Dysregulation of Tumor Necrosis Factor Alpha-Induced Protein 3 mRNA Expression in Lupus Nephritis in Relation to Clinic-pathologic Characteristics and Disease Activity of Systemic Lupus Erythematosus

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ABSTRACT
Background: Lupus nephritis (LN) is one of the most dangerous manifestations of systemic lupus erythematosus (SLE). LN is a complex interplay between genetics, immunological, and environmental factors.
Objective: Our study aimed to evaluate tumor necrosis factor α-induced protein-3 (TNFAIP3) mRNA expression level as a noninvasive predictive test of LN and to assess its correlations with clinic-pathologic characteristics as well as disease activity of SLE.
Subjects and Methods: Among 150 studied subjects; 80 had SLE and 70 were healthy controls. Patients were stratified into LN group (n=35) and the non-LN group (n=45). TNFAIP3 mRNA expression level was measured using a quantitative real-time PCR.
Results*:* TNFAIP3 mRNA expression level was upregulated in the SLE group compared to the control group. While TNFAIP3 mRNA expression level was downregulated in the LN group of SLE patients compared to the non-LN group. Our results show that the lowest values of TNFAIP3 mRNA expression level were in Class V compared to Class IV, Class III, and Class II. According to the current study results, the effectiveness and strength of TNFAIP3 mRNA expression level for differentiating SLE a from the control group we applied ROC curve, the sensitivities and specificities were 96.8% and 83.3%, respectively. Regards discriminating LN among SLE the sensitivities and specificities were 91.7% and 82.2%, respectively. Thus, TNFAIP3 mRNA expression level could be a useful diagnostic test to discriminate between SLE patients in particular LN patients.
Conclusion: Non-LN group had statistically significant higher values of TNFAIP3 mRNA expression level compared to LN and control groups. However, the values decreased with more damage to kidney tissues and progression of SLE activity thus, TNFAIP3 mRNA expression level could be used as a genetic marker of LN susceptibility and severity.
Keywords: SLE; lupus nephritis; TNFAIP3; SLEDAI..

INTRODUCTION

As a matter of fact, systemic lupus erythematosus (SLE) is termed a diffused autoimmunological disease [(1)], and affects many organs and tissue, for instance, the kidney. Several pieces of evidence have shown that SLE is characterized by the formation of autoantibodies [(2)]. Lupus nephritis (LN) is a major variety of SLE [(3)]. The prevalence of LN is about ~30–50% of patients with SLE present with renal damage [(4)]. Several pieces of evidence have shown that the clinical manifestations of LN include proteinuria, hematuria, and pyuria [(5)].

Tumor necrosis factor α-induced protein-3 (TNFAIP3) is a zinc finger and ubiquitin-editing protein that regulate the inflammatory and immunological reactions via the NF-κB signaling cascade [(6)]. Several research has reported that TNFAIP3 inhibits both TNF and NF-κB [(7)]. There is growing evidence that dysregulated TNFAIP3 is correlated with many autoimmune diseases, for example, SLE [(8)]. In this context, a study by **Oeckinghaus and Ghosh**[(9)] detected that dysregulated NF-κB, and genetic polymorphism of TNFAIP3 was correlated with SLE risk[(10)].

A preponderance of evidence suggests that the etiology of LN is a complex interplay between genetics, immunological, and environmental factors. Despite intense research efforts in the field, the exact etiopathogenesis of LN remains elusive. It may be assumed that many crucial factors perform a significant role in the pathogenesis of LN. Renal biopsy is the gold standard for LN diagnosis as well as assessment of LN activity and severity. Identification of noninvasive LN predictors or diagnostic, markers are of importance for enhancing early diagnosis and proper treatment. Thus, we aimed to explore TNFAIP3 mRNA expression level as a noninvasive predictive test of LN and to assess its correlations with clinic-pathologic characteristics as well as disease activity of SLE.

SUBJECTS AND METHODS

Our study enrolled 80 SLE patients who were recruited from Internal Medicine Departments at Zagazig University Hospitals and 70 healthy control subjects. The enrolled subjects were matched regarding age, sex, and race. We selected the SLE patients who met the criteria for SLE[(11)], LN[(12)], and disease activity [(13)] and categorized them according to the flowchart of the study as illustrated in figure 1.

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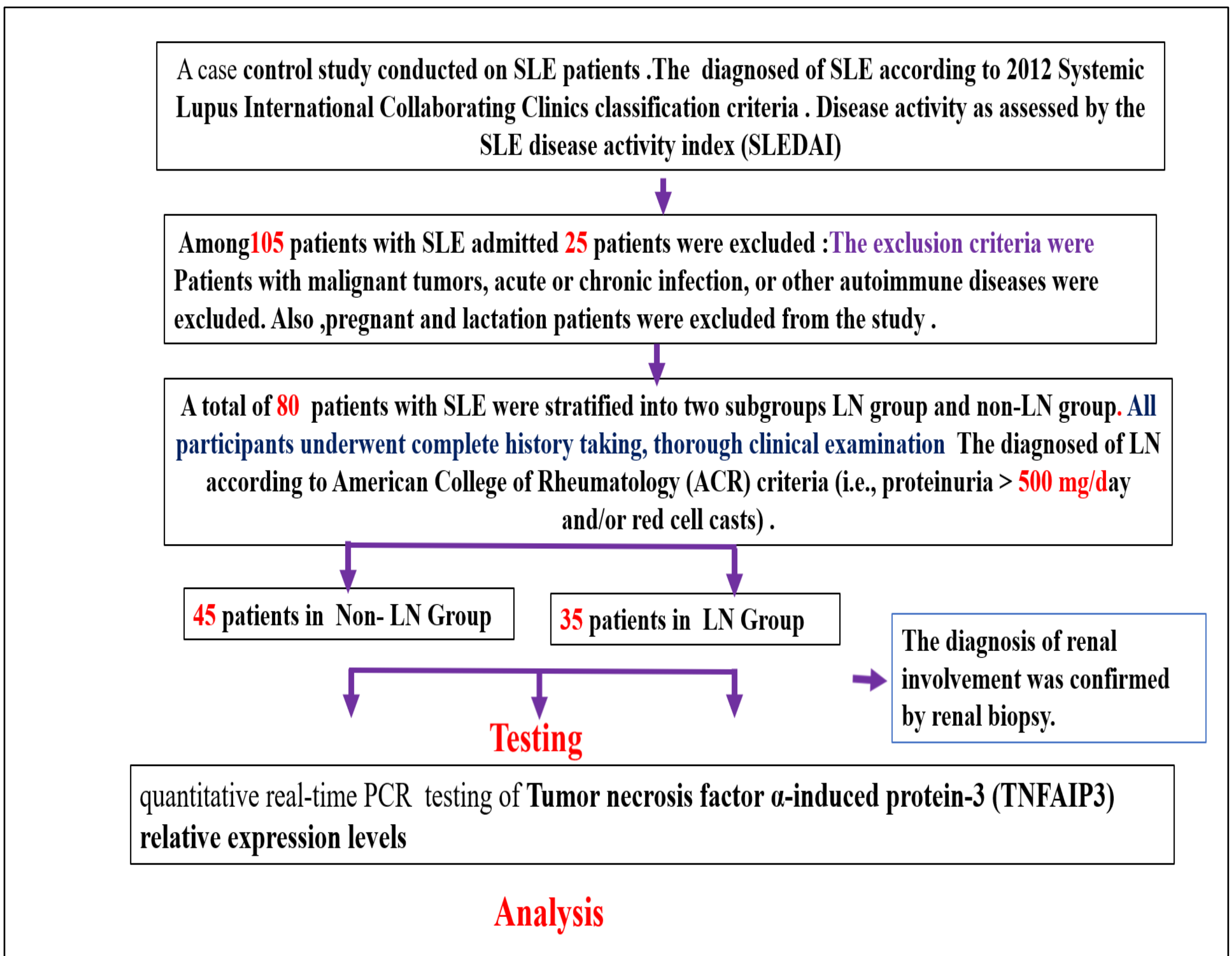

Figure (1): Flowchart of the study

Blood sampling and testing

Blood samples were drawn from all subjects after an overnight fast. Sera were separated after 1 hour (hour) longstanding and stored at -80°C. Serum creatinine, ANA, anti-dsDNA, and ESR, were determined manually. C- reactive protein (CRP), complement C3, and C4 were measured according to operating procedures in Zagazig University Hospital laboratories. Twenty-four h urine samples were collected from each participant in sterilized urine containers. Renal biopsies were reviewed and classified [(13)].

Gene expression analyses:

PBMC was separated from peripheral blood and anticoagulated with sodium citrate. Total RNA was extracted from PBMC (5×10^5) by use of TRIzol (Invitrogen, CA) and quantified by photometric measurement. RNA was reverse transcribed to cDNA by use of QuantiTect Reverse Transcription Kit as recommended by the manufacturer.

The expression of TNFAIP3 mRNA was evaluated by quantitative real-time PCR using 5 uL of the cDNA, 10 pmol/uL of each primer, 10 uL of SYBR Green 2x Master Mix Green (QuantiTect SYBR Green PCR Kits, Qiagen). Amplification conditions were as follows: 95°C for 10 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 40 sec. The level of β-actin mRNA was also detected as an internal control for each sample. Primers used in real-time PCR were as in table 1.

Table (1): Primers used in real-time PCR.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
TNFAIP3	CGTCCAGGTTCCAGAACACCATTC	TGCGCTGGCTCGATCTCAGTTG
β-actin	GACTACCTCATGAAGATCCTCACC	TCTCCTTAATGTCACGCACGATT

The expression of the TNFAIP3 gene was normalized to that of β-actin and determined using the comparative $2^{-\Delta\Delta CT}$ method, which is a relative quantification method.

Ethical consent:

An approval of the study was obtained from Zagazig University Academic and Ethical Committee. Every patient signed an informed written consent for acceptance of participation in the study. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

Statistical analysis:

The statistical analysis was carried out using IBM SPSS statistics program version 26. Quantitative data were presented as mean±standard deviation (SD) and were compared by one-way ANOVA test, while qualitative data were presented as frequency and percentage and were compared by chi-square test. Correlation analyses were performed using the Pearson correlation test and linear regression was used to assess the relations between TNFAIP3 mRNA levels and studied parameters. Receiver operation coefficient (ROC) curve assessment was carried out to detect the predictive accuracy of TNFAIP3 mRNA. P-was considered significant if <0.05.

RESULTS

Clinical and laboratory characteristics of studied groups

Our study enrolled 150 subjects (healthy control =70, LN group=35, and non-LN group).

Regarding clinical findings, there was a significantly higher prevalence of clinical manifestations in the LN group compared to the non-LN group as illustrated in **figure 2**.

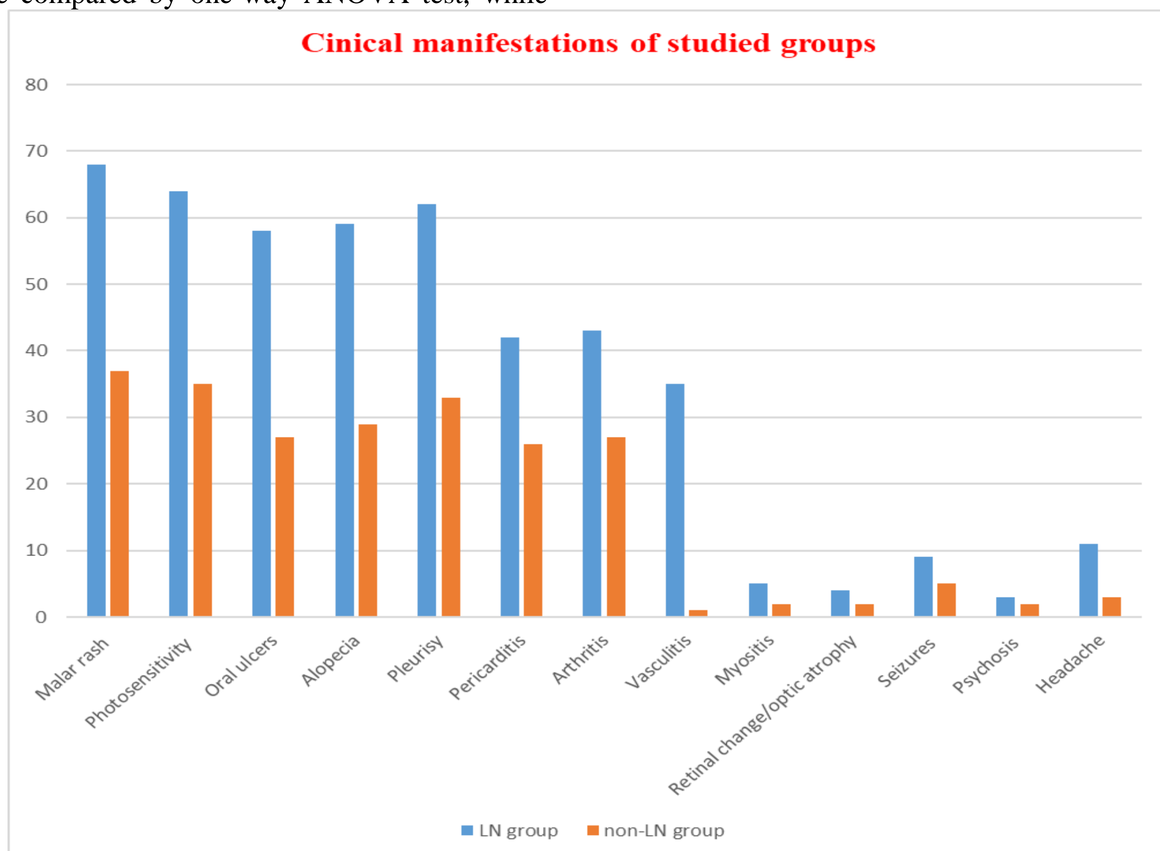

Figure (2): Clinical characteristics of SLE groups

TNFAIP3 mRNA expression level in the studied groups

Among SLE patients, the values of TNFAIP3 mRNA were upregulated in a patient with non-LN compared to the LN group and control group (T**able 2**).

Table (2): Clinical and laboratory characteristics SLE groups (n=80)

Variable	**Control (N=70)**	**Non-LN Group (n=45)**	**LN Group (n=35)**	*P* value
Age (y)	31.8±6.69	32.1±5.51	30.6±6.7	0.325
Male/female, number	8/70	4/41	3/32	0.541
Duration of disease (years)	---	7.14±1.23	11.86±3.82	<0.001*
SLEDAI	5.65±1.23	9.04±2.75	17.6±8.57	<0.001*
Fever	---	19 (42.2%)	31(88.6%)	<0.001*
Hypertension	---	5 (11.1%)	32 (71.1%)	<0.001*
Protein in urine (mg/24h)	64.47±12.7	241.23±14.16	256.66±122.9	<0.001*
Pus cell N (%)	---	3 (6.7%)	11(31.4%)	<0.001*
Cellular casts N (%)	---	7 (15.6%)	22 (62.9%)	<0.001*
Hematuria N (%)	----	8 (17.8%)	19 (54.2%)	<0.001*
Serum creatinine (mg/dL)	0.61±0.21	2.14±1.21	0.93±0.199	<0.001*
Serum urea (mg/dL)	28. 2±5.51	54.06± 3.12	38.06± 7.93	<0.001*
eGFR, ml/min/1.73m^2	81.7 ±17.51	64.47± 6.87	43.7±4.9	<0.001*
Hemoglobin (g/dl)	12.69±1.6	10.12±1.18	9.19±1.61	<0.001*
PLTs (10^6 ml/μl)	311.61±26.2	242.14±52.3	198.3±38.2	<0.001*
Lymphocytes ×10^9/L	2.51±0.8	2.14±0.24	1.93±0.14	<0.001*
Leukocytes, ×10^9/L	7.41±1.21	5.14±1.34	4.83±1.12	<0.001*
Neutrophils, ×10^9/L	4.33±1.4	3.14±0.4	3.93±0.1	<0.001*
CRP (mg/dL)	2.82±0.21	5.92±1.68	9.69±1.61	<0.001*
ESR (mm/h)	24.5±4.6	34.87± 4.9	86.47± 10.3	<0.001*
C3 (mg/dl)	85.87± 6.9	55.87± 6.2	43.16±1.4	<0.001*
C4 (mg/dl)	24.2±1.4	13.2±2.95	8.93±2.09	<0.001*
TNFAIP3 mRNA expression level	1.15±0.19	3.1±0.64	1.51±0.35	<0.001*

LN; lupus nephritis, SLEDAI; systemic lupus erythematosus disease activity index, ESR; erythrocyte sedimentation rate, CRP; C-reactive protein, C3; complement 3, C4; complement *; significant

The current study results show that there were significantly higher values of TNFAIP3 mRNA expression levels in SLE patients compared to the control group **(Figure 3a)**.

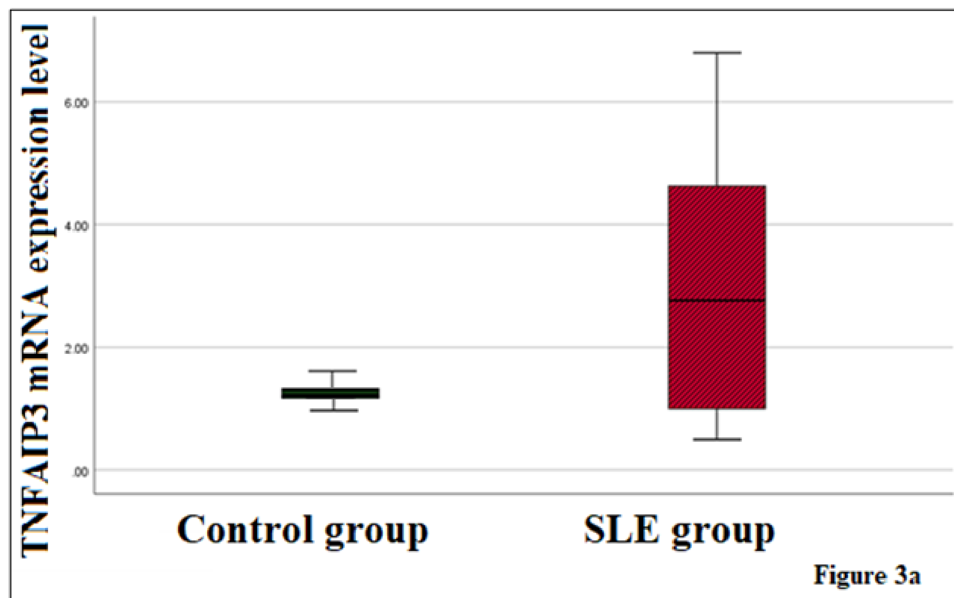

Figure (3a): Comparison of TNFAIP3 mRNA expression level between studied groups

Comparison of TNFAIP3 mRNA expression level with histopathological characteristics of LN group

Our results show that there were statistically significant differences between LN stages with the lowest values of TNFAIP3 mRNA expression level in Class V compared to Class IV, Class III, and Class II**, figure 3b, p <0.001.**

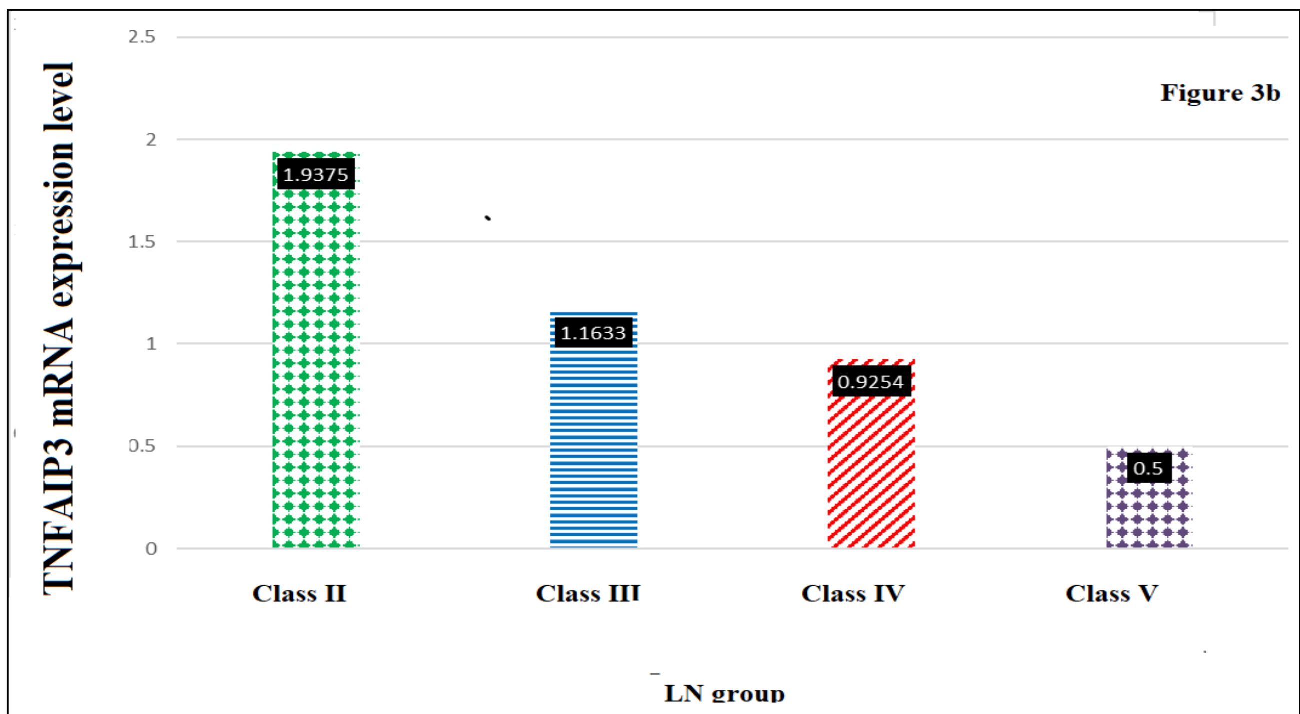

Figure (3b): Comparison of TNFAIP3 mRNA expression level among LN group according to histopathological classes

Correlation between TNFAIP3 mRNA expression level and laboratory characteristics as well as disease activity of SLE patients

The findings of the current study demonstrated significant positive associations between TNFAIP3 mRNA expression and SLEDAI, proteinuria, serum creatinine, as well as ESR **(Table 3).**

Table (3): Correlation between TNFAIP3 mRNA expression level and laboratory characteristics as well as disease activity of SLE patients

Variable	TNFAIP3 mRNA expression level	
	r	**P value**
SLEDAI	0.619	<0.001*
Proteinuria (mg/24h)	0.552	<0.001*
Serum creatinine (mg/dL)	0.787	<0.001*
Hemoglobin (g/dl)	-0.022	0.686
PLTs (10^6 ml/μl)	-0.320	0.057
CRP (mg/dL)	0.133	0.104
ESR (mm/h)	0.801	<0.001*
C3 (mg/dl)	- 0.086	0.295
C4 (mg/dl)	- 0.019	0.777

ESR; erythrocyte sedimentation rate, CRP; C-reactive protein و C3; complement 3, C4; complement 4. *; significant

Linear regression analysis in SLE patients

The interesting finding of the present study is that ESR and serum creatinine were independently correlated with TNFAIP3 mRNA expression level (**Table 4).**

Table (4): Linear regression analyses to test the influence of the main independent variables against TNFAIP3 mRNA expression level (dependent variable) in lupus nephritis group

Model	Unstandardized Coefficients		Standardized Coefficients	t	P	95% CI	
	B	**SE**	**Beta**			**Lower Bound**	**Upper Bound**
(Constant)	3.357	5.205		0.645	0.524	-7.305	14.020
CRP	-0.154	0.56800	-0.120	0.271	0.788	-1.317	1.009
ESR	0.206	0.084	1.234	2.461	<0.001*	0.035	0.378
SLEDAI	-0.153	0.197	-0.180	-0.775	0.445	-0.557	0.252
Creatinine	9.884	3.903	1.815	2.532	<0.001*	1.889	17.880
Proteinuria	-0.375	0.206	-1.914	-1.825	0.079	-0.796	0.046

ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; 95% C.I.: 95% confidence interval. *; significant

ROC analysis for testing the diagnostic power of TNFAIP3 mRNA in diagnosis SLE among studied participants revealed that the cutoff value of the TNFAIP3 mRNA expression level was 0.68 and the AUC was 0.754 (95% CI=0.670-0.838). Additionally, the sensitivities and the specificities of TNFAIP3 mRNA were 95.1% and 63.3% respectively (Figure 4a).

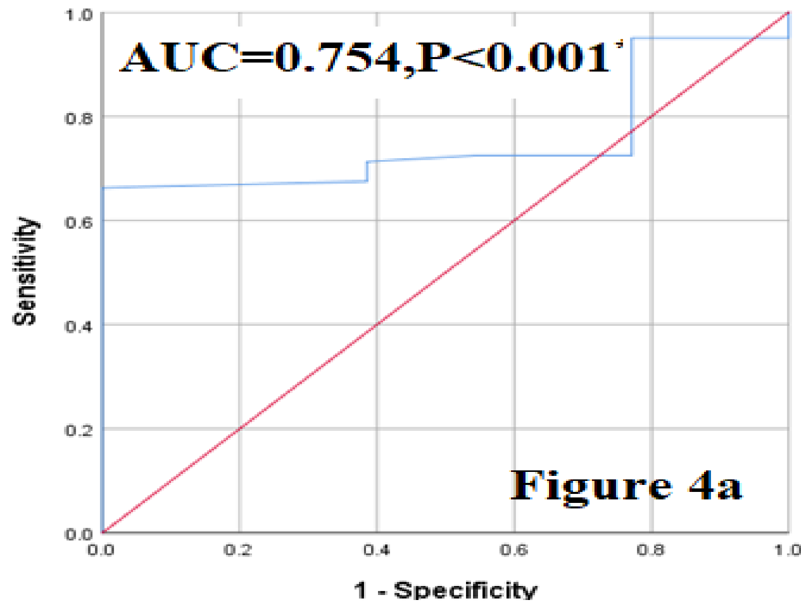

Figure (4a): Receiver operator characteristic (ROC) curve for TNFAIP3 mRNA expression level for differentiating SLE a from control group

ROC analysis for investigating the analytical power of TNFAIP3 mRNA in diagnosis LN among SLE detected that the cutoff value of TNFAIP3 mRNA expression level was 2.1 and the AUC was 0.963 (95% CI =0.929-0.997). Additionally, the sensitivities and the specificities of TNFAIP3 mRNA expression levels were 91.7% and 82.2%, respectively (Figure 4b). Thus, TNFAIP3 mRNA expression level could be a useful diagnostic test to discriminate between SLE patients in particular LN patients.

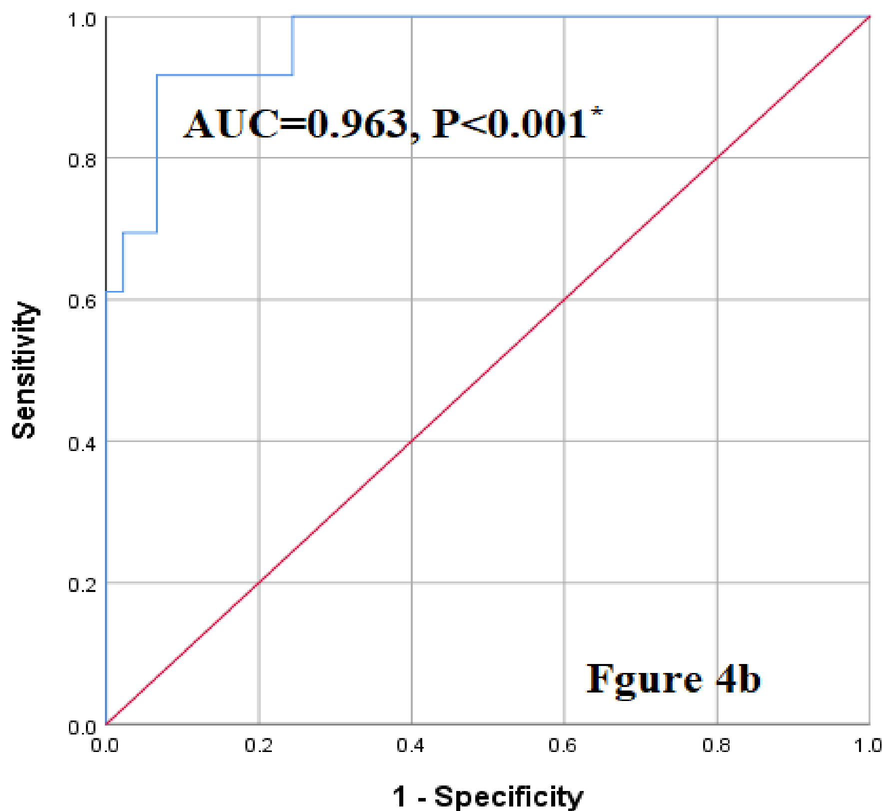

Figure (4b): Receiver operator characteristic (ROC) curve for TNFAIP3 mRNA expression level for discriminating LN among SLE

DISCUSSION

Autoimmunity refers to dysregulation of the immune system leading to the detriment of many organs and tissues such as SLE and in particular kidneys [14]. There is a lot of evidence emphasizing the morbidities and mortalities among SLE and they detected that LN is the most dangerous manifestation of SLE [15]. A preponderance of evidence suggests that the epidemiological distribution of LN worldwide varies according to ethnicity [16].

It may be assumed that TNFAIP3 is implicated in numerous autoimmune and rheumatologic disorders, including SLE as it is considered one of the key regulators of inflammatory signal transduction [17]. It has been suggested that it negatively regulates the NF-κB pathway [18].

There is little evidence about the association between TNFAIP3 gene polymorphisms and SLE [19]. Though, the association between the TNFAIP3 mRNA and SLE and of particular importance LN is still unclear. Despite the availability of aggressive immunotherapies, the outcome of LN is still poor [20]. Therefore, we are in need to explore the immune mechanism behind LN, to recognize genetic predictors and prognostic markers of LN. To the best of our knowledge this study is the first Egyptian study investigated TNFAIP3 mRNA expression level as a noninvasive predictive test of LN and investigated correlations with clinic-pathologic characteristics as well as disease activity of SLE.

The results of the current study demonstrated that among 80 patients with SLE, only 35 patients had renal biopsy proved LN to increase the reliability of the present study. We enrolled 70 healthy controls additionally; age, sex, and ethnicity were matched. Consequently, we compared the studied parameters of each group and we found that, LN patients had significantly higher values of inflammatory and renal impairment risk factors compared to other studied groups. The interesting result of our study was that TNFAIP3 mRNA expression level was upregulated in the non-LN group of SLE patients compared to the control group. While TNFAIP3 mRNA expression level was downregulated in the LN group of SLE patients compared to the non-LN group.

According to the results of a recent Egyptian study, that enrolled 160 subjects (80 controls and 80 patients with SLE), the Egyptian population carrying TNFAIP3 rs5029939 polymorphism has no susceptibility to developing SLE [19].

To further study the potential association between TNFAIP3 mRNA expression level severity of LN, we compared TNFAIP3 mRNA expression levels in different classes of LN according to histopathological findings. Our results showed that there were statistically significant differences between LN stages with the lowest values of TNFAIP3 mRNA expression level in Class V compared to Class IV, Class III, and Class II. Thus, TNFAIP3 mRNA expression level is inversely correlated with LN severity.

Majumdar *et al.*[21] conducted their study on UC patients to assess TNFAIP3 mRNA concerning disease severity and they detected downregulation of TNFAIP3 mRNA in moderate and severe forms of UC compared to mild forms, which could be due to a lack of TNFAIP3 expression resulting in significant up-regulation of NFκB.

Similarly, reports by **Jiang and his colleagues**[22] detected that the TNFAIP3 mRNA level in the mild group was higher than that in the severe group of psoriasis vulgaris. Consequently, they explained their findings that this upregulation may have been due to chronic inflammation, which leads to elevation of proinflammatory cytokines, including TNF-α and this could activate NF-κB [23]. Convincing evidence suggested that the activated NF-κB increased TNFAIP3 gene expression [24]. However, downregulation of TNFAIP3 in severe disease may contribute to the inefficiency and dysfunction of TNFAIP3 expression in severe disease[25].

Even more importantly, we investigate the correlations between TNFAIP3 mRNA expression levels and activity index as well as laboratory characteristics, we found significant positive correlations between TNFAIP3 mRNA expression and SLEDAI, proteinuria, serum creatinine, and ESR. Interestingly, our results showed that ESR and serum creatinine were independently correlated with TNFAIP3 mRNA expression levels.

As a consequence of our studies, we evaluated the effectiveness and strength of TNFAIP3 mRNA expression level for differentiating SLE from the control group. We applied the ROC curve. and the AUC was 0.754 (95% CI=0.670-0.838, with sensitivities and specificities of 96.8% and 83.3% respectively. We further investigated the effectiveness and strength of TNFAIP3 mRNA expression level for discriminating LN among SLE, AUC was 0.963 (95% CI =0.929-0.997) with sensitivities and specificities of 91.7% and 82.2%, respectively. Thus TNFAIP3 mRNA expression level could be a useful diagnostic test to discriminate between SLE patients, in particular, LN patients.

CONCLUSION

The non-LN group had statistically significant higher values of TNFAIP3 mRNA expression level compared to LN and control groups. However, the values decreased with more damage to kidney tissues and progression of SLE activity, thus TNFAIP3 mRNA expression level could be used as genetic biomarker of LN susceptibility and severity.

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Author contribution: Authors contributed equally in the study.

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