Whole peripheral blood miR-146a and miR-155 expression levels in systemic lupus erythematosus patients

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ABSTRACT

Objective: To evaluate the diagnostic value of peripheral blood microribonucleic acid (miRNA, miR)-146a and miR-155 expression in systemic lupus erythematosus (SLE).

Methods: Expression levels of miR-155 and miR-146a in whole peripheral blood samples from 40 SLE patients and 32 healthy controls (HCs) were determined by quantitative reverse transcription-polymerase chain reaction qRT-PCR (SYBR Green technology) and $2^{-\Delta\Delta Ct}$ method was used for analysis. SPSS v20 was used for receiver operating characteristic (ROC) curve and Spearman correlation analysis.

Results: Whole peripheral blood expression levels of miR-146a and miR-155 were overexpressed in 62.5% and 50%, respectively, of the SLE patients compared to HCs. The ROC curve analysis showed that the expression levels of miR-146a could discriminate SLE patients from HCs with area under the curve (AUC)=0.711 (95% CI: 0.585÷0.837, p=0.002, with 82.5% sensitivity and 56.2% specificity. The diagnostic accuracy of miR-155 was lower with AUC=0.691 (95% CI: 0.566÷0.817, p=0.005, with 77.5% sensitivity and 50.0% specificity. The diagnostic accuracy did improve when combination of the studied miRNAs was used in multimarker ROC curve analysis (AUC=0.716, 95% CI: 0.590÷0.842, p=0.002, 82.5% sensitivity and 56.2% specificity). miR-146a and miR-155 showed correlation with the diagnosis (rs=0.363 and 0.330, respectively) and the age of the patients (rs =0.239 and 0.366, respectively), and miR-155 showed correlation with the presence of secondary Raynaud syndrome (Spearman correlation coefficient=0.250).

Conclusions: Our data showed that the expression levels of miR-146a and miR-155 in PB could be used as diagnostic biomarkers for SLE patients but larger study is needed to confirm these results.

Keywords: Systemic lupus erythematosus; miRNA expression; Peripheral blood; Biomarker;

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a complex autoimmune disease characterized by autoantibody production, complement activation and deposition of immune complexes in tissues and organs[1,2]. T cells play a crucial role in the pathogenicity of SLE through stimulating the autoantibody producing B cells[3]. Alterations in the phenotype and functional activity of T cells and T cells subtypes lead to their failure to suppress autoreactive lymphocytes, the proinflammatory milieu and thus to lupus-related inflammation[4-6].

Although the pathogenesis of SLE is still unknown recent studies reveal the biological and clinical relevance of epigenetic changes related to the development of SLE including alterations in deoxyribonucleic acid (DNA) methylation, histone modification and micro-ribonucleic acid (microRNA, miRNA) expression[7-11]. miRNAs are endogenous short noncoding RNA molecules that negatively regulate the gene expression on posttranscriptional level by targeting the messenger RNAs (mRNAs). In SLE miRNAs control the differentiation and immunological functions of B cells, induction pathways in T cells, activation, function and maintenance of regulatory T-cells (Tregs)[12-19]. miRNAs target several interferon (IFN) signaling mediators and IFN--inducible genes in lupus derived peripheral blood monocytes (PBMC) as well as molecules related to T cell hyperactivity and abnormality[20].

miRNAs as well as molecules involved in their biogenesis has been linked to the levels of autoantibody in

SLE. Interestingly, anti-Su autoantibodies patients with SLE could recognize the catalytic enzyme in miRNA pathways thus indicating a possible relationship between miRNA and the pathogenesis of SLE[21].

Two of the most studied miRNAs in autoimmune rheumatic diseases including SLE are miR-146a and miR-155. It has been found that the altered expression of miR-146a in lupus PBMCs correlates with the activity of SLE and IFN scores. On molecular level miR-146a targets the signal transducer and activator of transcription (STAT) 1 and interferon regulatory factor (IRF) 5 and thus negatively regulates the IFN signaling pathway[20,22]. In lupus macrophages miR-146a also modulates the production of type I IFN by targeting tumor necrosis factor (TNF) receptor-associated factor (TRAF)-6, interleukin-1 receptor-associated kinase (IRAK)-1, and IRAK2 in the retinoic acid-inducible gene (RIG)-I-dependent type I IFN production pathway[23]. miR-155 has been found to be overexpressed in lupus Tregs[24]. miR-155 has a contrary regulatory function in SLE - it could inhibit inflammatory response by targeting the myeloid differentiation primary response gene 88 (MYD88) and the adapter molecule TAB2 but also promotes inflammatory response and type I interferon signaling by targeting the suppressor of cytokine signaling (SOCS)-1 in macrophage. miR-155* has an opposite effect on the regulation of type I interferon production compared to that of miR-155 in plasmacytoid dendritic cells (pDCs) and both forms cooperatively regulate the production of type I IFN by human plasma cells[25,26]. Altered miR-155 expression has been described also in SLE B cells. Overexpression of miR-155 in SLE B cells contributes to enhance B cell survival and higher titer of IgG autoantibodies by downregulation of SH2 domain containing inositol-5--phosphatase (SHIP)-1 protein[27-29].

The aim of our study was to evaluate the diagnostic value of whole peripheral blood (PB) miR-146a and miR-155 expression levels in SLE patients. This is the first study to demonstrate the expression pattern of these two miRNAs in whole PB samples of SLE.

MATERIAL AND METHODS

PREPARATION OF PERIPHERAL BLOOD SAMPLES

Ethical approval for this study was granted by the ethics committee of the Medical University – Sofia. Informed consent was obtained from 72 participants from European ancestry (40 SLE patients and 32 healthy controls (HCs), Table I). According to the ethics committee, HCs were over 18 years of age, with not known rheumatic conditions. SLE was diagnosed according to the 1997 update of the 1982 American College of Rheumatology revised criteria for classification of SLE[30]. PB samples were collected in PAXgene RNA tubes and stored at -80°C prior isolation. All samples were thawed only one time.

RNA ISOLATION, cDNA SYNTHESIS AND REAL-TIME PCR

Total RNA from the PB samples was extracted with PAXgene Blood miRNA kit. The concentrations and quality of RNA samples were evaluated spectrophotometric by NanoDrop. Equal concentrations of RNA samples were used in reverse transcription reactions for synthesis of complementary DNA (cDNA) with miScript II RT kit (Qiagen®). Before use, every cDNA was diluted as recommended. Quantitative real-time polymerase chain reaction (PCR) was done on the Applied Biosystems 7900HT Fast Real-Time PCR System with miScript SYBR Green PCR kit and miScript Primer Assays (Qiagen®). RNU6B was used as reference control for normalization as described before[31]. Relative changes of gene expression levels of studied miRNAs were calculated by the $2^{-\Delta\Delta Cq}$ method. HCs were used as calibrators in the analysis. Real-time experiments were performed in triplicates and the mean Ct values were calculated.

CLINICAL MEASUREMENTS

Clinical disease activity was calculated using the systemic lupus erythematosus disease activity score (SLEDAI). Lupus activity profile (LAP) was determined by measurement of the titer of antinuclear antibodies, anti-double stranded DNA antibodies and levels of complement components – C3 and C4. Testing for anti-extractable nuclear antigen antibodies (ENA) was performed by using ANA immunoblot including IgG antibodies against 15 extractable nuclear antigens – RNP/Sm, Sm, ssA, ssB, Ro52, Scl70, PM/Scl70, Jo1, Cent B, PCNA, dsDNA, Nucleosomes, Histones, Ribosomal P-protein, Mi2 (Euroline 3, Euroimmun®, Germany). Testing for antiphospholipid antibodies was performed by in-vitro diagnostic (IVD) test kits [anti-b2GPI (IgA, IgG, IgM) Human®; anti-prothrombin (IgG/IgM) Alegria, Orgentec®); anti-cardiolipin (IgA, IgG, IgM) Euroimmun® EA)]. Laboratory activity was measured by elevated acute phase reactants (ery-

TABLE I. CLINICAL CHARACTERISTICS OF THE PARTICIPANTS

Characteristics	SLE	HCs
Number of pts	40	32
Gender, men/women	0/40	10/22
Age in years (range) mean age	24÷67 (43.6±12.01)	26÷72 (39.15±12.07)
Disease duration, months (mean, range)	99.02 (1÷336)	n/a
ANA (+), number (%)	36 (90.0 %)	0 (0.0 %)
a-dsDNA Ab (+), number (%)	12 (30.0 %)	n/a
a-nucleosomes Ab (+), number (%)	4 (10.0 %)	n/a
a-histones Ab (+), number (%)	3 (7.5 %)	n/a
a-Sm Ab (+), number (%)	4 (10.0 %)	n/a
a-ssA Ab (+), number (%)	11 (27.5 %)	n/a
a-Ro52 Ab (+), number (%)	13 (32.5 %)	n/a
a-ssB Ab (+), number (%)	7 (17.5 %)	n/a
aCLs Ab (+), number (%)	5 (12.5 %)	n/a
a- 2GP1 Ab (+), number (%)	7 (17.5 %)	n/a
Anti-protrombin Ab (+), number (%)	0 (0.0 %)	n/a
Low C3 franction (+), number (%)	3 (7.5 %)	n/a
Low C4 franction (+), number (%)	11 (27.5 %)	n/a
ESR mm/h (mean, range)	29.27 (2 ÷ 80)	n/a
CRP mg/dl (mean, range)	5.59 (0.05÷24.78)	n/a
SLEDAI (mean, range)	5.53 (1÷13)	n/a
Secondary Raynaud syndrome	12 (30.0 %)	n/a
Secondary Sjögren's syndrome	8 (20.0 %)	n/a
Secondary APS	4 (10.0 %)	n/a
Corticosteroids, n (pts), mean dose (mg)	40 (10.2)	n/a
DMARD	37 (92.5 %)	n/a
Biological DMARD	5 (12.5 %)	n/a

Ab: antibody; a- 2GP1 Ab: anti-beta2 glycoprotein 1 antibodies; a-CL Ab: anti-cardiolipin antibodies; a-dsDNA Ab: anti-doublestrained deoxyribonucleic acid antibodies; ANA: antinuclear antibodies; a-Sm Ab: anti-Smith antibodies; APS: antiphospholipid syndrome; CRP: C-reactive protein; SLEDAI: systemic lupus erythematosus disease activity index; DMARD: disease modifying antirheumatic drug; ESR: erythrocyte sedimention rate; HCs - healthy controls; n/a - not applicable; pts: patients; SLE: systemic lupus erythematosus.

throcyte sedimentation rate – ESR, C-reactive protein--CRP). The frequency of secondary Raynaud syndrome, Sjögren's syndrome and antiphospholipid syndrome was determined and correlated with disease variables.

STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS Statistics v. 20.0. The Student's t-test and the Spearman's rank correlation coefficient were used for comparison and estimation of correlations between miRNA expression levels and clinico-pathological characteristics such as diagnosis, age, ESR, CRP, SLEDAI, immunological parameters, presence of secondary Raynaud syndrome and Sjögren's syndrome. Receiver operating curve (ROC) analysis was performed for evaluation of specificity and sensitivity of PB miRNA expression levels for discriminating SLE patients from HCs. Diagnostic accuracy for combination of biomarkers was also determined by calculating weight coefficients for every biomarker obtaining the largest possible area under the curve (AUC) in ROC analysis. Calculation of coefficients was performed according to Pepe and Thomson[32]. Two-tailed p-values were taken into account.

RESULTS

Relative miRNA expression levels in PB samples of SLE patients were obtained by using RNU6B as a reference gene for normalization and HCs as calibrator samples.

Relative quantification (RQ) values were calculated by the $2^{-\Delta\Delta Ct}$ method for evaluation of the expression levels. RQ values between 0.500 and 1.999 mean no significant difference in expression, values ≤ 0.499 mean decreased expression and values ≥ 2.00 mean increased expression.

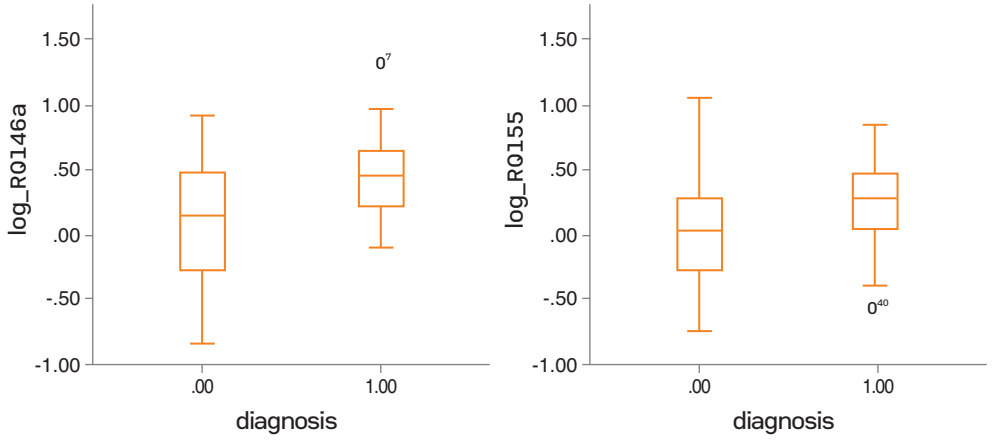
PERIPHERAL BLOOD miRNA EXPRESSION

Peripheral blood expression levels of miR-146a and miR-155 were overexpressed in 62.5% and 50.0%, respectively, of the SLE patients compared to HCs (Figure 1).

The ROC curve analysis showed that the expression levels of miR-146a could discriminate SLE patients from HCs with AUC=0.711 (95% CI: 0.585÷0.837, p=0.002), with 82.5% sensitivity and 56.2% specificity. The diagnostic accuracy of miR-155 was lower with AUC=0.691 (95% CI: 0.566÷0.817, p=0.005), with 77.5% sensitivity and 50.0% specificity. The diagnostic accuracy did improve when combination of the studied miRNAs was used in multimarker ROC curve analysis (AUC=0.716, 95% CI: 0.590÷0.842, p = 0.002), 82.5% sensitivity and 56.2% specificity (Figure 2, Table II).

CLINICAL PARAMETERS AND PERIPHERAL BLOOD miRNA EXPRESSION LEVELS

miR-146a and miR-155 showed correlation with the diagnosis (rs=0.363, p=0.002 and 0.330, p=0.005, respectively) and the age of the patients (rs=0.239, p=0.043 and 0.366, p=0.002, respectively), and miR-155 showed correlation with the presence of secondary Raynaud syndrome (Spearman correlation coefficient was 0.250, p=0.035). None of studied miRNA correlated with SLEDAI (rs -.098, p=0.547 and rs -.022, p=0.894) nor with the immunological activity according to the presence of ANA, a-dsDNA, a-Sm, a-b2GPI,



Peripheral blood miRNA expression in SLE patients vs HCs	miRNA-146a	miRNA-155
	Number of SLE patients (%)	
Without differences in expression	15 (37.5)	17 (42.5)
Decreased expression	0 (0.00)	3 (7.5)
Increased expression	25 (62.5)	20 (50.0)

FIGURE 1. Peripheral blood miRNA expression in SLE patients compared to healthy controls.
HCs: healthy controls; miR: microribonucleic acid; SLE: systemic lupus erythematosus

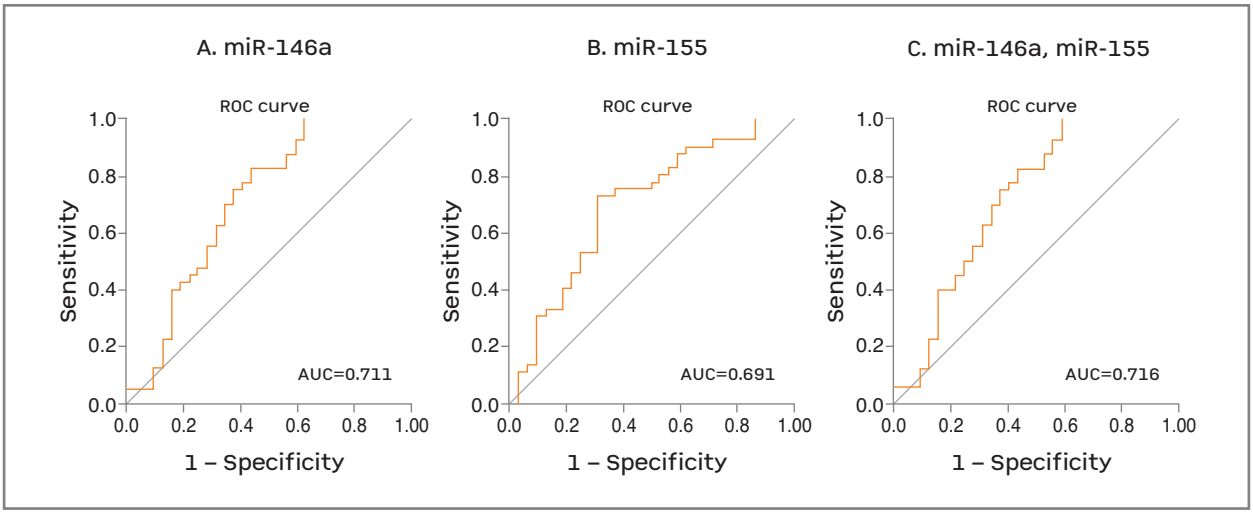


FIGURE 2. ROC curve analysis for determination the sensitivity and specificity of the studied miRNAs in peripheral blood for discriminating SLE patients from HCs.
AUC: area under the curve; HCs: healthy controls; miRNAs: microribonucleic acids; ROC: receiver operating curve; SLE: systemic lupus erytematosus

TABLE II. RECEIVER OPERATING CURVE ANALYSIS BY USING miRNAS IN PERIPHERAL BLOOD TO DIFFERENTIATE SLE PATIENTS FROM HEALTHY CONTROLS

PB miRNA SLE vs HCs	AUC (95 % CI)	p-value	Se (%)	Sp (%)
miR-146a	0.711 (0.585 ÷ 0.837)	0.002	82.50	56.20
miR-155	0.691 (0.566 ÷ 0.817)	0.005	77.50	50.00
miR-146a and miR-155	0.716 (0.590 ÷ 0.842)	0.002	82.50	56.20

AUC: area under the curve; HC: healthy controls; miR: microribonucleic acid; PB: peripheral blood; Se: sensitivity; Sp: specificity; SLE: systemic lupus erythematosus;

a-CL, a-ssA, a-ssB antibodies as well as C3 and C4 complement levels (Table III).

DISCUSSION

Deregulated expression of circulating and tissue specific miRNAs has been described in SLE patients compared to HCs indicating that miRNA could be used as biomarkers in the clinical practice. miRNAs signatures in the circulation or urine have been reported to be associated with the disease itself or different disease manifestations such as lupus nephritis, proteinuria and disease activity[33].

To evaluate the expression of miRNAs as noninvasive diagnostic biomarkers in SLE we checked the expression levels of two miRNAs in whole PB samples of SLE patients compared to HCs for the first time and found that there was a significant difference between the studied groups. Both miR-146a and miR-155 have been found to be overexpressed in patients compared to HCs and the expression levels could be used to differentiate the studied groups. A possible reason for the different miRNA expression among the patients could be due to the heterogeneity of the analyzed group in regard to disease duration, severity and treatment.

Most of the previous studies of miRNA expression in SLE have been focused on PBMC or subtypes of cells – B cells, T cells, serum or urinary cell-free miRNAs. Data about the expression levels of miR-146a and miR-155 differ among the studies. It is known that miR-146a is a negative regulator of autoimmunity[34]. It has been demonstrated that miR-146a levels in PBMCs of SLE patients negatively correlate with the disease activity and

TABLE III. CLINICAL PARAMETERS AND PERIPHERAL BLOOD miRNA EXPRESSION LEVELS

Clinical parameter		Expression profile of miR-146a in PB	Expression profile of miR-155 in PB
Diagnosis	Correlation coefficiency	.363	.330
	p-value	.002	.005
	N	72	72
Smoking	Correlation coefficiency	-.024	.100
	p-value	.847	.416
	N	68	68
Raynaud syndrome	Correlation coefficiency	.157	.250
	p-value	.189	.035
	N	72	72
Sjogren syndrome	Correlation coefficiency	.038	.168
	p-value	.750	.158
	N	72	72
Age	Correlation coefficiency	.239	.366
	p-value	.043	.002
	N	72	72
ESR	Correlation coefficiency	-.218	-.164
	p-value	.177	.311
	N	40	40
CRP	Correlation coefficiency	-.201	-.216
	p-value	.213	.181
	N	40	40
Lupus band test	Correlation coefficiency	.184	.227
	p-value	.255	.159
	N	40	40
SLEDAI	Correlation coefficiency	-.098	-.022
	p-value	.547	.894
	N	40	40
ANA	Correlation coefficiency	.144	.137
	p-value	.374	.399
	N	40	40
aRNP/Sm	Correlation coefficiency	-.070	-.275
	p-value	.668	.085
	N	40	40
a-Sm	Correlation coefficiency	-.108	.173
	p-value	.506	.285
	N	40	40
a-ssA	Correlation coefficiency	-.308	.056
	p-value	.053	.732
	N	40	40
a-Ro52	Correlation coefficiency	-.109	.118
	p-value	.504	.469
	N	40	40
a-ssB	Correlation coefficiency	-.265	.083
	p-value	.098	.612
	N	40	40

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TABLE III. CONTINUATION

Clinical parameter		Expression profile of miR-146a in PB	Expression profile of miR-155 in PB
a-dsDNA	Correlation coefficiency	.090	.038
	p-value	.582	.817
	N	40	40
C3	Correlation coefficiency	.111	.298
	p-value	.495	.062
	N	40	40
C4	Correlation coefficiency	.140	.097
	p-value	.389	.550
	N	40	40
aCL	Correlation coefficiency	-.095	.029
	p-value	.560	.857
	N	40	40
a-b2GPI	Correlation coefficiency	.185	.020
	p-value	.252	.903
	N	40	40

Ab: antibody; a-β2GP1 Ab: anti-beta2 glycoprotein 1 antibodies; a-CL Ab: anti-cardiolipin antibodies; a-dsDNA Ab: anti-doublestrained deoxyribonucleic acid antibodies; a-Sm Ab: anti-Smith antibodies; ANA: antinuclear antibodies; CRP: C-reactive protein; ESR: erythrocyte sedimention rate; miRNA: micro ribonucleic acids; N: number; PB: peripheral blood; SLEDAI: systemic lupus erythematosus disease activity index

activity of IFN signaling pathway and the expression of miR-146a in lupus macrophages negatively regulated the IFN type I production by targeting toll like receptors (TLRs) signaling molecules[22]. Levels of miR--146a have been reported to be downregulated in PBMCs as well as in plasma and serum of SLE patients including lupus nephritis patients[11,35,36]. Zheng C et al. demonstrated a decreased expression of miR-146a in renal tissues with an enhanced transcriptional activity of TRAF6 while Lu J et al. found an overexpression of miR-146a in the glomeruli but not in the tubulointerstitium of lupus nephritis patients which degree of change correlated with the clinical disease severity[37,38]. Perez-Hermandez J et al. reported much higher levels of miR-146a in the urinary exosomes of patients with active lupus nephritis when compared to whole urine or exosome-depleted fractions[39]. In our study only 5 patients had lupus nephritis and in two of them this important disease manifestation was active and required change of treatment regimen. In three of the samples from lupus nephritis patients the expression levels of both studied miRNAs was not changed compared to HCs. In one of the samples miR-146 was elevated but miR-155 levels were not changed and in one sample miR-155 was downregulated and miR-146 levels were not changed. The small number of lupus nephritis patients doesn't allow us to draw any conclusions regarding this disease manifestation and levels of miRNAs expression in the PB. Interestingly, Charrier E et al. described higher levels of miR-146a and miR-155 in human umbilical cord blood pDCs when compared to adult blood pDCs and supposed that miR-146a could be a negative modulator in pDC survival and function[40]. Löfgren S et al. found that the expression of miR-146a in SLE patients with European ancestry was associated with a single-nucleotide polymorphism (SNP) - rs2431697 and the risk allele of this SNP correlates with the downregulation of the miR-146a[41]. In our study all the patients were from European ancestry, but we didn't perform a genetic study for SNPs.

Similarly to Thai T et al. who found overexpression of miR-155 in SLE B cells in our study levels of miR--155 were higher in the peripheral blood of SLE patients compared to HCs[29]. The possible role of miR-155 in B cell survival and autoantibody production has been linked to targeting SH2 domain-containing inositol 5'-phosphatase 1 (SHIP1) protein, which is an inhibitory signaling transducer responsible for B cell activation[27]. miR-155 has been also shown to regulate Tregs pheno-

type. Using mice models of SLE Divekar A et al. demonstrated that the insufficiency in the Dicer expression as well as the upregulation of miR-155 promote the defect in Tregs phenotype by targeting CD62L[24].

According to Chen J et al. levels of miR-146a and miR-155 were increased in SLE PBMC of European patients and the elevated expression levels of miR-155 in PBMC correlated with the increased peripheral DN B cells percentage in lupus[42]. Similarly, in our study levels of miR-146a and miR-155 were overexpression in whole PB samples.

The differences in the expression pattern of the studied miRNAs according to the different studies could be due to the number of studied SLE patients, to their genetic background as studies using European and Chinese patients showed controversial results, as well as the influence of environmental factors – including dietary factors, on miRNA expression which are different in the different ethnicities[43]. The discordance between the expression pattern of miRNAs in whole PB and PBMCs or their subtypes could also be attributed to the presence of regulatory mechanisms that affect the expression of miR-146a and miR-155 when examining whole PB samples, the different methodology of RNA isolation in the samples as well as the blood cell counts[44]. Finally, the conflicting data about the levels of expression of the studied miRNAs in PB, cell compartments or exosomes in SLE patients confirms that the expression of miRNA differs according to the used body fluids because of the origin of the miRNAs, their concentrations, procedural artifacts and variation of sample processing as well as in the data normalization. To our knowledge our study is the first to investigate the expression patterns of miR-146a and miR-155 in whole PB samples of SLE patients.

We didn't find a correlation between the levels of miR-146a and miR-155 and the disease activity in the studied SLE patients as a whole as well as with the immunological activity which might reflect the variants of SLE disease activity in the patients who participated in the study, the difference in their genetic background or in the used medications. Measuring the concentrations of specific miRNAs at a specific disease state might miss the dynamically modulated miRNAs thus indicating that larger study is needed to confirm our results in the clinical practice.

CONCLUSION

Although SLE has an unknown etiology in the recent years many studies are trying to understand the trigger factor and mechanisms behind the autoimmunity in SLE. The research on the epigenetic deregulated expression of SLE related genes including studies on miRNA expression and function have proved the important role of miRNAs in regulating the innate and adaptive immune responses in the pathogenesis of autoimmune diseases. The lower complexity, the stability, no known postprocessing modifications, simple detection and amplification methods of miRNAs in the body fluids and tissues as well as the correlation between the expression of certain miRNAs and clinical variables suggests a potential role of miRNAs profiling as biomarkers for disease activity in SLE. Larger multicenter studies including patients with different ancestry and genetic background are needed to confirm the diagnostic utility of the studied miRNAs in SLE.

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