

# Journal of Nephropathology



## Assessment of microRNA profile of kidney biopsies of patients with lupus nephritis

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### ARTICLE INFO

*Article type:*  
Original Article

*Article history:*  
Received: 11 November 2016  
Accepted: 20 January 2017  
Published online: 12 February 2017  
DOI: 10.15171/jnp.2017.53

*Keywords:*  
SLE  
Kidney  
Biopsy  
microRNA  
Lupus nephritis

### ABSTRACT

**Background:** The changes in some epigenetic elements such as microRNAs result in aberrant immune responses leading to production and secretion of nephritogenic autoantibodies as the main fundament of lupus nephritis (LN).

**Objectives:** The present study aimed to assess the miRNA profile of kidney biopsies in patients with LN with the purpose of describing the critical role of these elements in LN creation.

**Patients and Methods:** In this case-control single center study 11 patients who suffered LN (as the case group) and 11 patients with normal kidney function who were candidate for nephrectomy due to cancer or cyst (as the control group) were included. Kidney biopsies were taken from all LN and control subjects. RNA was extracted and converted to cDNA, then the cDNA was evaluated using NANODROP and then intra-renal expression of candidate miRNAs were quantified in the two groups. In the present study, four top-ranked miRNAs, miR-638, miR-146a, miR-198, and miR-731 were selected for qRT-PCR.

**Results:** Consistent with the microarray data, we found no significant difference in the expression of all miRNAs between LN and control groups. Using REST 2009 software, we did not also reveal any difference in expression of four miRNAs studied between the patients with LN and those without LN in both parametric and nonparametric patterns.

**Conclusions:** The expression of miR-638, miR-146a, miR-198, and miR-731 may not be related to occurrence of LN in Iranian population.

### *Implication for health policy/practice/research/medical education:*

Lupus nephritis is one of the most important complications of systemic lupus erythematosus (SLE). Kidney biopsy is gold standard for diagnosis, however, microRNAs evaluation is a novel way of diagnosis while it is noninvasive. The change in these epigenetic elements results in aberrant immune responses leading production and secretion of nephritogenic autoantibodies as the main fundament of lupus nephritis.

*Please cite this paper as:* Malakoutian T, Hajian S, Ebrahimi A, Kamali K. Assessment of microRNA profile of kidney biopsies of patients with lupus nephritis. J Nephropathol. 2017;6(4):333-337. DOI: 10.15171/jnp.2017.53.

### 1. Background

Lupus nephritis (LN) is a common serious complication of systemic lupus erythematosus (SLE) evident in most affected patients even before appearing clinical symptoms of renal impairment (1). The LN is frequently evident with proteinuria, hypertension, and even may be progressed to end-stage renal failure (2). The main pathogenesis of LN refers to autoimmunity related to production and secretion of nephritogenic autoantibodies against nuclear elements (3-6). In

this regard, the cross-reaction of some mutated and class-switched anti-double-stranded DNA (dsDNA), anti-histone, and anti-ribonucleoprotein secreted by plasma cells with the glomerular basement membrane is the fundament of disease pathogenesis (7,8). These cationic autoantibodies highly tend to form intravascular immune complexes that deposit in anionic glomeruli basement membrane and thus activate elements of immune reactions as well as complements. In severe LN forms, fibrosis may be appeared due to

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proliferation of epithelial and endothelial cells and accumulation of matrix proteins (9). Histologically, LN is typed according to the antigen specificity as well as type of inflammatory response (10). Moreover, glomerular thrombosis due to autoantibodies secretion against phospholipid-protein complexes has been revealed to be another pathological process. Based on the pointed mechanisms, early evaluation of renal functioning is very important leading improvement of renal outcome. While histological changes of LN may be appeared from the first episodes of disease, renal biopsy is potentially considered for all SLE patients with any clinical evidences of LN.

Overall, the development of LN is dependent to both genetic and environmental factors. In this regard, genetic deregulation due to gene polymorphisms is strongly related to developing LN. In fact, the main sources of producing disease-related autoantibodies include some specific gene variants appeared by specific polymorphisms (11). Recently, it has been demonstrated that, the changes in some epigenetic elements such as microRNA result to aberrant immune responses leading production and secretion of autoantibodies (12,13). In fact, the central role of miRNAs as the main mediator of autoimmunity is now suggested in LN. MiRNAs are small RNAs that can regulate protein expression by inhibition of mRNA stability. These particles involve in large range of physiological and pathological processes such as immune responses. Thus, any abnormal change in these miRNAs may be diversely linked to pathological immune conditions such as autoimmunity.

## 2. Objectives

The present study as the first population-based study in Iran aimed to assess the miRNA profile of kidney biopsies in patients with LN with the purpose of describing the critical role of these elements in LN creation.

## 3. Patients and Methods

### 3.1. Study population

In this case-control, single center study 11 patients suffering LN who referred to Hashemi-Nejad hospital from 2014 to 2015 constituted our study population. All subjects fulfilled the American College of Rheumatology (ACR) definitive criteria for SLE. The control group (n = 11) was selected from patients with normal kidney function who were candidate for nephrectomy due to cancer or cyst.

### 3.2. Study procedure

Kidney biopsies with 0.3 mm thickness were taken from all LN and control subjects in procedure room of

nephrology ward. The samples were then washed with normal saline 0.9% and placed in microtubes containing RNAlater, and stored in 4°C. After depressor removing, RNA was extracted and converted to cDNA, then the cDNA was evaluated using NanoDrop and then intra-renal expression of four candidate miRNAs (or miRNAs profile) including miR638, miR-146a, miR-198 and miR-371 were quantified in the two groups using particular primers. These miRNA targets were reported to be differentially expressed in peripheral blood or urine between the LN and control groups across different racial subgroups (7-9). To proliferation of microRNA, a tail of polyA was added to miRNA in PASAGENOME MiR-Amp kit in three steps. In the first step, 10 x Reaction Buffer, 2 µL, ATP 1 µL, **poly** A enzyme 0.5 µL and RNA 1.5-2 µg were mixed using pipet and finally were spun in 37°C and was incubated for 10 minutes. In second step, 5 x Reaction Buffer 2 µL, DNTP 1 µL (10mM), RT enzyme 0.5 µL, miR cDNA Syn specific primer 0.5 µL (15 pmol) and RNA poly A tail up to 2 µg were mixed together using pipet and finally were spun. The mixture was incubated in 43-45°C and for 60 minutes was incubated, then was incubated in 85°C for 1 minute (to inactivate RT). Then, SYBER Green master mixture vials were shaken before using, and other materials including Rox dye, cDNA (diluted) 7-8 µL, the mix of Mir specific primers 1each 10 pmol, were mixed and spun speedily and were prepared for consumption. The PCR steps were a denaturing cycle at 95°C for 5 minutes and 95°C for 5 seconds followed by 40 cycles of proliferation at 62-65°C for 20 seconds and 72°C for 30 seconds.

### 3.3. Ethical issues

The research followed the tenets of the Declaration of Helsinki. The research was approved by the ethical committee of Iran University of Medical Sciences. This study was conducted as a nephrology fellowship thesis in this university.

### 3.4. Statistical analysis

For statistical analysis, the results were expressed as mean ± standard deviation (SD) for quantitative variables and were illustrated by absolute frequencies and percentages for categorical variables. Normality of data was analyzed using the Kolmogorov-Smirnoff test. Categorical variables were compared using chi-square test or Fisher's exact test when more than 20% of cells with expected count of less than 5 were observed. Quantitative variables were also compared with *t* test or Mann-Whitney U tests. For the statistical analysis, the statistical software SPSS version 16.0 for windows (SPSS Inc., Chicago, IL) was used. We also used Rest 2009 software as specific software to compare genes

expression. *P* values of 0.05 or less were considered statistically significant.

#### 4. Results

In total, 11 LN patients and 11 controls were included. The mean age of subjects in LN group was significantly higher than that of control group (23 versus 56 years old,  $P < 0.001$ ). The mean duration of disease in LN patients was 2.3 months. The mean systolic blood pressure of subjects was 147 mm Hg in cases and 123 mm Hg in controls. The average glomerular filtration rate was 72 cc/min in LN group and 80 cc/min in control group, indicating significantly lower in former group ( $P < 0.001$ ). In the present study, four top-ranked miRNAs—miR-638, miR-146a, miR-198, and miR-731—were selected for qRT-PCR. House keeper was applied as internal control. Consistent with the microarray data, we found no significant difference in the expression of all miRNAs between LN and control groups (Table 1). Using Rest 2009 software, we did not also reveal any difference in expression of four miRNAs studied between the patients with LN and those without LN in both parametric and nonparametric patterns (Table 2).

#### 5. Discussion

Targeted therapy is now accepted as an important medical approach that is achieved by modulating abnormal miRNA expression patterns. The technical basis of this treatment is to determine specific miRNA expression related to the genes involved in the disease. In this regard, specific genes are involved in pathogenesis of LN that encodes some specific proteins in disease pathway. In this line, some miRNAs can inhibit special loci of these genes and thus can be applied as the source of gene therapy in LN patients. In other word, deregulated expression of these miRNAs represents an underlying trigger inducing LN and its pathogenesis (14). Some of these miRNAs directly involve in expression of pro-inflammatory cytokines that dictate magnitude of immune response (15-17). Within the recent two decades, more than 140 conserved miRNAs were discovered that have specific target site on 72 candidate genes for lupus and LN (18). The investigations could show that some miRNAs play a central role in the pathogenesis of LN by reducing production of pro-inflammatory mediators as well as with inhibiting lymphocyte function (19-23). In the

**Table 1.** Comparison of miR-638, miR-146a, miR-198, and miR-731 expression in LN and control groups

microRNA	Group	Minimum	Maximum	Percentiles			<i>P</i>
				25th	50th (Median)	75th	
ff	Control	20.96	40.00	22.77	32.30	35.91	0.94
	Lupus nephritis	21.95	35.00	31.46	32.25	34.65	
miR-146a	Control	20.96	40.00	22.45	32.29	34.92	0.74
	Lupus nephritis	21.96	34.23	31.91	31.98	32.93	
miR-198	Control	19.88	34.96	21.78	30.68	32.63	0.89
	Lupus nephritis	20.93	33.26	29.96	30.71	31.73	
miR-371	Control	19.94	33.97	21.93	30.45	32.64	0.56
	Lupus nephritis	21.94	32.99	30.68	31.00	31.82	
HK control	Control	25.99	40.00	29.39	33.02	35.59	0.69
	Lupus nephritis	27.97	40.00	30.98	32.29	33.95	

**Table 2.** Comparison of miR-638, miR-146a, miR-198, and miR-731 expression in LN and control groups using REST 2009 software

Gene	Type	Reaction efficiency	Expression	Standard error	95% CI	<i>P</i> (H1)
Parametric pattern						
miR-638	TRG	1.0	0.384	0.003 - 144.986	0.000 - 2,457.541	0.545
miR-146a	TRG	1.0	0.250	0.002 - 61.017	0.000 - 2,393.656	0.423
miR-198	TRG	1.0	0.273	0.003 - 15.084	0.000 - 2,035.264	0.386
miR-371	TRG	1.0	0.259	0.004 - 10.792	0.000 - 1,005.014	0.336
HK	REF	1.0	1.000			
Nonparametric pattern						
miR-638	TRG	1.0	0.216	0.001 - 9.302	0.000 - 214.525	0.257
miR-146a	TRG	1.0	0.141	0.001 - 7.823	0.000 - 260.114	0.205
miR-198	TRG	1.0	0.154	0.001 - 4.254	0.000 - 20.450	0.147
miR-371	TRG	1.0	0.146	0.001 - 2.899	0.000 - 7.841	0.134
HK	REF	1.0	0.563	0.022 - 8.373	0.001 - 343.937	0.564

*P* (H1) - Probability of alternate hypothesis that difference between sample and control groups is due only to chance.

Abbreviations: TRG, Target; REF, Reference; HK, Control.

present study, we assessed the expression of three introduced genes including miR638, miR-146a, miR-198 and miR-371 in LN patients. There are numerous evidences for involvement of some of these miRNAs in LN pathogenesis (miR638 and miR-146a), but some others have been studied very little.

As shown in our experiment, miR-638 expression was similar in both LN patients and control group. Pathophysiological studies could demonstrate increased expression of miR-638 in both glomerular and tubule-interstitial tissues that is directly associated with proteinuria and thus with SLE disease activity index. However, it remains uncertain whether inhibition of this miRNAs may introduce as a target therapeutic approach in LN patients (24).

More evidences are available in involvement of miR-146a in pathogenesis of LN, however we could not demonstrate its role in pathogenesis of disease. It has been revealed that the expression of miR-146a involves in several pathological pathways including tubular atrophy and interstitial fibrosis by infiltrating inflammatory cells such as macrophage and T cell as well as with secretion of some cytokines such as IL-1 $\beta$ , IL-10, and CXCL leading progressive renal inflammation (25). Similar to our study, some authors demonstrated the role of miR-146a in LN pathogenesis (24). In a study by Lu et al, miR-146a is not overexpressed in LN tubulointerstitial tissue, however effectively up-regulate in glomerular tissue (24). In another study, expression of miR-146a was shown to be involved in level of inflammation and thus can determine histological activity index (25). It has been also identified a functional variant in the promoter of miR-146a associated with LN risk (26,27). We could not show any significant role for miR-198 in pathogenesis of LN. A few studies assessed the relation between miR-198 and LN. As shown by Lu et al (28), both glomerular and tubulointerstitial expression of miR-198 were higher in LN patients than controls, but we could not show this relationship. Also, contrary to our finding, Te et al (29) showed significant association between miR-371 and LN, however it has been also pointed the different expression of this miRNA in different ethnic groups. In fact, the major ethnical differences are responsible for the difference in miRNA variants. Finally, it seems that small sample size of the present study might also responsible for insignificant causalities in our research.

## 6. Conclusions

In conclusion, our study could not show the relationship between the four miRNAs studied and appearance of LN in a sample of Iranian population. In fact, these miRNAs may not play a role in LN, but the potential limitations of the study such as small size or ignoring

ethnic diversity may be also the main reasons for these findings.

## Strengths and limitations of the study

Present study introduced a novel method of evaluation in patients with LN in our country however it was a single center study with small sample size due to cost. Results would be reliable for judgment if research be done in the form of multi-center study with larger sample size.

## Acknowledgements

The authors are grateful to the “Genetics Center” of Shahid-Beheshti University of Medical Sciences for cooperation in this research.

## Conflicts of interest

The authors declare no conflict of interest.

## Authors' contribution

TM and SH conducted the research. TM and SH and KK collected the data. AE analyzed the data. SH and AE prepared the primary draft. TM edited the final draft. All authors signed the manuscript.

## Funding/support

This paper was extracted from nephrology fellowship thesis of Sepideh Hajian affiliated to Iran University of Medical Sciences.

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