Serum Level Determination of Some Immunological Parameters and STAT Genes in Pediatrics SLE Patients Compared to Healthy Control

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Abstract

Systemic lupus erythematosus was defined as an autoimmune chronic systemic rheumatic disease. it has many disease phenotypes and clinical symptoms varying from one patient to another, which include mild mucocutaneous manifestations to severe involvement of the central nervous system and various organs in the body. Several pathogenic pathways of immunity play a part in the progression of SLE. The aims of this study are to determine the serum levels of C3, C4, IL-9, IL-10, IL-12 and INF-gamma and disease activity in pediatric Iraqi (pSLE) patients additionally in this study we aim to find the levels of STAT-alpha and beta gene expression change in patients.30 samples were collected from pediatric SLE patients and 30 healthy samples. The serum levels of all the immunological parameters were evaluated by ELISA and the gene expression of STAT-alpha and beta were evaluated by RT-PCR. The results showed lower level in both complements components while increased levels in all interleukins. The gene expression showed increased levels of both genes.

Key Words: C3, C4, IL-9, IL-10, IL-12, INF-Gamma.

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Introduction

SLE, or systemic lupus erythematosus, is an autoimmune disease that mostly affects women. Children account for about 15 - 20% of all SLE patients. At the onset, they have a more severe disease than adults (Kinsey *et al*., 2018). The important medical and psychosocial cases that have a relationship with a pediatrician who specializes in children with SLE is caused by antibodies attacking cells in host organs such as the joints, brain, blood, kidneys, skin and muscles (Shaikh *et al*., 2017; Rekvig, 2018).

Pediatric SLE (pSLE) is an uncommon disease presenting with a rate of 3.3-8.8 per 100.000 children\ year and a frequency of 0.3-0.9 per 100.000 children\ year. A higher frequency of pSLE has been reported in the populations of Asia, Africa, America, Hispanics and Native Americans Asians, Africans, Americans, Hispanics and Native Americans (Kinsey *et al*., 2018; Mok, 2015). Also, several recent studies indicated that the average age at which pSLE expresses itself is between 11 and 12 years; and its appearance was very rare under the age of 5 years.

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While studies have proven that the presence of SLE in adults has registered nearly 80% of the patients, and most of them were female (Aggarwal and Srivastava, 2015; Mok, 2015).

Many factors play a crucial role in pSLE development. Many susceptibility sites have been discovered by genome-wide interaction studies (Webber *et al*., 2020), this is partly because it occurs in a wide range of SLE. There are several monogenic disorders that appear to cluster in children and families (Kwon *et al*., 2019).

Th1 plasma levels (IL-2, IFN-γ, TNF), Th2 (IL-4), Th17 (IL-17A, IL-6), and Treg (IL-10) cytokines were measured in a cohort of pSLE patients and healthy controls, the results showed a connection between these cytokines and disease progression (Postal *et al*., 2014). Patients with active disease had higher increased IL-6, IL-10, and IL-17A as candidate biomarkers for disease activity in pSLE patients (Cavalcanti *et al*., 2017; Robert and Miossec, 2019).

A connection has been discovered between a signal transducer and activator of transcription 4 (STAT4) and lower serum IFN- α activity and improved IFN-α signaling sensitivity (Nageeb *et al*., 2018). It's one of the STAT family's six main members, all of which play essential roles in cytokine signaling. STAT4 is required for IL-12 signaling in both T and N cells, increasing IFN- γ production and CD4 T cell differentiation (Piotrowski *et al*., 2012; Goropevšek *et al*., 2016).

This study aims to find the serum concentrations of C3, C4, IL-9, IL-10, IL-12 and INF-gamma and disease activity in pediatric Iraqi (pSLE) patients additionally in this study we aim to find the levels of STAT-alpha and beta gene expression change in patients.

Methodology

Subjects

This study was carried out in Baghdad teaching Hospital and Teaching Laboratories in Medical City. It was conducted during the period from December/ 2019 to December/ 2020.This study involved thirty Iraqi patients who had been diagnosed with Systemic Lupus Erythematosus (SLE). Their age ranged between (6-16) years. They were sequentially visited Baghdad Teaching Hospital. The diagnosis based upon the patients' medical history, physical examination of the consultant and laboratory findings, which included immunological tests (Anti-nuclear antibody (ANA), ds-DNA, Anti-cardiolipin antibody (aCL) by ELISA technique in addition to hematological parameters such as ESR, Hb, WBCs count. Thirty-four blood samples have collected from apparently healthy volunteers with age range from (20-60) years; as a healthy control group. This group constituted of 23 males and 7 females.

Measurement of the Serum Immunological Parameters Levels

The levels of the immunological parameters which included C3, C4, IL-9, IL-10, IL-12, and INF, have been tested by using ELISA technique following the manufacturer procedure provided by the Kits (CAT# Numbers; CSB-E08665h, CSB-E08705h, CSB-E04642h, CSB-E04593h, and CSB-E04599h, respectively).

Measurement of STAT Alpha and Beta Gene Expression Level

The RNA extraction was done by following the procedure provided by the commercial kit (Direct-zol™ RNA MiniPrep, Zymo/USA) and then the conversion of the RNA to cDNA were also done by using the 8µl of the eluted RNA added to the 2 µl of the master mix provided by the kit (PrimeScriptTM RT reagent Kit, TAKARA/ Korea). After that, it was incubated at 37 °C for 30 minutes, the prepared cDNA then was used to do the real time –PCR to evaluate the gene expression level. 3 µl of the previously prepared cDNA were added to 10 µl of the commercial sybr green master mix (Kappa sybr Fast universal, Kappa/ USA) and 0.5 µl of forward and reverse primers, followed by a volume that completed to 20 µl by adding nuclease free water. The real time- PCR procedure was done for both STAT- alpha and beta genes and for GAPDH gene as reference gene. The primers sequences that are used are mentioned in table (1).

Table 1. Primers sequences

NO	Name	Oligo Nucleotide	Tm (Cº)	GC %	Sequence (5′ to 3′.)
1	Stat alpha	Forward	50.4	50	CATCTCAACAATCCGAAGTGATTCA
		Reverse	51.0	44.4	GTCAGAGTTTATCCTGTCATTCAGCAG
2	Stat beta	Forward	50.5	50	TGACCTTGTTATCTCTTTAAGCCGA
		Reverse	51.0	54.4	GTCAGAGTTTATCCTGTCATTCAGCAG
3	GAPDH	Forward	51.5	50	GTCTCTTCTCACTTCATTCAGCG
		Reverse	51.0	44.4	ACCACCCAGTTCGTGTAGCCAA

Results

The serum level of both C3 and C4 showed a significant higher result in control (25.1 and 30.1, respectively) than patients (18.6 and 22.0, respectively). The analysis of variance (T-test) showed a significant difference with respect to serum IL-9 levels between the patients and control ($p < 0.01$). The levels were distinguished to be increased in SLE patients compared to healthy controls (284.0 ± 72.5 vs. 151.1± 50.6, p= 0.001). while the serum level of IL-10 showed a higher level in patients (75.3± 21.2) group than in control (45.1± 13.6). IL-12 recorded with a higher level in patients than in control the IL-12 in patients equal to (91.7± 32.7) and the control level equal to (51.7± 19.9). Analysis of variance (T-test) showed a significant difference with respect to serum IFN-γ levels between the patients and control ($p < 0.01$). The levels were distinguished to be increased in SLE patients compared to healthy controls (132.9 ± 53.7 pg/mL *vs.* 42.8± 35.0 pg/mL). the results are summarized in table (2).

Table 2. The serum level of immunological parameters

test	group	N	Mean	Std. Deviation	p. value
C3 (ng/m)	**patient**	**30**	**18.6667**	**4.76578**	**0.001**
	control	**30**	**25.1**	**3.24143**	
C4 (ng/m)	**patient**	**30**	**22.0667**	**7.24418**	**0.001**
	control	**30**	**30.1667**	**11.7535**	
IL9 (pg/ml)	PATIENTS	30	13.25444	72.59758	< 0.001
	CONTROL	30	9.24262	50.62393	
IL10 (pg/ml)	PATIENTS	30	75.3	21.2151	< 0.002
	CONTROL	30	45.1333	13.64	
IL12 (pg/ml)	PATIENTS	30	91.7	32.7774	< 0.001
	CONTROL	30	51.7333	19.974	
INF (pg/ml)	PATIENTS	30	132.933	53.7054	< 0.001
	CONTROL	30	42.8667	35.0672	

Comprehensive analysis was performed for 3.9.1 Stat- α gene in whole blood from patients and compared to healthy controls. Analysis of the Stat- α gene expression data after normalization with GAPDH revealed that increase level of Stat- α gene in SLE total patients. The fold change (2-ΔΔCt) was (5.8 ± 4.15) indicating a significant overexpression of the gene as given in table (3). Analysis of the Stat – β gene expression data after normalization with GAPDH gene revealed that there is a significant up regulation of stat- β patients compared with controls, the fold change was (2.8± 1.9 *vs.* 1).

Table 3. The gene expression levels comparison between patients and control

test	group	N	Mean	Std. Deviation	p. value
stat- alpha	**patient**	**30**	**5.8379**	**4.15429**	**0.001**
	control	**30**	**1**	**0**	
statbeta	**patient**	**30**	**2.8593**	**1.9511**	**0.001**
	control	**30**	**1**	**0**	

Discussion

The results of this study showed lower level of C3 and C4 were accompanied with the SLE disease, The decrease level of complement C3 and C4 in the serum of SLE patients means that the patient has higher autoantibodies concentration, which is the manifestation of immune activation (Vasilev *et al.*, 2015). This may be attributed to a pro-inflammatory cytokine environment, since pediatric systemic lupus erythematosus has an active clinical phenotype with serious or severe complications (Arora *et al.*, 2012). Since SLE is characterized by the deposition of immune complexes and the formation of autoantibodies, cytokines play a key role in immune system dysregulation in SLE by affecting the differentiation, maturation, and activation of many effector cells, resulting in inflammation and tissue harm. Although there have been many studies of cytokine profiles in adult SLE patients, there have been few studies of cytokines in children with SLE. A previous analysis of pediatric patients found that the levels of IL-10 and INF-gamma were similar to those found in this study (Cavalcanti *et al.*, 2017).

In SLE, IL-10 promotes B-lymphocyte proliferation and differentiation, as well as the formation of autoantibodies by these cells. Continuous anti-IL-10 antibody administration to NZB/W F1 mice postponed the onset of autoimmunity in SLE animal models (Ishida *et al.*, 1994). IFN-gamma has been the archetypal inducer of organ-specific autoimmunity due to the fact that Th1-mediated effects can clarify certain features of autoimmune diseases [64]. IFN-gamma can play a role in autoimmune disease by stimulating the development of complement-activating IgG2a and IgG3 antibodies, as well as triggering macrophage activation and tissue inflammation (Ohl and Tenbrock, 2011). In respect to the IL-9 level there is a lack of studies that targeted the level of IL-9 in pediatric SLE, a previous study agreed with the results of this study that IL-9 increased in patients but in adults and they mentioned that it could be a promising therapeutic agent (Leng *et al.*, 2012), they also mentioned that the symptoms of SLE ranged from rash and arthritis to neuropathies and severe renal disease and those symptoms strongly indicated an influence of mast cells, and Mast cell recruitment and/or aggregation are linked to IL-9.

The Janus kinase (JAK)—signal transducer and activator of transcription (STAT) signaling pathway—has recently been discovered to be abnormal in inflammatory conditions and autoimmune disorders, like SLE. Interferon (IFN)-dependent gene expression requires STAT proteins as a key component and regulate essential cellular processes such as survival, proliferation, and differentiation by signaling over 50 cytokines, hormones, and growth factors (Goropevšek, Holcar and Avčin, 2017). For those reasons it has thought that it has indirect effect on the disease susceptibility. SLE that begins before the age of 18 is known as pediatric -SLE. Early-onset SLE patients have a higher genetic aspect of their condition, more multi-systemic intervention, and a more severe disease path, which has a higher chance of nephritis and end-stage kidney disease. Adult-onset SLE has a lower five- to ten-year mortality rate (Wenderfer and Eldin, 2019).

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