

Pro-apoptotic Bax mRNA expression: A novel predictor for systemic lupus erythematosus disease flare-up

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ABSTRACT

Objectives: In this study, we aimed to better understand the expression of pro-apoptotic Bad and Bax in the pathogenesis of systemic lupus erythematosus (SLE) and their relationship with the disease activity.

Patients and methods: Between June 2019 and January 2021, a total of 60 female patients with SLE (median age 29 years; IQR, 25.0-32.0) and 60 age- and sex-matched healthy female controls (median age: 30 years; IQR, 24.0-32.0) were included. The Bax and Bad messenger ribonucleic acid (mRNA) expression was measured by real-time polymerase chain reaction.

Results: The expression of Bax and Bad was significantly lower in SLE group than the control group. The median value of mRNA expression of Bax and Bad was 0.72 and 0.84, respectively versus 0.76 and 0.89 in the control group. The median value of (Bax*Bad)/ β -actin index was 17.8 in the SLE group and 19.64 in the control group. The expression of both Bax, Bad and (Bax*Bad)/ β -actin index had a good significant diagnostic utility (area under the curve [AUC]= 0.64, 0.70, and 0.65, respectively). The Bax mRNA expression showed a significant upregulation with disease flare-up. The efficacy of Bax mRNA expression in predicting SLE flare-up was good (AUC= 73%). In the regression model, the probability of flare-up reached 100%, with increasing Bax/ β -actin as well, and the likelihood of flare-up increased 10,314 times with every unit increase of Bax/ β -actin mRNA expression.

Conclusion: Deregulation of the mRNA expression of Bax may have a role in the susceptibility to SLE and may be associated with disease flare. A better understanding of the expression of these pro-apoptotic molecules may carry a great potential for the development of specific effective therapies.

Keywords: Apoptosis, bad, bax, flare, systemic lupus erythematosus.

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease with multisystem involvement. The disease has different phenotypes, with different clinical manifestations ranging from mild cutaneous manifestations to multi-organ involvement.¹

Although the definitive cause of SLE is not clearly identified, various studies have shown that defective apoptosis during central and peripheral tolerance process is implicated in the pathogenesis of several autoimmune disease due to escape of autoreactive cells from immune

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tolerance mechanisms.² The role of apoptosis in the pathogenesis of SLE is believed to consist mainly of accelerated apoptosis and improper clearance of cellular debris from apoptotic cells, resulting in an increase in the amount of intact nuclear antigens that can be presented by dendritic cells. These self-antigens are presented to autoreactive T lymphocytes, inducing an autoimmune response.³

Apoptosis is a conserved form of cell death that eliminates unhealthy and unwanted cells. It is tightly regulated and essential for tissue development and homeostasis. The central tolerance depends on the intrinsic pathway that is regulated by the B-cell lymphoma-2 (Bcl-2) family of proteins which is encoded in humans by Bcl-2 gene and it is the principle member of Bcl-2 family proteins which regulate cell death. They are organized functionally and structurally into pro-survival proteins (Bcl-2, Bcl-xL, Bcl-w, A1/Bfl-1, and Mcl-1), multi-domain pro-apoptotic effectors (Bax, Bak, and Bok), and BH3-only pro-apoptotic initiators (Bad, Bid, Noxa, Puma, Bim, Bik, Bmf, Moap-1, and Hrk). The interaction between members of this family of proteins through physical interaction determines the fate of the cell, which of them would survive or suicide.⁴

The Bad, the pro-apoptotic BH3-only protein, is activated transcriptionally, post-transcriptionally and/or post-translationally through a wide range of cytotoxic stimuli. This process is crucial to initiate apoptosis signaling. The Bax, the multi-BH domain pro-apoptotic protein, is required for the permeability of the outer mitochondrial membrane, which is necessary for triggering caspase-mediated cell degradation. It is believed that Bax is activated directly through the binding of certain BH3-only proteins (e.g., tBid, Bim), or indirectly when released from their restraint by pro-survival protein Bcl-2, after being neutralized by the BH3-only proteins.⁵

Abnormal apoptosis with decreased pro-apoptotic gene expression plays a key role in the pathogenesis of many different autoimmune diseases, including SLE.⁶

In the current study, we aimed to examine the expression of pro-apoptotic Bad and Bax and their role in pathogenesis of SLE and to investigate their association with disease activity paving the way for the establishment of new

therapies targeting these molecules, resulting in a more effective and specific disease response.

PATIENTS AND METHODS

This cross-sectional study was conducted at Kasr Alainy Educational Hospital, Department of Internal Medicine, Rheumatology and Clinical Immunology Unit, between June 2019 and January 2021. A total of 60 female patients with SLE (median age 29 years; IQR, 25.0-32.0) and 60 age- and sex-matched healthy female controls (median age: 30 years; IQR, 24.0-32.0) were enrolled. All patients with SLE were recruited from the outpatient clinic and inpatient ward and met the American College of Rheumatology (ACR) classification SLE criteria.⁷ Patients younger than 18 years old and those with other autoimmune diseases, overlap syndromes and mixed connective tissue diseases were excluded from the study.

Clinical and laboratory data

All recruited patients were subjected to full history taking and clinical examination and routine laboratory data. Disease activity was assessed by Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2K) score and patients were categorized according to the degree of disease activity into inactive (SLEDAI-2K=0), low disease activity (SLEDAI-2K<4) and high disease activity (SLEDAI-2K≥4).⁸

Ribonucleic acid (RNA) extraction and complementary deoxyribonucleic acid (cDNA) synthesis

Total RNA from fresh venous blood samples was extracted using the Qiagen QIAamp RNA Blood Mini Kit (Catalog number: 52304; Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The final RNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and RNA purity was verified by an average A260/A280 ratio of 1.98 (range, 1.97 to 2.01).

The cDNA synthesis: RNA was reverse transcribed to cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems®, Branchburg, NJ, USA) in a final volume of 20 µL. We included negative control samples in each set

of reactions. Reactions were incubated at 25°C for 10 min, followed by 37°C for 120 min and final denaturation at 85°C for 5 min. The reaction was carried out in the using Bio-Rad T100™ Thermal Cycler. The cDNA was stored at -20°C

Relative quantification of gene expression by real-time polymerase chain reaction (PCR)

Gene expression of Bad and Bax was measured by using the TaqMan® Amplification System (Applied Biosystems®, Branchburg, NJ, USA). All samples were run in a final reaction volume of 20 µL. The reaction mix was combined using 10 µL TaqMan® Universal PCR Master Mix, 3 µL of cDNA, 6 µL of DNase-free water and 1 µL of specific primers and probes 20×20 (Applied Biosystems®, Branchburg, NJ, USA). Expression of genes Bad Hs00188930_m1 4331182 and

Bax Hs00180269_m1 4331182 were normalized using endogenous housekeeping gene beta-actin and the relative expression of the studied target genes were obtained after normalizing using the geometric average of the housekeeping gene messenger RNA (mRNA) levels.

The PCR run was carried out using the thermal profile 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min on the Rotor gene (Applied Biosystems®, Branchburg, NJ, USA).

The data from TaqMan® gene expression assays obtained at the end of the run were analyzed by viewing the amplification plots for all samples followed setting the baseline and threshold values. The relative standard curve or the comparative cycle threshold (CT) methods was used to analyze data.

Table 1. Demographic character of patients with SLE (n=60)

Characteristics	n	%	Median	IQR
Age (year)			29	25.0-32.0
Disease duration in months			24	20.0-48.0
Positive family history	4	6.7		
High C-reactive protein	6	10.0		
Erythrocyte sedimentation rate			27	20.0-40.0
Oral ulcer	25	41.7		
Ocular manifestations	2	3.3		
Arthritis	33	55.0		
Cutaneous manifestations	6	10.0		
Malar rash	29	48.3		
Nephritis	27	45		
Anemia	13	21.7		
Lymphopenia	16	26.7		
Thrombocytopenia	2	3.3		
Neurologic disorder	3	5.0		
Positive anti-nuclear antibody	60	100		
Positive anti dsDNA antibody	43	71.0		
Low complement	24	40.0		
Positive antiphospholipid antibodies	10	16.7		
Medications				
Cyclophosphamide intake	8	13.3		
Mycophenolatemofetil	4	6.7		
Azathioprine	26	43.3		
SLEDAI score			5.5	2.3-9.0

SLE: Systemic lupus erythematosus; IQR: Interquartile range; anti-dsDNA: Anti-double-stranded deoxyribonucleic acid; SLEDAI: Systemic Lupus Erythematosus Disease Activity Index

Statistical analysis

Statistical analysis was performed using the Minitab for Windows version 17.1.0.0 (Minitab Inc., 2013, PA, USA). The normality of the data was examined using the Shapiro-Wilk test. Continuous data were presented in mean $\pm$ standard deviation (SD) or median (interquartile range [IQR]), while categorical data were presented in number and frequency. Comparison between two groups of continuous data was performed using the independent t-test or Mann-Whitney U test, for parametric and non-parametric variables, respectively, and one-way analysis of variance (ANOVA) for comparison between more than two groups with the Tukey methods as *post-hoc* test. The accuracy of Bax/ β -actin and Bad/ β -actin mRNA expression for predicting SLE and SLE flare-up were assessed using the receiver operating curve (ROC) analysis; assuming that the area under the ROC curve (AUC)= 0.6 was significant with a margin of type 1 error 0.05 and type 2 error 0.2. Probability of SLE flare-up was evaluated using logistic regression models. A two-sided p value of <0.05 was considered statistically significant.

RESULTS

Demographic and clinical characteristics of all participants are summarized in Table 1. The median duration of the disease was 24 (range, 20 to 48) months with a severity score index level of SLDAI 5.5 (range, 2.3 to 9).

The mRNA expression of pro-apoptotic agents (Bax/ β -actin and Bad/ β -actin) was significantly lower in SLE group than the control group (p=0.008 and p<0.001, respectively), as well

as the new modified index, which is a result of (Bax*Bad)/ β -actin (p=0.004) (Table 2). The median value of Bax/ β -actin and Bad/ β -actin was 0.72 and 0.84, respectively compared to 0.76 and 0.89 in the control group. The median value of (Bax*Bad)/ β -actin index was 17.8 in the SLE group and 19.64 in the control group. Detailed descriptive statistical data are provided in Supplementary file (Table 1). The mRNA expression of both pro-apoptotic agents had a good significant diagnostic utility illustrated in Figure 1, as the AUC= 0.64 and 0.70 (p=0.008 and p<0.001, respectively). The (Bax*Bad)/ β -actin index also showed good utility and the AUC was 0.65 (p=0.003) (Figure 1). Furthermore, at a cut-off point below 16.63, 0.66 and 0.8 for index, Bax/ β -actin and Bad/ β -actin, respectively, the specificity and negative predictive value (NPV) were more than 90% and 85%, respectively (Table 3).

On correlating the level of pro-apoptotic agents with disease flare-up, Bax/ β -actin, Bad/ β -actin mRNA expression and their index showed significant downregulation during remission phase of the disease (p=0.01, p=0.002, and p=0.01, respectively) (Table 4). However, the level of those proteins during mild/moderate and severe flare-up phase showed up regulation, although it did not reach statistical significance with the control level (p<0.05) (Table 4). Moreover, the level of pro-apoptotic agents (Bax/ β -actin) only during active disease whatever the grade of activity showed significant upregulation compared to remission phase (p=0.03) (Supplementary File, Table 2). The efficacy of Bax/ β -actin mRNA expression in predicting SLE disease flare-up is shown in Figure 2 with the AUC= 69% (p=0.03).

The sensitivity at cut-off value above 0.58 reached to 96% with NPV about 94% (Table 5).

Table 2. Pro-apoptotic Bax and Bad in correlation with SLE

Factors	Control (n=60)		SLE (n=60)		p†
	Median	IQR	Median	IQR	
Autophagy protein					
Bax/ β -actin	0.76	0.69-0.80	0.72	0.65-0.74	0.008
Bad/ β -actin	0.89	0.84-0.98	0.84	0.74-0.89	<0.001
Index	19.64	17.74-20.9	17.80	15.22-20.53	0.004

Index= (Bax*Bad)/ β -actin; † Mann-Whitney U test; p<0.05 considered significant; IQR: Inter quartile range.

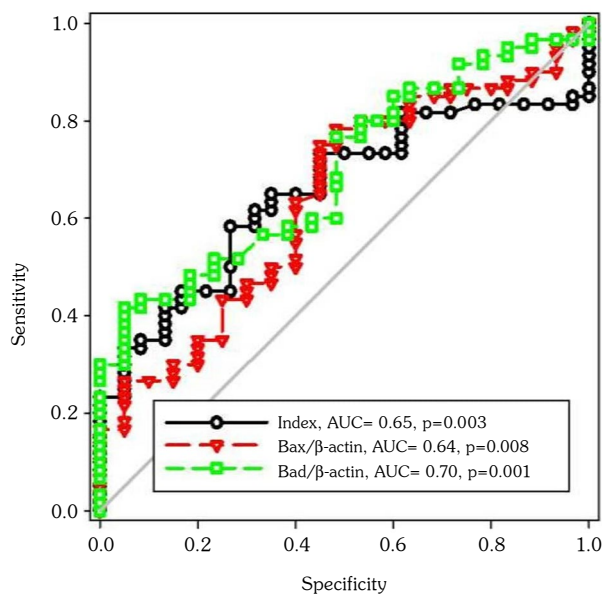


Figure 1. ROC curve of Bax, Bad, mRNA expression and index for discriminating SLE from control subjects. AUC: Area Under the Curve; ROC: Receiver operating curve; mRNA: Messenger ribonucleic acid; SLE: Systemic lupus erythematosus.

In the regression model, the probability of flare-up reached to 100% with increasing the expression of Bax/ β -actin; ($Y' = -7.02 + 9.24 \text{ Bax}/\beta\text{-actin}$), as well, the likelihood of flare-up increased 10,314 times with every unit increase of Bax/ β -actin mRNA expression (odds ratio [OR]=10313.9, $p=0.004$) (Figure 3).

DISCUSSION

Systemic lupus erythematosus is an autoimmune disease of the connective tissue associated with overactive T-lymphocytes, abnormal antigen-presenting cells, and overproduction of autoantibodies with subsequent involvement of many organs.⁹ While the exact etiology of the disease is not known, researchers have advocated that it is likely due to the interaction of several factors, such as hormones, genetic makeup, and environment that includes chemicals, pathogens and radiation. These

Table 3. Diagnostic performance of pro-apoptotic autophagy agent (Bax and Bad) in discriminating SLE from control subjects

Test	Cut-off	Sensitivity (%)	95% CI	Specificity (%)	95% CI	PPV (%)	NPV (%)
Bax/ β -actin	<0.66	27	0.1607 to 0.3966	90	0.7949 to 0.9624	37	85
Bad/ β -actin	<0.80	43	0.3059 to 0.5676	92	0.8161 to 0.9724	53	88
Index	<16.36	35	0.2313 to 0.4840	92	0.8161 to 0.9724	48	87

Index= (Bax*Bad)/ β -actin, SLE: Systemic lupus erythematosus; CI: Confidence interval; PPV: Positive, predictive value; NPV: Negative predictive value.

Table 4. Pro-apoptotic Bax and Bad mRNA expression and their index in correlation with disease flare up phase

Test	Category	n	Mean $\pm$ SD	95% CI	$p^\dagger$
Bax/ β -actin	Remission*	13	0.67 $\pm$ 0.06	0.6139-0.7211	0.01
	Mild/Moderate	38	0.73 $\pm$ 0.11	0.6991-0.7618	
	Severe	9	0.69 $\pm$ 0.17	0.6287-0.7574	
	Control	60	0.75 $\pm$ 0.08	0.7298-0.7796	
Bad/ β -actin	Remission*	13	0.80 $\pm$ 0.07	0.7198-0.8804	0.002
	Mild/Moderate	38	0.79 $\pm$ 0.23	0.7424-0.8363	
	Severe	9	0.83 $\pm$ 0.10	0.7374-0.9304	
	Control	60	0.90 $\pm$ 0.08	0.8628-0.9376	
Index	Remission*	13	16.65 $\pm$ 3.04	14.543-18.757	0.01
	Mild/Moderate	38	17.51 $\pm$ 5.63	16.273-18.738	
	Severe	9	18.27 $\pm$ 5.03	15.74-20.80	
	Control	60	19.24 $\pm$ 1.93	18.259-20.221	

mRNA: Messenger ribonucleic acid; SD: Standard deviation; CI: Confidence interval; † One way ANOVA test with post hoc test "Dunn's methods", $P<0.05$ considered significant

Table 5. Diagnostic performance of pro-apoptotic Bax in predicting SLE flare up

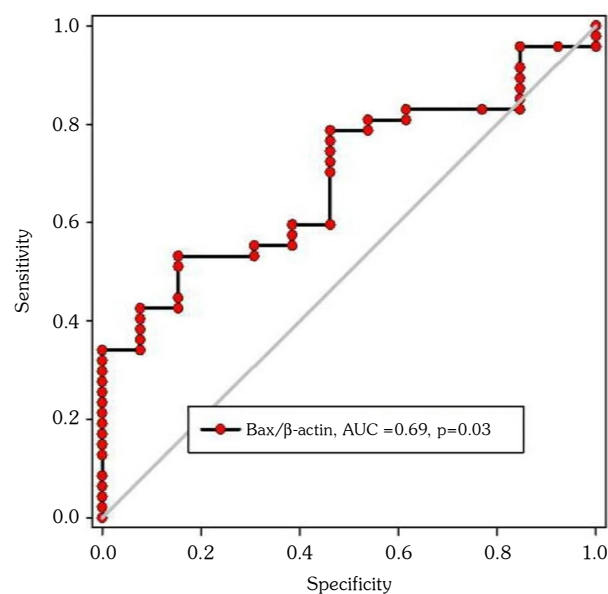
Test	Cut-off	Sensitivity (%)	95% CI	Specificity (%)	95% CI	PPV (%)	NPV (%)
Bax/ β -actin	>0.58	96	0.8546-0.9948	15	0.01921-0.4545	20	94

SLE: Systemic lupus erythematosus; CI: Confidence interval; PPV: Positive, predictive value; NPV: Negative predictive value.

factors can provoke immune dysfunction leading to the pathogenesis of SLE.⁵

Apoptosis is a form of programmed cell death that clears senescent, diseased, and dead cells. The main features of apoptosis are cellular shrinkage, membrane blebbing, and chromatin condensation. To date, researchers have identified two different apoptotic signaling pathways: internal and external. Several death factors, such as tumor necrosis factor- α (TNF- α) TRAIL, and FasL can activate the extrinsic pathway, while the intrinsic pathway is triggered by DNA damage, cytokine withdrawal, endoplasmic reticulum stress or deficiency of nutrient support.⁴

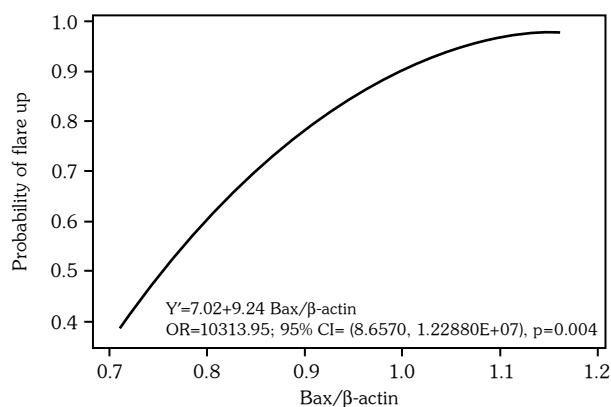
Pro-apoptotic proteins, Bad and Bax, play crucial roles in the intrinsic pathway of apoptosis. Their activity is essential for the control of cell survival during lymphocyte development and homeostasis.⁶

**Figure 2.** ROC curve of Bax/ β -actin for predicting SLE flare up.

AUC: Area Under the Curve; ROC: Receiver operating curve; SLE: Systemic lupus erythematosus.

The relevance of our current work is to confirm the involvement of defective apoptosis in the pathogenesis of SLE and to find the association with disease activity for gene expression results. To the best of our knowledge, this is the first study to investigate Bax and Bad mRNA expression in SLE in our population. In this study, we found that the mRNA expression of pro-apoptotic Bax and Bad were significantly lower in SLE group than the control group, and these findings are consistent with previous studies which showed decreased expression of Bad and Bax in rheumatoid arthritis¹⁰ and multiple sclerosis patients.¹¹ On the other hand, the study by Mason et al.⁶ found that autoimmune manifestations were not detected in Bax-deficient mice.

Several triggering factors such as viral infection or exposure to ultraviolet rays may induce disease flare-up, in which both innate and adaptive immune pathway involved in disease pathogenesis,¹² leading to the production of chemical mediators as TNF- α and inflammatory cytokines as interleukin (IL)-2 which play a vital role in disease activity and loss of cellular

**Figure 3.** Probability of flare up using Bax mRNA expression for prediction.

mRNA: Messenger ribonucleic acid; OR: Odds ratio; CI: Confidence interval.

regulatory pathway.^{13,14} During disease activity, the level of immune mediators, as well as the expression of mRNA regulating protein spikes to its higher level in the blood and subsides with disease remission either due to the effect of immune suppressive medication or through the prolonged self-remission pathway.¹⁵

The severity of the disease flares in SLE ranges from mild/moderate to severe life-threatening that can cause significant irreversible damage to organs and can even lead to death. Controlling and predicting flares and disease exacerbations have been promptly required in the management of SLE, with many studies focused on the development of flare prediction biomarkers.¹ It is important to identify patients most at risk of flares and exacerbations for close follow-up, make early diagnosis, and start prompt treatment or even consider preventive treatments.¹⁶

Many researchers have attempted to identify predictors of SLE flare, and previous studies investigated the prevalence of and risk factors for British Isles Lupus Activity Group (BILAG) flare in patients with SLE. The authors found that the increased baseline anti-double-stranded DNA (anti-dsDNA) level was an independent predictor of moderate-to-severe flares (hazard ratio [HR]=1.83, 95% confidence interval [CI] 1.29-2.60) for any new (BILAG) A domain at Week 52¹⁷ or a risk factor only for hematological flares (OR=2.33, 95% CI: 1.34-4.04, $p=0.0033$).¹⁸ Petri et al.¹⁷ also confirmed the usefulness of B-lymphocyte-stimulating factor (BLyS) as a predictor for consequent SLE flare.

In the current study, we observed that Bax/ β -actin mRNA expression showed significant upregulation with disease flare-up. Moreover, the expression downregulated significantly with stability of the disease, and become nearer to control level, this can be explained by the presence of large numbers of lymphocytes and monocytes/macrophages, which release large amounts of cytokines and reactive oxygen species (ROS) and may stimulate the upregulation of apoptotic factors such as Bax during disease flare-up.¹⁹

Our analysis using the ROC curves provided us with cut-off values for Bad and Bax. Thus,

we could discriminate SLE patients from control subjects, if their mRNA expression levels were as follows: Bad <0.8, Bax <0.66. The study by Motawi et al.²⁰ that investigated Bad and Bax mRNA expression in hepatitis C virus (HCV) patients predicted that HCV patients were more likely to have late-stage fibrosis, if their mRNA expression levels were as follows: Bad <52.57, Bax <58.2. Data using the ROC curves provided us with cut-off values for Bax. Therefore, we could predict that SLE patients were more likely to flare-up, if their mRNA expression levels were Bax >0.81, making it a potential biomarker of disease activity. Furthermore, we found that the probability of flare-up reached to 100% with increasing the expression of Bax/ β -actin as well, the likelihood of flare-up increased 10,314 times with every unit increase of Bax/ β -actin mRNA expression.

The main limitation of this study is the small sample size and, therefore, further researches with larger samples are needed to gain a better understanding of disease pathogenesis and the usefulness of this pro-apoptotic molecule as possible biomarkers of SLE disease activity and therapeutic target.

In conclusion, deregulations of the expression of pro-apoptotic Bax may have a role in the susceptibility to SLE and may be associated with disease flare. A better understanding of the expression of these pro-apoptotic molecules involved in SLE pathogenesis may carry a great potential for the development of specific effective therapies.

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Ethics Committee Approval: The protocol of the study was approved by ethics committee of the National Research Centre (date/no: January 2018/16385). The study was conducted in accordance with the principles of the Declaration of Helsinki.

Patient Consent for Publication: A written informed consent was obtained from each patient.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions: Made the study design, supervised the project, participated in the molecular genetic studies, wrote the manuscript, and submitted the manuscript: R.N.Y.; Participated in the molecular genetic studies, participated in sample collection, data collection and analysis; A.R., E.A.; Collected samples and data of SLE patients and control subjects: A.R.A., N.M.K., M.E.B.; Made the statistics and analyzed the results; A.A.; Participated in molecular genetic studies, made study design, supervised the work and analyzed data, this manuscript was revised and approved by all authorm: H.G.E.D.

Conflict of Interest: The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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Supplementary Table 1. Descriptive statistics of Bax/ β -actin, Bad/ β -actin and index in SLE patients and control group

	Mean $\pm$ SD	Minimum	Q1	Median	Q3	Maximum
Bad/ β -actin						
Control	0.75 $\pm$ 0.08	0.61	0.70	0.76	0.80	1.03
SLE	0.71 $\pm$ 0.11	0.27	0.65	0.72	0.75	1.12
Bax/ β -actin						
Control	0.90 $\pm$ 0.08	0.76	0.84	0.89	0.97	1.09
SLE	0.80 $\pm$ 0.19	0.13	0.74	0.84	0.89	1.34
Index						
Control	19.24 $\pm$ 1.93	15.11	17.74	19.64	20.90	22.18
SLE	17.43 $\pm$ 5.04	2.99	15.22	17.80	20.53	33.53

SLE: Systemic lupus erythematosus; SD: Standard deviation; Q1: Quartile one; Q3: Quartile 3.

Supplementary Table 2. Bax/ β -actin, Bad/ β -actin and index in SLE patients in correlation with flare up phase

Test	Category	n	Mean $\pm$ SD	<i>p</i> *
Bax/ β -actin	Remission	13	0.67 $\pm$ 0.06	0.03
	Flare up	47	0.72 $\pm$ 0.12	
Bad/ β -actin	Remission	13	0.80 $\pm$ 0.07	0.91
	Flare up	47	0.80 $\pm$ 0.21	
Index	Remission	13	16.65 $\pm$ 3.04	0.39
	Flare up	47	17.65 $\pm$ 5.48	

SLE: Systemic lupus erythematosus; SD: Standard deviation; * Independent t-test; *p*<0.05 considered significant.