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Noninvasive diagnostic value of urinary mir-663a in pediatric lupus nephritis

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Abstract

Background and objectives Lupus nephritis (LN) is a severe clinical manifestation seen in individuals with systemic lupus erythematous (SLE). It has a poor long-term prognosis in pediatric patients with high morbidity and mortality rates. MicroRNAs (miRNAs) are noncoding small RNAs that act as epigenetic modulators, regulating gene expression, and modulating the understanding of mechanisms and pathogenesis of human diseases. Depending on bioinformatics analysis, we aimed to investigate urinary expression of miR-663a in LN among SLE children and discriminate between proteinuria of LN versus chronic renal disease without SLE.

Methods The urinary miR-663a expression levels were estimated in cellular pellets from 15 SLE patients, 15 SLE and biopsy-proven active LN patients, 15 chronic kidney disease (CKD) patients rather than LN and 15 healthy controls.

Results LN patients had significantly higher urinary miR-663a expression levels compared to other groups ($p < 0.0001$). Urinary miR-663a at a cutoff of 8.61 had a diagnostic value of 93.3% for LN among pediatric SLE with 100% specificity ($p < 0.0001$). Moreover, miR-663a was upregulated in advanced grades and LN classes V, IV, and III compared to class II. Furthermore, miR-663a was positively correlated with the duration of SLE, activity index, chronicity index, urinary protein, anti-dsDNA, and SLEDAI score, and negatively correlated with serum complement C3 ($p < 0.05$).

Conclusion miR-663a could be related to the pathogenesis of kidney damage in LN; that could provide a specific noninvasive diagnostic and follow -up tool for LN patients.

Keywords Systemic lupus erythematous, Lupus nephritis, Urinary microRNA

Introduction

Systemic lupus erythematous (SLE) is a multi-systemic autoimmune disease of unknown etiology. Involvement of the kidneys by lupus nephritis (LN) occurs in over 50% of children and is considered a major risk factor of morbidity and a key contributor to end-stage renal disease; in approximately 10–17% [11]. The development of LN among SLE patients is diagnosed by proteinuria of > 0.5 g/24 h or a urine protein: creatinine ratio > 50 mg/mmol in an early morning sample [8]. However, renal biopsy till now remains the 'gold standard' of LN diagnosis showing immune-complex-mediated nephritis with complement deposition associated with varying degrees of cell injury [11]. An early diagnosis of LN is essential for directing treatment and halting the course of chronic kidney disease (CKD). However, because of the invasive nature of renal biopsy and its average wait time for may be more than 50 days, the early diagnosis of LN and

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monitoring disease progression and response to therapy is suboptimal [6].

MicroRNAs (miRNAs) are 18–22 nucleotides RNAs in length and regulate gene expression at the mRNA level. In most cases, miRNAs interact with the 3′ UTR of target mRNAs to suppress expression. However, interaction of miRNAs with other regions, including the 5′ UTR, coding sequence, and gene promoters, has also been reported. Consequently, they could mediate gene regulation functions via the inhibition of protein translation or the promotion of mRNA decay [5]. Numerous studies have demonstrated that alterations in miRNA expression are very vital to disease processes and that miRNAs are essential for renal development and homeostasis. So et al. [15] reported altered levels of different miRNAs in LN patients, suggesting their modulation of underlying immune response and inflammatory pathways in LN.

MiR-663a, located on chromosome 20q11.1, has been linked to many biological processes such as cell differentiation, autoimmune disorders, cancer, and inflammation [10]. Wang et al. [19] reported increased level of miR-663a/miR-423-5p in renal biopsy tissues from LN patients as compared to SLE patients and normal tissues. Meanwhile, forced over-expression of miR-663a could enhance the expression of p65 protein level, and secretion of TNFα, IL-1β and IL-6 through NF-κB activation.

The aim of this study was to evaluate the noninvasive diagnostic value of miR-663a in urine of pediatric LN compared to SLE without renal activity and validate its prognostic value. Furthermore, to evaluate urinary miR-663a in the discrimination between proteinuria of LN versus chronic renal disease without SLE.

Materials and methods

Subjects

This study was conducted on 60 subjects from Allergy and Nephrology Pediatric Department, Faculty of Medicine, Ain Shams University. Sample size was calculated using PASS 11 program for sample size calculation, setting power at 99% and alpha error at 0.05. The participates in the study were divided into four groups:

Group 1 Fifteen SLE patients. SLE disease activity was assessed by the SLE Disease Activity Index 2000 update (SLEDAI-2Ks; range 0–105). SLEDAI-2K includes evaluation of specific manifestations in nine organ systems and contains 24 items, of which 16 are clinical and 8 are based solely on laboratory test results. High disease activity has been defined as an SLEDAI-2K score greater than 6 [1].

Group 2 Fifteen SLE patients with LN confirmed by renal biopsy and classified according to British Isles Lupus Assessment Group (BILAG) index [14]. BILAG index classified disease activity into A to C according to disease severity level, while grade D represents no current disease activity with history of previous affection and Grade E represents no current or previous disease activity.

Group 3 Fifteen CKD patients with stage 2–4 according to National kidney foundation Practice Guidelines; with no activity of SLE [4].

Group 4 Fifteen healthy volunteers matched age and gender, with normal renal function, urine analysis and no history of urinary tract infection, renal stones, or other renal or genitourinary diseases.

Inclusion criteria (1) Male or female cases with 2–18 years, (2): SLE patients were evaluated based on clinical and laboratory evaluation within 10 days before enrollment, (3) LN patients were evaluated based on clinical and laboratory evaluation within 30 days before enrolment, and (4) patients with renal biopsy to groups LN & CKD.

Exclusion criteria (1) Patients with clinical and laboratory features suggestive of autoimmune diseases other than SLE or infections at time of enrollment, (2) patients who have never done renal biopsy were excluded from groups LN & CKD and (3) patients with insignificant renal damage (stage 1) and end-stage renal disease in regular hemodialysis (stage V). All subjects were subjected to complete medical history taking and complete physical examination.

The study was ethically approved by Research Ethics Committee, Faculty of Medicine, Ain Shams University, no: FMASU MS 839/2022, and an informed consents were taken from parents of the provided subjects.

Sample collection

Our patients did not receive any LN-related therapy at the time the samples were taken, which was close to the time of the biopsy. Four milliliters of venous blood were drawn from the subjects and placed in vacutainers with the anticoagulant ethylenediaminetetraacetic acid for laboratory measurements of creatinine, urea, complement C3, anti-dsDNA antibodies, Erythrocyte sedimentation rate (ESR). Fresh urine samples for measurements of urinary creatinine and estimated glomerular filtration rate (eGFR) according to Schwartz formula [13]. Proteinuria levels were all assessed in 24-h urine samples. Renal biopsies were taken from the patients with LN and CKD synchronized with laboratory interventions. Patients chronicity index (score from 0 to 12) and activity index (score from 0 to 24) were determined by renal biopsies [3]. All biopsies were classified according to the modified WHO classification into six classes [20].

Urinary mirna-663a expression levels

Sample preparation

Fresh urine samples were collected (50 mL) and immediately followed by centrifugation at 4000×*g* for 10 min; the supernatant was discarded, and urine pellets were immediately stored at −80 °C.

Extraction of mirna from urine pellet samples

Total miRNAs were extracted from all urine pellets using miRNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's recommendations. RNA integrity was determined using formaldehyde denaturalization agarose gel electrophoresis. RNA concentrations were measured using a smartspecTM plus spectrophotometer (BIO-RAD, Hercules, CA, USA).

Detection of mirna-663a by reverse transcription quantitative polymerase chain reaction (RT-qpcr)

First, the cDNA was synthesized from the total miRNAs of the urine pellets using miRCURY LNA RT kit according to the manufacturer's instructions (Qiagen, Germany). The resultant cDNA was subjected to real-time quantitative polymerase chain reaction (qPCR) using miRCURY LNA SYBR® Green PCR Kit with miRCURY LNA miRNA Primer assays that complimentary with all 22 nucleotide within gene sequence (Qiagen, Germany, Catalog No.339306). U6 was used as an internal control for miRNA. Specific primer oligonucleotides (Qiagen, Germany) were used (Table 1). The relative expression of target genes was calculated with the 2−△△Ct method.

Bioinformatics analysis

The present study used scanning algorithms to search for miRNA specific to pathophysiology of LN through several public databases. miRNA-663a (RefSeq: NR_030386.1 and NCBI GeneID: 724033) with sequence AGGCGGGGCGCCGCGGGACCGC. The bioinformatics data was conducted on DisGeNET database (available at https://www.disgenet.org/browser/0/1/0/C0024143/), miRDB database (available at https://mirdb.org/cgi-bin/target_detail.cgi?targetID=1353107), miRNet database (available at https://www.mirnet.ca/miRNet/Secure/MirNetView.xhtml) and the Human microRNA Disease Database (HMDD) (available at http://www.cuilab.cn/hmdd). This was performed to confirm proposed associations in previous studies [7, 12, 18, 19].

Table 1 Sequences of miR-663a primers

Gene name	qRT-PCR primers sequence (5′–3′)
U6	Forward: 5′-CTCGCTTCGGCAGCACA-3′ Reverse: 5′-ACGCTTCACGAATTTGCGT-3′
miR-663a	Forward: 5′-AGGCGGGGCGCCGCGGGACCGC-3′ Reverse: Universal primers (miScript SYBR Green PCR kit, Qiagen, Germany, Catalog No.339306)

Statistical analysis

Statistical Package for the Social Sciences (SPSS) version 25 was used for coding and analysis of data. Results are presented as means ± standard error of mean (SE). Student's t-test and one-way ANOVA were used to compare between groups. The potential significant associations between urinary miR-663a and other investigated parameters were calculated using Spearman correlation analysis. Evaluation of the diagnostic performance of miR-663a and determination of the best cutoff value was done using Receiver operating characteristic (ROC) curve. p-value ≤ 0.05 was considered statistically significant.

Results

This study was conducted on 60 subjects, divided into four groups; group 1:15 SLE patients; group 2:15 SLE patients with active LN, group 3:15 chronic kidney disease (CKD) patients' stage 2–4, and group 4:15 age and sex matched healthy controls. The demographic and clinical characteristics of these involved subjects are presented in Table 2. ESR and urinary proteins were significant higher in LN patients compared to other groups, $p < 0.001$. Moreover, CKD patients showed high levels of serum creatinine, urea and urinary protein/creatinine ratio, and lower levels of Hb and GFR, $p < 0.001$. SLE and LN patients showed no significant correlations regards complement-3 and anti-dsDNA while SLEDAI-2K score was significantly higher in SLE (15.8 ± 2.57) compared to LN (7.53 ± 0.66), $p < 0.05$. The mean time between the diagnosis of SLE and the development of renal disease of LN was just 0.53 ± 0.27 years. LN patients were classified into A (very active disease) 33.3% and B scores (moderate disease activity) 66.7% according to BILAG index. Moreover, LN patients were categorized according to renal biopsy into 20% class II, 13.3% class III, 20% class IV and 46.7% class V with 5.07 ± 0.75 activity index and 1.8 ± 0.28 chronicity index.

Determination of the relative expression of mir-663a

LN patients showed the highest levels of miR-663a expression in comparison with the other three groups ($p < 0.0001$). Moreover, miR-663a was upregulated in SLE cases in comparison with both CKD and control groups ($p < 0.0001$). Despite increased miR-663a expression in CKD group as compared to the healthy control group, no statistically significant difference was detected ($p > 0.05$) (Fig. 1).

Table 2 Demographic, laboratory data and disease index of all studied groups

Characteristics	Groups				*p*-value
	SLE (*n* = 15)	LN (*n* = 15)	CKD (*n* = 15)	Healthy control (*n* = 15)	
Age (years)	12.8 ± 0.85	12.9 ± 0.81	10.4 ± 1.01	11.7 ± 0.59	> 0.05
Females, *n* (%)	14 (93.3%)	14 (93.3%)	2 (13.3%)	10 (66.7%)	–
Hb (g/dl)	10 ± 0.33	11.9 ± 0.46	9 ± 0.34	12.2 ± 0.29	< 0.001*
Platelets (10^9/L)	218.7 ± 22.2	295.1 ± 19.1	146.6 ± 24.4	253.8 ± 8.28	< 0.001*
ESR (mm/h)	51.8 ± 9.86	57.9 ± 9.32	42.4 ± 1.95	9.3 ± 1.1	< 0.001*
TLC (10^9/L)	6.7 ± 0.4	6.9 ± 0.7	12.7 ± 0.9	5.46 ± 0.3	< 0.001*
Serum creatinine (mg/dl)	0.47 ± 0.02	0.51 ± 0.04	2.24 ± 0.24	0.49 ± 0.03	< 0.001*
GFR (ml/min/1.73 m^2)	120.1 ± 4.9	115.5 ± 9.8	28.59 ± 3.77	129.2 ± 5.18	< 0.001*
Urea (mg/dl)	20.13 ± 1.1	16.9 ± 1.1	63.5 ± 4.58	11 ± 1.05	< 0.001*
Urinary proteins (g/24 h)	0.19 ± 0.02	0.73 ± 0.19	0.56 ± 0.07	0.19 ± 0.02	< 0.001*
Urinary protein/creatinine ratio (g/g)	0.09 ± 0.09	8 ± 2.87	31.96 ± 3.79	0.07 ± 0.07	< 0.001*
Complement C3	106.8 ± 10.2	85.5 ± 12.1	81.5 ± 1.7	–	> 0.05
Anti dsDNA (IU/ml)	66.6 ± 11.9	77.22 ± 14.9	–	–	> 0.05
SLE duration (years)	1.35 ± 0.2	2.54 ± 0.4	–		–
Time between SLE diagnosis and development of renal disease (months)	–	6.36 ± 3.24	–	–	–
Renal BILAG					–
A		5 (33.3%)			
B		10 (66.7%)			
E	15 (100%)				
SLEDAI-2K score	15.8 ± 2.57	7.53 ± 0.66	–	–	< 0.05*
LN class, *n* (%)	–		–	–	–
Class II		3 (20%)			
Class III		2 (13.3%)			
Class IV		3 (20.0%)			
Class V		7 (46.7%)			
Activity index	–	5.07 ± 0.75	–	–	–
Chronicity index	–	1.8 ± 0.28	–	–	–

Values are means ± SE, *p-value < 0.05 is considered significant

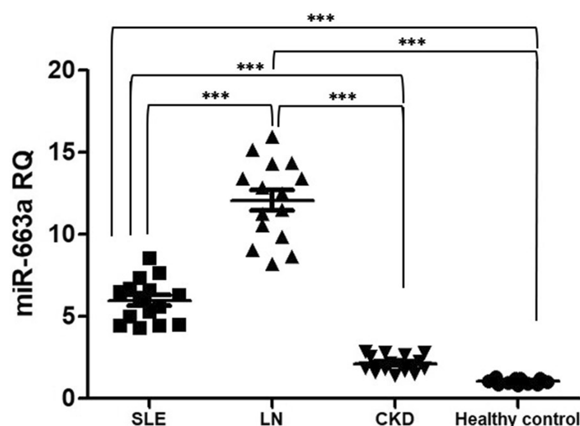

Fig. 1 Scatter plot showing relative expression of miR-663a among all studied groups. Data are presented as mean ± SE ($n = 15$). Groups were compared by one-way ANOVA followed by post hoc test, ***$p < 0.0001$

Evaluation of the diagnostic performance of mir-663a

ROC curve analysis was carried out to determine the best cutoff value for miR-663a discriminating LN from SLE cases (Fig. 2). At cutoff ≥ 8.61, miR-663a showed a sensitivity of 93.3% and a specificity of 100%.

Prognostic value of mir-663a among LN patients

A significant positive correlation was found between urinary miR-663a and duration of SLE, activity index, chronicity index, LN class, urinary protein and SLEDAI score ($p < 0.05$) and anti-dsDNA ($p < 0.001$). While miR-663a was significantly negatively correlated with serum C3 ($p < 0.05$) (Table 3). miR-663a showed higher expression level in group A (13.86 ± 0.77) compared to group B (11.18 ± 0.72) according to Renal BILAG, $p < 0.05$. Regarding LN class miR-663a was increased in class V compared to other groups $p < 0.05$ (Table 4).

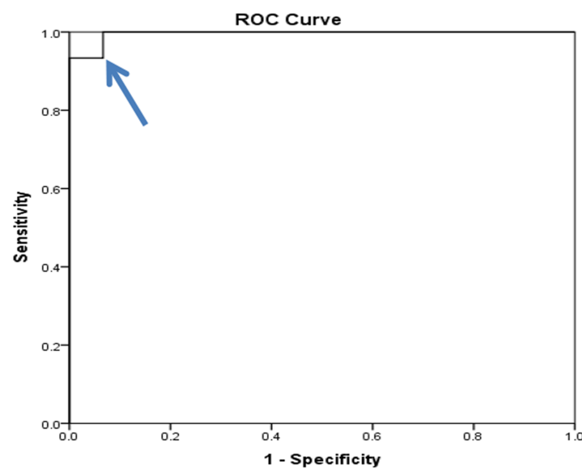

Fig. 2 ROC curve analysis for urine miR-663a to discriminate LN from SLE patients. Area under the curve (AUC) of 0.996

Table 3 Correlations of urinary miR-663a with different variables in LN

Variables in patients with LN	R	*p*-value
Urinary miR-663a		
Hb	0.097	0.731
TLC	– 0.166	0.554
Platelets	0.097	0.731
ESR	−0.220	0.430
Creatinine	0.116	0.682
eGFR	−0.128	0.650
Urea	0.130	0.645
Urinary proteins (g/24 h)	0.726	< 0.05*
Complement C3	−0.690	< 0.05*
Anti dsDNA (IU/ml)	0.788	< 0.001*
SLE duration (months)	7.43	< 0.05*
SLEDAI score	0.703	< 0.05*
Activity Index	0.577	< 0.05*
Chronicity Index	0.575	< 0.05*

**p*-value < 0.05 is considered significant

Table 4 Expression levels of miR-663a in LN patients

	Mean ± SE	Median	*p*-value
Renal BILAG, *n* (%)			
A, 5 (33.3%)	13.86 ± 0.77	14.3	< 0.05*
B, 10 (66.7%)	11.18 ± 0.72	11.04	
LN class, *n* (%)			
Class II, 3 (20%)	6.65 ± 0.24	8.65	< 0.05*
Class III, 2 (13.3%)	10.91 ± 0.37	10.91	
Class IV, 3 (20%)	12.89 ± 0.79	12.85	
Class V, 7 (46.7%)	13.53 ± 0.75	13.43	

**p*-value < 0.05 is considered significant

Discussion

To our knowledge, our study is one of the first to evaluate the urinary levels of miR-663a in pediatric patients with LN as a diagnostic marker among those with SLE. Additionally, we aimed to evaluate its levels in relation to renal damage from LN and chronic renal disease without SLE.

In our study, we found that urinary miR-663a expression levels were significantly higher in pediatric patients with newly diagnosed untreated LN compared to other studied groups. The upregulated miR-663a levels in LN among SLE patients might reflect the association of this marker with the pathogenesis of LN renal damage. At cutoff value ≥ 8.61, urinary miR-663a could discriminate LN from SLE cases with diagnostic sensitivity of 93.3% and 100% specificity, $p < 0.0001$. Moreover, the significant increase of miR-663a in LN compared to CKD of non-lupus patients who had proteinuria suggests it as a specific marker for renal damage associated with LN rather than other causes. This is consistent with So et al. [15] who recorded that miR-663a levels were overexpressed in kidney biopsies from LN patients. Rashad et al. [12] reported that serum TGFβ1 as one of the underlying pathogenic factors of LN that was significantly decreased with more renal damage. Meanwhile, miR-663a has been shown to be closely associated with SLE disease activity, where it inhibits the proliferation and migration of bone marrow-derived mesenchymal stem cells by targeting TGF-β1 expression and activates SMAD family [7]. Other researchers reported that miR-663a/miR-423-5p fundamentally contributed to LN induced NF-κB activation in HEK293T culture cells [19].

Our results showed that complement-3 and anti-dsDNA had no significant correlations between SLE and LN patients, while the SLEDAI-2K score was significantly higher in SLE (15.8 ± 2.57) compared to LN (7.53 ± 0.66), $p < 0.05$. This may be explained as the SLEDAI-2K score is a generalized disease activity index that give a high score in SLE compared with selective criteria of renal affection in LN [1, 3]. As expected, urinary proteinuria was significantly higher in LN compared to SLE patients which raises concerns for complicated SLE with LN as reported by Touma et al. [17]. However, the lack of other significant laboratory parameters between SLE and LN patients in this study indicates the absence of clear evidence for early diagnosis and follow-up of the disease.

Renal biopsies are crucial for the confirmation of the diagnosis, assessment of disease activity, prognosis, and treatment of LN patients [2]. We found that miR-663a levels were elevated with advanced classes of LN; class V (13.53 ± 0.75), class IV (12.89 ± 0.79), III (0.91 ± 0.37) compared to class II (6.65 ± 0.24), $p < 0.05$. The renal disease activity of our enrolled LN patients was graded according to the BILAG index, and they were classified into A

and B grade. We found that miR-663a showed 1.3 times higher levels in advanced disease activity, renal BILAG A (13.86 ± 0.77) compared to BILAG B (11.18 ± 0.72), $p < 0.05$. Other previous researches had clarified the role of urinary miRNAs in LN patients; Li et al., [9] reported that urinary miR-3201 and miR-1273e and urinary exosomal miR-654-5p and miR-3135b could distinguish between LN renal biopsy classes. Furthermore Tan et al. [16] found that downregulated exosomal miR-451a expression is significantly associated with SLE activity and associated renal damage of LN.

It was reported that anti-DNAds, serum C3 complement, creatinine clearance, urine sediment microscopy for urinalysis, proteinuria and 24-h proteinuria could be considered as the international guidelines for lupus nephritis [21]. In the current study, miR-663a was positively correlated with the duration of SLE, activity index, chronicity index, urinary protein, SLEDAI score and anti-dsDNA, and negatively correlated with serum C3 among LN patients, highlighting its value in monitoring activity and following up on disease progression.

In conclusion, the urinary miR-663a expression levels correlate with the extent and specific activity of renal damage in LN disease and are associated with aggressive clinical behavior, highlighting the usefulness of this marker as a potential noninvasive specific diagnostic and prognostic factor for LN among pediatric SLE patients. Larger sample size in future studies could help increase the reliability and accuracy of miR-663a in diagnosis of LN. Future studies should focus on the analysis of the pathogenic mechanism of miR-663a and its correlation with proteins of the target genes. This could be valuable in both suggesting a diagnostic panel that could enhance the accuracy and reliability, and also for exploration of its possible therapeutic intervention and better management of LN patients.

Abbreviations

LN	Lupus nephritis
SLE	Systemic lupus erythematous
miRNAs	MicroRNAs
CKD	Chronic kidney disease
BILAG	British isles lupus assessment group
ESR	Erythrocyte sedimentation rate
eGFR	Estimated glomerular filtration rate
qPCR	Quantitative polymerase chain reaction

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Author contributions
TA contributed to methodology, resources, writing-original draft, MT contributed to conceptualization, methodology, formal analysis, investigation, resources, writing-original draft, writing—review & editing, visualization, SA contributed to methodology, investigation, resources, validation, writing—review & editing, MA contributed to conceptualization, methodology, writing—review & editing, visualization, supervision. RE contributed to methodology, investigation, writing—review & editing, DS contributed to methodology, validation, writing—review & editing, and MA contributed to conceptualization, methodology, writing—review & editing, visualization, supervision. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate
The study was ethically approved by Research Ethics Committee, Faculty of Medicine, Ain Shams University, no: FMASU MS 839/2022 and an informed consents were taken from parents of the provided subjects.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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