Urinary Foxp3 mRNA As A biomarker For Lupus Nephritis Activity in Children with Systemic Lupus Erythematosus at Benha University Hospitals

Abstract

Background and Aim: Despite the improvement in the medical care of the systemic lupus erythematosus (SLE) in the past two decades, the prognosis of lupus nephritis (LN) remains unsatisfactory. The aim of this study is to assess the relationship of urinary Foxp3 mRNA with disease activity in children with lupus nephritis as a noninvasive biomarker. **Methods:** This study included 40 children with SLE; 25 JSLE patients with clinical, pathological or laboratory evidence of renal disease and in renal nephritis activity (Active LN group), besides 15 patients were clinically, laboratory and biopsy diagnosed renal diseased but are not in renal activity (Inactive LN group) and 40 healthy children (control group). Disease activity was assessed by SLEDAI-2k score. Assessment of urinary Foxp3 messenger RNA (mRNA) expression was done in all subjects. **Results**: Active LN group had statistically significant higher Foxp3 mRNA compared to inactive LN and control groups (16.1±2.5, 5.9±2.1 and 1.1±0.6, respectively), $p<0.001$. Foxp3 mRNA had a significant positive correlation with (SLEDAI.2k, rSLEDAI, ESR, urea and creatinine). ROC analysis was done to assess the performance of Foxp3 mRNA; At a cutoff point > 3.3, it could detect cases of LN from controls with sensitivity 97.5% and specificity 87.5%. At a cutoff point > 10.3, it could detect active LN with sensitivity 96% and specificity 93.3%. **Conclusion:** Urinary FOXP3 mRNA expression was found to be significantly up regulated in patients with active LN, and was significantly associated with systemic and histological disease activity.

Keywords: Foxp3; Lupus Nephritis; SLE

Introduction

Systemic lupus erythematosus (SLE) is life long, life-limiting, multisystemic autoimmune disease, whose etiology and pathogenesis are incompletely understood (1). SLE has a negative impact on quality of life and is associated with high health-care

costs and significant productivity loss. In consequence, SLE incurs a great burden on both the patient and society (2).

Glomerulonephritis is one of the most serious manifestations of SLE. Despite the improvement in the medical care of the SLE in the past two decades, the prognosis of lupus nephritis (LN) remains unsatisfactory (3).

Juvenile-onset systemic lupus erythematosus (JSLE) is characterized by more renal involvement than disease presenting in adulthood. The renal biopsy is the gold standard in confirming the diagnosis and class of lupus nephritis (LN). However, it is invasive and serial biopsies are required which is impractical in monitoring lupus nephritis (4).

The conventional biomarkers used for LN [proteinuria, anti-double stranded deoxyribonucleic acid antibodies (anti-dsDNA), complement C3 and C4 levels] cannot imitate the real-time renal pathological changes. Biologic, genetic, and chemical characteristic biomarkers are currently studied to detect LN and its flare (5).

Recently, there is special interest in urinary biomarkers as the sample is easily obtained and not invasive. The ideal biomarker in SLE patients with suspicion or confirmation of LN should have certain properties including ; to be specific for renal involvement ,to have a good correlation with kidney activity or damage, to be useful for serial monitoring, to be superior to conventional clinical or laboratory parameters, possess the ability to assess the severity of renal involvement, be cost-effective and to be easy to perform and available in most clinical laboratories (6).

Forkhead box P3 (FoxP3) is a crucial transcription factor in the regulation of the development and function of regulatory T cells. This molecule is very important in the homeostasis of the immune system. Mutation of the FoXP3 gene causes a complex multi-organ autoimmune disease called immune dysregulation, poly endocrinopathy, enteropathy, X-linked syndrome (IPEX syndrome). The participation of Foxp3 in the pathogenesis of SLE has been reported, and the expression of this transcription factor in CD4+ T cells has been correlated with the disease activity (7).

Increased levels of FoXP3 mRNA in the urine have been described in patients with proliferative LN, associated with high rSLEDAI and high titters of anti-dsDNA. Additionally, FoXP3 has been correlated with the histological activity index, and its

levels decreased significantly in SLE patients who received treatment with a complete response. In fact, it is reported that FoXP3 starts to elevate approximately 8 weeks before the renal flare. According to the results found in the reviewed studies, the measurement of this transcription factor could be useful for determining the activity of the disease, predicting relapses, and monitoring the response to treatment (8).

The aim of this study is to assess the relationship of urinary Foxp3 mRNA with disease activity in children with lupus nephritis as a noninvasive biomarker.

Patients and methods

This study is a case-control study included 40 children with systemic lupus erythematous attended the paediatric rheumatology outpatient clinic at Benha University Hospitals from February 2022 to February 2024.

Inclusion criteria:

- All children with systemic lupus erythematous attended the pediatric rheumatology outpatient clinic at Benha University Hospitals from February2022 to February 2024.
- JSLE patients were diagnosed according to the revised American College of Rheumatology (ACR) classification criteria for SLE.
- Children diagnosed to have SLE and LN proven with:
    - Hematuria with RBC > 5 per high-power field (HPF) and /or proteinuria > 0.5 g per 24 h.
    - Renal biopsy with at least 10 glomeruli in the renal biopsy specimens

Exclusion criteria:

- Patients have any associated autoimmune diseases such as familial Mediterranean fever (FMF).
- Patients with End-Stage Renal Disease (ESRD) or had undergone renal transplantation.

- Patients in whom a renal biopsy cannot be performed.

Approval code: MD: 16-1-2022

Ethical considerations

- The whole study design was approved by the local ethics committee, Faculty of Medicine, Benha University.
- After explaining the value of the study and the procedures that would be commenced, an informed written consent was obtained from the guardian of every participant before being included in the study.

Patients were divided into two groups: Group I (patient group), this group was subdivided into: Subgroup A (Active LN group): This subgroup included 25 JSLE patients (7 males and 18 females) with clinical, pathological or laboratory evidence of renal disease and in renal activity; Subgroup B (Inactive LN group): This subgroup included 15 JSLE patients (3 males and 12 females) were clinically, laboratory and biopsy diagnosed renal diseased and not in renal activity (in remission after receiving treatment by at least 6 months); Group II (control group); This group included 40 children age and sex matched healthy subjects as a control group.

All children were subjected to full history taking, complete clinical examination, laboratory investigations as complete blood count, aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea, creatinine, Erythrocyte sedimentation rate (ESR), complement 3 and 4 levels and urine analysis; urine samples were tested for protein creatinine ratio at the requested time. Activity was assessed by the SLE Disease Activity Index (SLEDAI-2k), and renal SLEDAI (9)**.**

Assessment of urinary Foxp3 mRNA; At 48 degrees Celsius, urine samples were centrifuged at 3000 g for 30 minutes and 13 000 g for 5 minutes. After discarding the supernatant, the urinary cell pellet were lysed with RNA lysis buffer (Qiagen,Ontario,Canada). Specimens were held at 80C before their use. Complete RNA was extracted using RNeasy Mini Kits (Qiagen) according to the manufacturer's instructions. DNase was used to remove any potentially genomic DNA. The relative absorbance at 260/280 nm ratio of the spectrometer were used to confirm the purity of

the RNA. For reverse transcription, 5 µl total RNA were mixed with 1 µL random primers (150 ng), 1 µL dNTP mix (10 mM each), 4µL 5× first_ strand buffer, 2 µL dithiothreitol (0.1 M), 1 µL Superscript II RNase H Reverse Transcriptase (all from Intrivogen, Life Technologies, Philadelphia, PA, USA) and made up to 20 µl with H2O. Reverse transcription were performed at 65 C for 5 min, 25C for 10 min, 42 C for 50 min, and then inactivate reaction at 70 C for 10 min. The resulting cDNA were stored at - 80C until use. Using the ABI Prism 7700 Sequence Detection System, we measured FOXP3 gene expression in the urine (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

The collected data was revised, coded, tabulated using Statistical package for Social Science (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.). Data were presented and suitable analysis was done according to the type of data obtained for each parameter. Descriptive statistics: Mean, Standard deviation (± SD) for parametric numerical data, while Median and range for non-parametric numerical data. Frequency and percentage of non-numerical data. Analytical statistics: Student T Test was used to assess the statistical significance of the difference between two study group means. For the comparison of more than two groups' means, one way analysis of variance (ANOVA) was used. Chi-Square test was used to examine the relationship between two qualitative variables. Fisher's exact test: was used to examine the relationship between two qualitative variables when the expected count is less than 5 in more than 20% of cells. Correlation analysis: To assess the strength of association between two quantitative variables. The correlation coefficient defines the strength and direction of the linear relationship between two variables. The ROC Curve (receiver operating characteristic) provides a useful way to evaluate the sensitivity and specificity for quantitative diagnostic measures that categorize cases into one of two groups. The optimum cut off point was defined as that which maximized the AUC value.

Results

This study included 40 children with SLE; 25 JSLE patients with clinical, pathological or laboratory evidence of renal disease and in renal activity (Active LN group) , and 15 patients were clinically, laboratory and biopsy diagnosed renal diseased and not in

renal activity (Inactive LN group) besides 40 healthy children age and sex matched healthy subjects as a control group. There was no statistically significant difference between the studied groups as regards to age, sex or consanguinity. Active SLE group had statistically higher frequency of previous hospital admission compared to other groups (table 1).

There was statistically significant difference between active and inactive SLE groups as regards to LN duration. While there was no significant difference between groups as regards to presence of lupus nephritis at presentation or regards class of LN in renal biopsy (Table 2).

The most common presentation was oliguria with edema (28%), followed by oliguria alone (20%). the most common finding in urine analysis was combination of hematuria, proteinuria and raised urinary protein–creatinine ratio (24%) (Table 3).

Active LN group had a statistically significant higher SLEDAI-2k score and rSLEDAI score compared to inactive LN group (figure 1).

Active LN group had a statistically significant higher Foxp3 mRNA compared to

in active LN and control groups (16.1±2.5, 5.9±2.1 and 1.1±0.6, respectively), $p<0.001$ (figure 2).

Foxp3 mRNA increased significantly in grade V compared to grade II, III and IV, $p=0.044$ (Table 4).

Foxp3 mRNA had a significant positive correlation with (SLEDAI.2k, rSLEDAI, ESR, urea and creatinine) and had a significant negative correlation with (LN duration, hemoglobin, platelets, C3 and C4). While there was no significant correlation between Foxp3 mRNA and (age, disease duration, anthropometric measurements, TLC, AST, ALT) (Table 5).

ROC analysis was done to assess the performance of Foxp3 mRNA to detect cases of LN from controls; AUC was 0.991 (95% confidence interval: 0.950-1), $p<0.001$. At a cutoff point > 3.3, the sensitivity was 97.5% and specificity was 87.5% (Figure 3). ROC analysis was done to assess the performance of Foxp3 mRNA to detect cases of lupus

nephritis; AUC was 0.995 (95% confidence interval: 0.981-1), p<0.001. At a cutoff point > 10.3, the sensitivity was 96% and specificity was 93.3% (Figure 4).

Discussion

The pathogenesis of LN remains incompletely understood. Recent studies revealed that the fork head transcription factor FOXP3, a key element in the development and homeostasis of $CD4^{+}CD25^{+}$ regulatory T cells (Tregs), plays an important role in the pathogenesis of SLE. A number of quantitative and functional studies have proved altered number of FOXP3-positive cells in SLE and expression of FOXP3 might be related to the activation of $CD4^{+}$ T cells induced by disease activity. The intra-renal expression of FOXP3 and Tregs in LN, however, has not been specifically studied (10).

Kidney biopsy is usually regarded as the 'gold standard' for diagnosis and histological classification of LN. Nonetheless, there are important problems with kidney biopsy. Sampling bias remains a real problem because the kidney may not be uniformly affected in LN. Serial biopsy is practically difficult and is not without complication. On this respect, urinary biomarkers may be an immediate indicator of kidney status and thus an attractive candidate to reflect the activity of LN (11). Urinary expression of FOXP3 has recently been detected in renal-allograft recipients and has been found to be a biomarker of treatment outcome for acute rejection (12).

In the current study, active LN group had statistically significant higher Foxp3 mRNA compared to in active LN and control groups (16.1±2.5, 5.9±2.1 and 1.1±0.6, respectively), p<0.001.

In the same way, a previous study (13), observed that patients with active SLE had a significantly increased level of urinary FOXP3 mRNA expression compared with patients with inactive lupus and healthy controls ($P < 0.001$). Other researchers (14), reported that active lupus group had statistically significant higher levels of Foxp3 than inactive group (21.3 versus 3.6 respectively) (p<0.001). A more recent study (10), reported that FOXP3 was significantly higher in urine of patients with active LN than of patients with inactive lupus and healthy controls (22.93±4.13 vs 5.66± 0.47 vs 0.57±0.15; P < 0.001).

In the present study, Foxp3 mRNA increased significantly in grade V compared to grade II, III and IV, p=0.044. Foxp3 mRNA had a significant positive correlation with (SLEDAI.2k, rSLEDAI, ESR, urea and creatinine) and had a significant negative correlation with (LN duration, hemoglobin, platelets, C3 and C4). While there was no significant correlation between Foxp3 mRNA and (age, disease duration, anthropometric measurements, TLC, AST, ALT).

Our results were in agreement with another study (10), which reported that a significant negative correlation was encountered between urinary FOXP3 and duration of lupus nephritis, serum C3, and serum C4. Urinary FOXP3 mRNA level significantly correlated with SLEDAI (0.000057), and with histological activity index (< 0.00001).

In the same way, other researchers (13), observed that urinary FOXP3 mRNA significantly correlated with SLEDAI ($r = 0.668; P < 0.001$), renal SLEDAI ($r = 0.735; P < 0.001$).

Similarly, another study (14), reported that there was a significant positive correlation between higher levels of Foxp3 and higher mean serum creatinine level (r=0.576, p=0.003), higher mean SLEDAI values (r=0.934, p<0.001).

In the present study, ROC analysis was done to assess the performance of Foxp3 mRNA to detect cases of LN from controls; AUC was 0.991 (95% confidence interval: 0.950-1), p<0.001. At a cutoff point > 3.3, the sensitivity was 97.5% and specificity was 87.5%. ROC analysis was done to assess the performance of Foxp3 mRNA to detect cases of lupus nephritis; AUC was 0.995 (95% confidence interval: 0.981-1), p<0.001. At a cutoff point > 10.3, the sensitivity was 96% and specificity was 93.3%.

This was comparable with a previous study (10), which reported that ROC curve analysis was done to explore the validity of urinary FOXP3 mRNA as a biomarker of lupus nephritis in SLE patients, at a cutoff value >6.3, the sensitivity and specificity were 100%

FOXP3 mRNA expression was found to be higher in blood T cells in patients with active SLE. This paradox is likely because FOXP3 is expressed by CD4þ T cells other than natural Tregs during activation inactive SLE, but whether this activation-induced FOXP3 mRNA suggests a functional Treg potential is still debated. It’s also possible

that the immune response during active LN, like that during acute transplant rejection, involves the activation of both destructive and defensive effector cells, resulting in activation induced FOXP3 mRNA (15). The above results are supported by our findings.

Conclusion

Urinary FOXP3 mRNA expression was found to be significantly up regulated in patients with active LN, and were found to be significantly associated with systemic and histological disease activity. These preliminary findings indicate that urinary FOXP3 mRNA expression could be used as a biomarker in LN to evaluate disease activity and risk stratification. The function of this marker needs to be defined in future research.

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Author contribution

Authors contributed equally to the study.

Conflicts of interest

No conflicts of interest

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Table 1: Sociodemographic data of the studied groups

		Groups						Test	P value
		Active LN group N=25	%	Inactive LN group N=15	%	Control group N=40	%		
Age (years)	**Mean±SD**	13.5±2.5		14.5±1.6		13.9±2.6		F=2.3	0.19
	Range	8-16		9-17		7-16			
Sex	**Male**	7	28.0%	2	20.0%	9	22.5%	X^2=0.45	0.79
	Female	18	72.0%	13	80.0%	31	72.5%		
Consanguinity	**Negative**	14	56.0%	9	60.0%	23	57.5%	X^2=0.06	0.93
	Positive	11	44.0%	6	40.0%	17	42.5%		
History of previous hospital admission	**Negative**	17	68.0%	14	93.3%	39	97.5%	X^2=12.8	0.002*
	Positive	8	32.0%	1	6.7%	1	2.5%		

F: F-value of one way ANOVA, X^2: Chi-square test, *: significant

Table 2: Clinical criteria of lupus nephritis (LN) history in the studied patients

		Groups				Test	P value
		Active LN group		Inactive LN group			
		N=25	%	N=15	%		
LN on presentation	**No**	2	8.0%	2	13.3%	X^2=0.29	0.58
	Yes	23	92.0%	13	86.7%		
Duration (years)	**Mean±SD**	2.9±1.1		2.1±0.7		t=1.8	0.046*
	Range	1-6		1-3			
Histological classification of renal biopsy	**II**	3	12.0%	2	13.3%	X^2=0.62	0.89
	III	8	32.0%	5	33.3%		
	IV	12	48.0%	8	53.3%		
	V	2	8.0%	0	0.0%		

X^2: Chi-square test, t: Student t-test, *: significant

Table 3: Presenting symptoms in patients with active LN.

		Active LN group	
		N=25	**%**
Clinical presentation	**Oliguria**	5	20.0%
	Generalized edema	3	12.0%
	Hypertension	2	8.0%
	Oliguria & edema	7	28.0%
	Oliguria & hypertension	4	16.0%
	Edema & hypertension	2	8.0%
	Oliguria, hypertension & edema	2	8.0%
Urine analysis	**Hematuria**	4	16.0%
	proteinuria	3	12.0%
	Raised urinary protein–creatinine ratio	2	8.0%
	Hematuria & proteinuria	4	16.0%
	Hematuria & Raised urinary protein–creatinine ratio	4	16.0%
	Proteinuroa & Raised urinary protein–creatinine ratio	2	8.0%
	All three	6	24.0%

Table 4: Foxp3 mRNA according to histological classification of renal biopsy

		Foxp3 mRNA Mean±SD	Min.	Max.	Test	P value
Histological classification of renal biopsy	**II**	10.9±5.8	2.6	18.2	F= 7.7	0.044*
	III	11.4±6.5	1.9	18.6		
	IV	12.1±5.9	2.5	19.1		
	V	18.2±1.1	17.4	19.5		

F: F-value of one-way ANOVA, *: significant

Table 5: Correlations between Foxp3 mRNA and other clinical data

	Foxp3 mRNA	
	r	**P value**
Age/ years	0.062	0.586
Disease duration	-0.107	0.511
LN duration	-0.341	0.003*
Weight/percentile	-0.115	0.467
Height/percentile	-0.065	0.566
Body mass Index/percentile	-0.135	0.234
SLEDAI.2k	0.852	<0.001*
rSLEDAI	0.784	<0.001*
Hemoglobin (g/dl)	-0.631	<0.001*
TLC (x106)	0.109	0.338
Platelets (10^3/l)	-0.462	<0.001*
ESR	0.548	<0.001*
C3 (g/L)	-0.372	0.002*
C4 (g/L)	-0.352	0.006*
AST (U/L)	0.121	0.213
ALT (U/L)	0.216	0.064
Urea (mg/dL)	0.514	<0.001*
Creatinine (mg/dL)	0.662	<0.001*

r: Correlation coefficient, *: significant

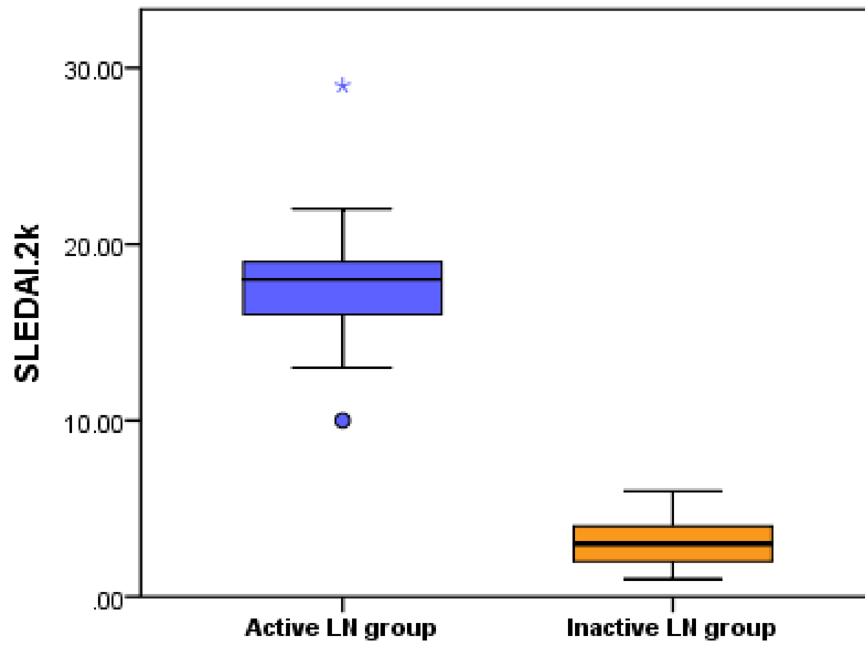

Figure 1: SLEDAI-2k score in the studied patients

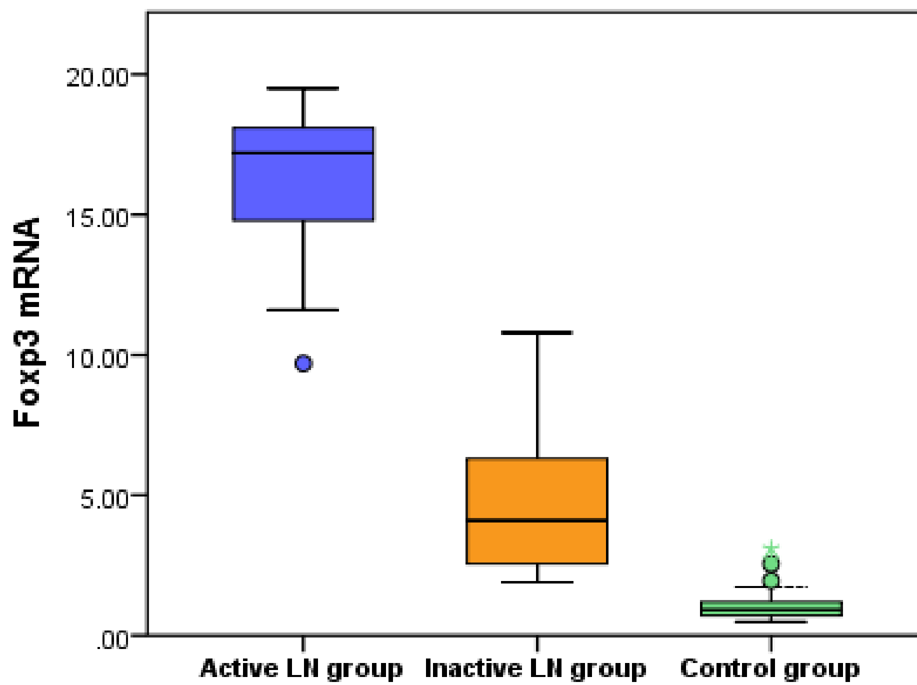

Figure 2: Foxp3 mRNA in the studied groups

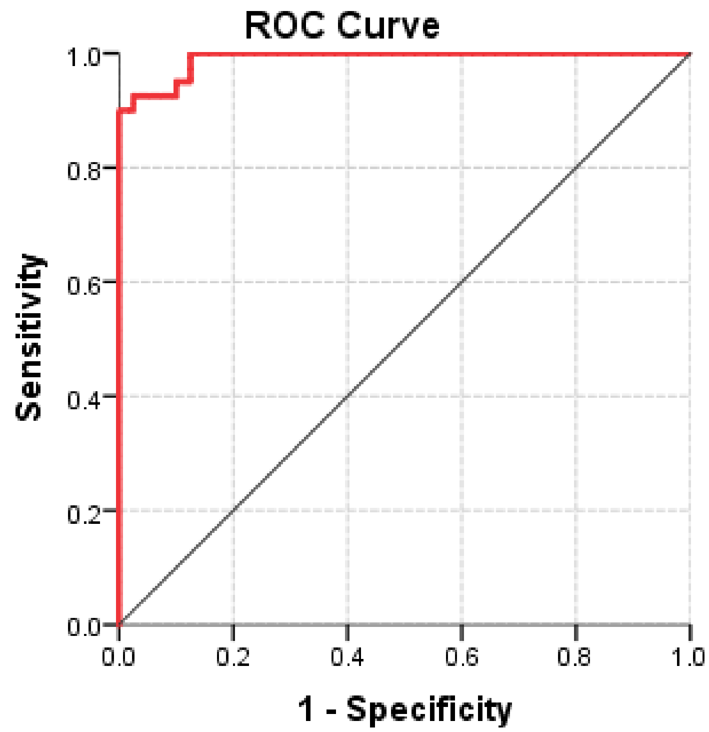

Figure 3: ROC curve of performance of Foxp3 mRNA to detect cases of LN from controls

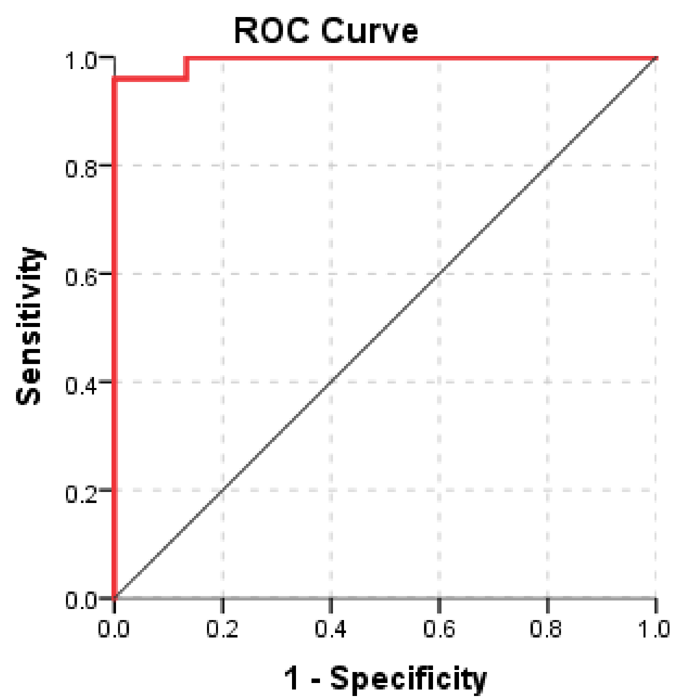

Figure 4: ROC curve of performance of Foxp3 mRNA to detect active cases of LN