Cells (PBMCs) from SSc patients. PBMCs were isolated from whole blood of 20 SSc patients and 50 healthy individuals. Total RNA content of leukocytes was extracted. Then, cDNA was synthesized from the mRNA of the cells. Afterward, Quantitative analysis was carried out through Real-Time PCR using the TaqMan Gene Expression Assays. An over expression of TLR5 mRNA in PBMCs from SSc patients was seen in comparison to healthy individuals. Nevertheless, the gene expression of TLR4 in SSc patients remained almost equal to controls. Our findings suggest that over expression of TLR5 in SSc patients may be involved in the pathogenesis of SSc.

Keywords: Scleroderma, TLR4, TLR5, PBMC, Gene Expression

Introduction

Systemic Sclerosis (SSc) is a systemic connective tissue disease, characterized by fibrosis of the skin and internal organs, injuries of the small arteries (microangiopathy) and the presence of autoantibodies (Varga and Abraham, 2007). A pathogenic role in the SSc has been suggested through interactions between modified endothelial cells and cells of immune system in eliciting changed fibroblast functional (Jimenez and Derk, 2004). Furthermore, interplay between environmental factors and genetic modification is thought to be contributing factor in the SSc (Tan and Arnett, 2000). Fibroblasts in SS are distinguished by high amount of collagen synthesis and excess fibrotic condition over degradation is thought to take place (Pattanaik *et al*., 2015).

Toll-Like Receptors (TLRs) are a group of type I integral membrane glycoproteins, which almost 11 families of them have been described in human (Botos *et al*., 2011). TLRs play an essential role in the innate immune system as Pathogen-Recognition Receptors (PRRs) to recognize conserved structures within microbes known as Pathogen-Associated Molecular Patterns (PAMPs) and self-cells derived Damage-Associated Molecular Patterns (DAMPs) (Janeway Jr, 1992). Several DAMPs in distinct pathogens are recognized by TLRs that leads to a signaling cascade and results in production of various pro-inflammatory cytokines, chemokines and type I IFNs (Akira *et al*., 2006).

Regarding to several autoimmune diseases, endogenous ligands or pathogens-activated immune cells and even fibroblasts and endothelial cells in SSc, have become more plausible to have role in such disease. Circulating TLR4 ligands have been identified in serum of SSc patients (Roelofs *et al*., 2005). Ligation of TLR4 can induce an increased production of IL-10 in monocyte-derived Dendritic Cells (moDCs) from SSc patients. Taking interleukin (IL)-10 production is related with the increased level of profibrotic CC Chemokine Ligand (CCL)-18, a profibrotic role for TLR-activated moDCs can be implied (van Lieshout *et al*., 2009). TLR4 has been shown to play a role in SSc patients and monocytes from SSc patients with interstitial lung disease have an enhanced pro-fibrotic phenotype. These

monocytes can differentiate into fibrocytes and secrete collagen in higher amounts after exposure to the LPS, the TLR4 agonist (Mathai *et al.*, 2010). Moreover, activation of fibroblast TLR4 signaling, triggered by damage-associated endogenous TLR4 ligands, results in increased transforming growth factor-β1 sensitivity with increased matrix production and progressive connective tissue remodeling. Accordingly, fibroblast TLR4 serves as the switch for converting self-limited tissue repair into intractable fibrosis (Bhattacharyya *et al.*, 2013). On the other side, TLR5 shows increment in SSc fibroblasts and may have a suppressive impression on collagen expression. Presumably, the over expression of TLR5 in SSc fibroblasts is a negative response against tissue fibrosis (Sakoguchi *et al.*, 2013).

TLRs pathway and overactivity, especially TLR4, have been related to SSc immunopathogenesis through involvement in secretion of chemokines and cytokines. Furthermore, the over expression of TLR4 and 5 genes among patients with SSc has already been observed. Given the before evidences, in this study we aimed to assess expression levels of TLR4 and 5 in Peripheral Blood Mononuclear Cells (PBMCs), of SSc patients as a confirmatory investigation of expression profile of these molecules.

Subjects and Methods

Patients and Healthy Control Subjects

The patients were recruited from Firouzgar Hospital Rheumatology Department and diagnosed as having systemic sclerosis according to the American College of Rheumatology criteria for SSc (LeRoy *et al.*, 1988). Twenty SSc patients and 50 healthy age- and sex-matched healthy volunteers were included in the study (Table 1). Patients had received no immunomodulatory therapy for at least 3 months before they were included in the study. Moreover, SSc patients who were suspicious of other plausible autoimmune disorders were excluded. None of the healthy controls had autoimmune diseases in neither themselves nor their immediate family members. The Human Research Ethics Committees from the Tehran University of Medical Sciences approved this study. Written informed consent was taken by all participants. Blood samples from SSc patients were obtained during clinical diagnosis; as such, samples were obtained from healthy controls. About 5 mL of blood from each subject was collected in EDTA-anticoagulated tubes and ESR blood collection test tubes using venipuncture.

PBMC Preparation and RNA Extraction

Ficoll-Hypaque density gradient centrifugation approach was used in order to isolate PBMCs from peripheral blood of the subjects. Total cellular RNA was extracted using High Pure RNA Isolation Kit (Roche, Germany) according to manufacturer's manuals. Yield and purity of RNA were determined using a NanoDrop spectrophotometer at 260/280 nm (Nano Drop ND-2000 C Spectrophotometer, Thermo Fisher Scientific, USA).

Reverse Transcription and Complementary DNA Synthesis

Complementary DNA (cDNA) was synthesized from the RNA of cells using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany) according to the manufacturer's instruction. Concisely, first 4 µL of isolated RNA (30 µg) through previous procedure were mixed with 1 µL of random hexamer primer and 7 µL RNASe-free H_2O and then incubated at 65°C for 5 min. Afterwards, micro-tubes were chilled on ice and a mixture of reaction buffer 4 µL, RNASe inhibitor 1 µL, dNTP mix 2 µL and reverse transcriptase 1 µL was added to each sample. Samples were immediately incubated at 25°C for 5 min followed by 42°C for 60 min; the reaction was finally terminated by heating at 70°C for 5 min. Reverse transcription was performed with the final volume of 20 µL pre tube.

Real-Time Quantitative Polymerase Chain Reaction

Quantitative analysis was carried out by Real Time PCR using the TaqMan Gene Expression Assays containing the FAM dye-labeled probes (Taq Man Pre-designed Gene Expression Products, Applied Bio systems, Foster City, CA, USA) and StepOnePlus Real-Time PCR System (Applied Bio systems, Foster City, CA, USA). Each reaction mixture contained a total volume of 20 µL (master mix 10 µL, cDNA 2 µL [5 ng mL], assay mix 1 µL and H_2O 7 µL). The Real Time PCR conditions were: 50°C for 2 min, 95°C for 10 min, then 40 cycles of 95°C for 15 sec and 60°C for 1 min. A widely used approach to represent relative gene expression, the comparative C_T method, was exploited to evaluate expression as previously described by (Schmittgen and Livak, 2008). Relative amounts of target mRNAs in test samples were calculated and normalized to the corresponding Beta-actin (ACTB) mRNA transcript level as a housekeeping gene. Then relative expression for each sample was calculated using the following equation: Relative mRNA expression = $(2^{-\Delta Ct}) \times 10^3$.

Statistical Analysis

Data analysis was fulfilled via SPSS software version 18 (SPSS, Chicago, IL, USA). Scale variables were calculated for normality using the Kolmogorov-Smirnov test. Through the independent sample *t*-test, group

comparisons of continues variables were carried out. If the variable was not normally distributed, Mann-Whitney nonparametric test was conducted. The GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com) was applied to illustrate data through graph. All results are expressed as mean ± Standard Error of Mean (SEM) with statistical significance set at 5%.

Results

Demographic traits of SSc patients are listed in the Table 1. Case subjects with the mean age of 40.7±6.9 were found to be age matched with healthy control group with that of 42±11.5 (p>0.05). The participants were also sex matched and of the 20 SSc patients, 15 (75%) and 5 (25%) were male and female respectively; healthy subjects were consisted of 37 (74%) males and 13 (26%) females. A significant difference ($p<0.001$) was observed between ESR values of SSc patients (21.26±16.1) and healthy individuals (4.4±3.82).

No significant difference was observed (fold change = 1.11; p = 0.34) in the expression level of TLR4 between SSc patients and healthy controls (Fig. 1A). Nevertheless, PBMCs from SSc patients expressed the TLR5 mRNA highly (2.49 times up regulated; p = 0.011) in comparison to healthy subjects (Fig. 1B).

Table 1. Baseline Specifications of SSc patients (N = 20)

Characteristic	Mean (%)	Standard deviation
Age	42	11.5
Gender		
Male	15 (75)	-
Female	5 (25)	-
Disease duration	7.05	7.77
Disease subset		
Limited	14 (70)	-
Diffuse	6 (30)	-
modified Rodnan Skin Score (mRSS)	9.5	6.6
Forced Vital Capacity (FVC)	2.5	0.9
Diffusing Capacity for Carbon Monoxide (DLCO)	6.5	2.8
Fibrosis		
Yes	3 (15)	-
No	17 (85)	-
human Placental Alkaline Phosphatase (hPAP)	30.5	7.9
Pulmonary Hypertension (PHT)		
Yes	4 (20)	-
No	16 (80)	-
Muscle weakness		
Yes	2 (10)	-
No	18 (90)	-
Myosis		
Yes	1 (5)	-
No	19 (95)	-
Gastrointestinal (GI) involvement		
Yes	16 (80)	-
No	4 (20)	-
Alveolit		
Yes	1 (5)	-
No	19 (95)	-
Pulmonary Arterial Hypertension (PAH)		
No	15 (75)	-
Primary	4 (20)	-
Secondary	1 (5)	-
Kidney involvement		
Yes	3 (15)	-
No	17 (85)	-
Fluorescent Antinuclear Antibodies (FANA)		
Positive	16 (80)	-
Negative	4 (20)	-
Anti-Centromere Antibodies (ACA) Titer	15.7	6
Anti-topo I (Anti-DNA topoisomerase I)		
Positive	10 (50)	-
Negative	10 (50)	-
Erythrocyte Sedimentation Rate (ESR)	21.26	16.1

Table 2. The correlations of the expressions of TLR4 and TLR5 mRNAs in PBMCs fromSSc patients with clinical manifestations

Parameter	TLR4		TLR5	
	r	P value	r	P value
Age	-0.214	0.379	0.171	0.485
ESR	0.244	0.314	0.275	0.255
mRSS	0.345	0.747	0.125	0.845
FVC	0.225	0221	0.227	0.350
DLCO	0101	0.681	-0.112	0.653
hPAP	-0.017	0.946	-0.103	0.596
ACA Titer	-0.210	0.387	-0.175	0.474

Toll-Like Receptor (TLR), Erythrocyte Sedimentation Rate (ESR), r: Pearson's Correlation Coefficient, Modified Rodnan Skin Score (mRSS), Forced Vital Capacity (FVC), Diffusing Capacity for Carbon Monoxide (DLCO), human Placental Alkaline Phosphatase (hPAP), Anti-Centromere Antibodies (ACA)
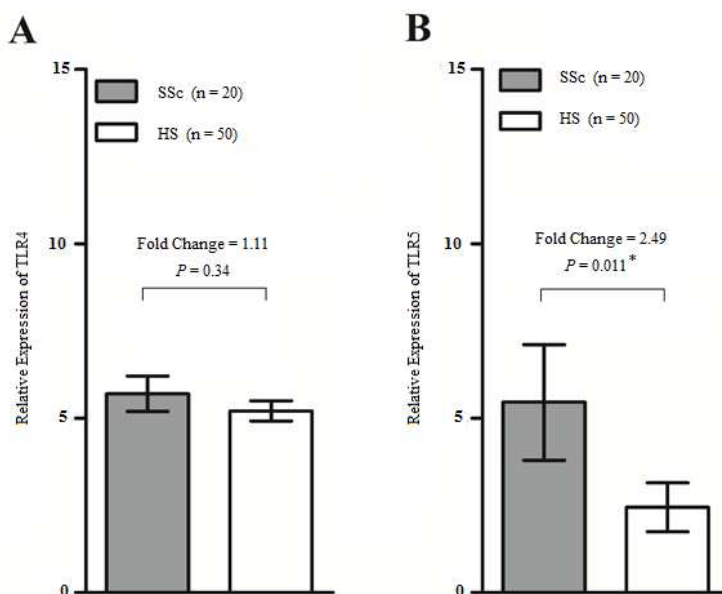

Fig. 1. Relative expression of TLR4 (A) and TLR5 (B) mRNA in PBMCs from SSc patients and healthy subjects is illustrated through Bar graph (SSc: Systemic Sclerosis, HS: Healthy Subjects)
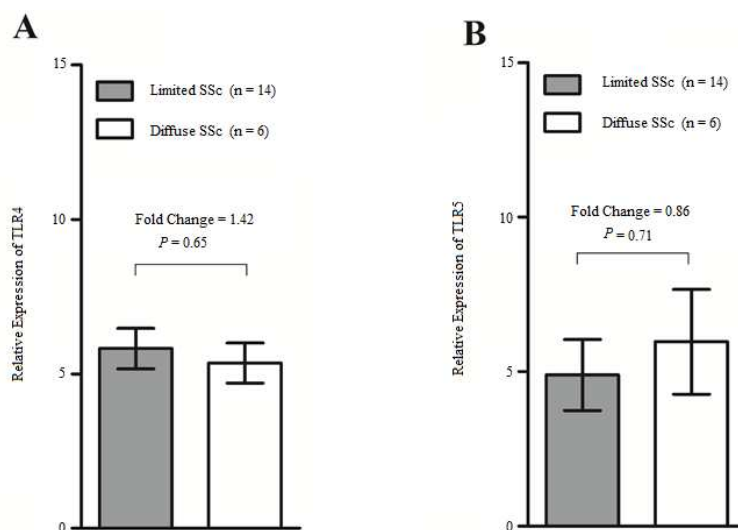

Fig. 2. Relative expression of TLR4 (A) and TLR5 (B) mRNA in PBMCs from Limited and Diffuse SSc patients is illustrated through Bar graph (SSc: Systemic Sclerosis)
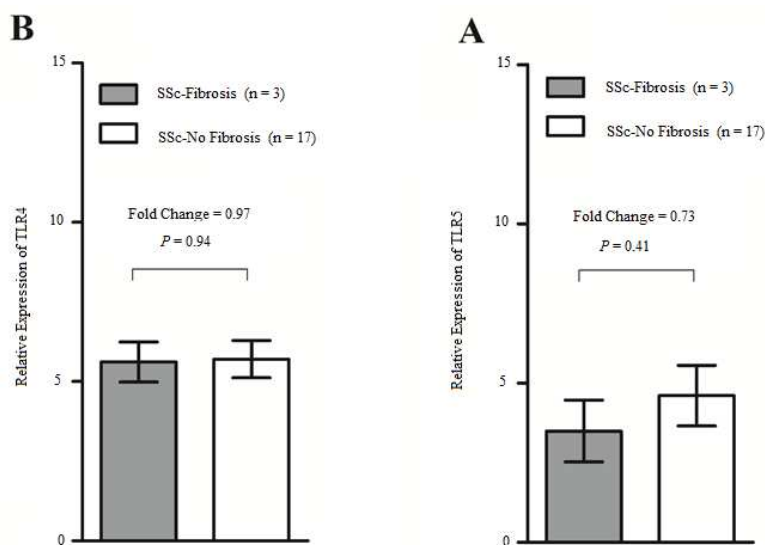
Fig. 3. Relative expression of TLR4 (A) and TLR5 (B) mRNA in PBMCs from SSc patients with and without Fibrosisis illustrated through Bar graph (SSc: Systemic Sclerosis)

Limited subset SSc patients expressed TLR4 higher (fold change = 1.42, p = 0.65) than diffuse subset, but expressed TLR5 lower (fold change = 0.86, p = 0.71; Fig. 2). Both TLR4 and 5 were down regulated (Fig. 3) in SSc patients with fibrosis compared with patients without fibrosis but this differences were not statistically significant (fold change = 0.97; p = 0.94, fold change = 0.73; p = 0.41, respectively).

Clinical specifications of age, ESR, mRSS, FVC, DLCO, hPAP and ACA titer of SSc patients correlated with neither TLR4 nor TLR5 mRNA expression level (Table 2).

Discussion

Current concentrations have been drawn toward the notion that systemic immune dysregulation is the central incident that ultimately may lead to fibroblast activation and development of SSc. Toll-like receptors are main molecules of innate immune system which recognize various DAMPs and PAMPs and relate them to adaptive immune system. A thorough immune response needs the interaction between both innate and adaptive immune systems and revolves around TLRs for activation of several pathways.

Outstanding clinical characteristics of systemic sclerosis are progressive fibrosis of the skin and internal organs. In the pathological point of view, SSc presents core manifestations are inflammation and autoimmunity, excessive deposition of Extracellular Matrix (ECM) and vasculopathy of small arteries (Varga and Abraham, 2007). Generally, the ECM contains fibrillins, collagens, proteoglycans, matrix molecules and fibroblasts and, myofibroblasts, which are pivotal effect or cells in

development and pathogenesis of SSc (Varga, 2007). Transforming growth factor β (TGF-β) can be produced and secreted by both resident and infiltrating cells in the dermis and can activate fibroblasts and induce differentiation into myofibroblasts (Kissin *et al.*, 2006; Tomasek *et al.*, 2002). The myofibroblasts, in turn, produce even more ECM, causing fibrosis and alongside with dermal fibroblasts secret various inflammatory cytokines and chemokines, like IL-6 and CCL-2 which are important inflammatory mediators in the pathogenesis of SSc (Feghali *et al.*, 1994; Distler *et al.*, 2001). Early skin biopsies of SSc patients show infiltration of mononuclear inflammatory cells around the vessels. Mononuclear cells through involvement of PRRs secret cytokines and chemokines that lead to over recruiting of inflammatory cells and promoting of the ECM deposition (Fleischmajer *et al.*, 1977).

Recent studies focused on patients with SSc have disclosed a dysregulation of type I interferon (IFN) pathways. The presence of a type I IFN signature in peripheral blood cells from patients with SSc has been described by expression profiling investigations (Tan *et al.*, 2006). Type I IFNs play role in the regulation and modulation of the immune system. Furthermore, they can modify several processes in the immune system such as differentiation, survival, proliferation and cytokine production of various immune cells. One of the immunoregulatory functions of IFNs is to stimulate immune cells to express the TLRs on themselves. TLRs are a group of Pattern Recognition Receptors (PRRs) in the innate immune system that sense common molecular patterns found on microbes and then initiate key inflammatory responses (Takeda and Akira, 2004). TLR activation can also trigger production and secretion of

various inflammatory mediators like type I IFNs. It has been demonstrated that the interaction between TLRs and IFNs plays a role in the pathogenesis of autoimmune diseases, such as systemic lupus erythematous, which shows overlapping autoantibody specificities and sometimes overlapping clinical manifestations with SSc (Crow *et al.*, 2003; Hua *et al.*, 2006).

Fineschi *et al.* (2008) demonstrated that autoantibodies in SSc patients, which bind to the surface of fibroblasts, might contribute to the pathogenesis of SSc. They concluded that fibroblasts-binding autoantibodies, through up regulation of production of profibrotic and proangiogenic chemokines, in a proteasome and TLR4-dependent approach, play a role in SSc development (Fineschi *et al.*, 2008). Van Lieshout *et al.* (2009) observed an increased serum level of CCL18 in SSc patients compared with healthy controls and this chemokine, which was mediated, at least partly, via IL-10 after TLR4 stimulation, was secreted by monocyte derived dendritic cells. The ongoing study investigated expression level of TLR4 mRNA in the PBMCs of SSc patients. Interestingly, we observed that TLR4 was not expressed differently in SSc patients compared with healthy subjects. Perhaps TLR4 on the specific cells other than a general immune cell population of PBMCs, such as monocyte derived dendritic cells and fibroblasts, contributes to the development of SSc.

Sakoguchi *et al.* (2013) investigated the expression profiles of TLR-related genes in SSc fibroblasts. They disclosed that TLR5 and TLR10 expressions were increased in SSc fibroblasts both *in vitro* and *in vivo*. Furthermore, they noted that TLR5 itself might have a suppressive impression on collagen expression and perhaps the over expression of TLR5 in SSc fibroblasts was the negative feedback against tissue fibrosis (Sakoguchi *et al.*, 2013). To concord with the evidence, in this investigation the expression level of TLR5 was realized to be 2.49 times up regulated in PBMCs from SSc patients compared with healthy individuals. However, probable impression and function of increased expression level of TLR4 in PBMCs from SSc patients need further studies.

Even though SSc patients over expressed TLR5 mRNA, ESR values did not correlate with expression level of TLR5 mRNA. This prematurely imply to the note that, despite increased expression of TLR5 suppresses collagen expression, this increment do not affect inflammatory situation according to ESR level. Furthermore, no correlation was observed between other parameters, namely Age, mRSS, FVC, DLCO, hPAP and ACA titer and TLR5 expression level.

Conclusion

The conclusion to be reached is that, our results imply that dysregulation of TLR5 expression, but not TLR4, may be associated with disease development in SSc patients. Hopefully, based upon beneficial effect of TLR5 increased expression in suppression of collagen production and increased ECM deposition, further studies will provide new strategies for TLR pathway intervention drug development to be therapeutic approaches for SSc patients.

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Author's Contributions

Simin Almasi: Conception and design the experiments, acquisition of data, drafting the article and final approve.

Saeed Aslani: Analysis and interpretation of data, drafting the article, critical review, final approve.

Hadi Poormoghim: Acquisition of data, critical review and final approve.

Ahmadreza Jamshidi: Acquisition of data, critical review and final approve.

Shiva Poursani: Acquisition of data, analysis and interpretation of data, drafting the article, final approve.

Mahdi Mahmoudi: Conception and design the experiments, analysis and interpretation of data, drafting the article, final approve, critical review and final approve.

Disclosure of Conflict of Interest

The authors declare that they have no financial or other conflicts of interest in relation to this research and its publication.

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