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Original Paper

MiRNA and mRNA Profiling in Systemic Lupus Reveals a Novel Set of Cytokine - Related miRNAs and their Target Genes in Cases With and Without Renal Involvement

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Key Words
Systemic *Lupus Erythematosus* • Renal Involvement • Cytokine • miRNAs • Microarray

Abstract
Background/Aims: MiRNAs transpire as promising elements in molecular medicine for the identification of new diagnostic, prognostic and targeting therapeutic biomarkers. This study consisted of four steps: First, to investigate one or a group of specific diagnostic miRNAs for Systemic Lupus Erythematosus (SLE) disease in patients with and without renal involvement, second, to identify cytokines genes' expression profiling, third, comparing the profiles with related amounts in the serum and finally, to study target-gene-mediated functional roles of miRNAs, which have been correlated to disease development and progression. ***Methods:*** In order to use in microarray assays total RNA and miRNAs were isolated from blood and serum samples that were obtained from 16 SLE patients (9 with renal involvement and 7 without renal involvement). Taking coexistence of factors such as hypocomplementemia, positive ANA and anti-DNA into account, obtained data were processed. For each differentially expressed miRNA, potential target genes were predicted by microRNAorg, TargetScan and PITA prediction tools. Obtained mRNA profiling data were interrogated for the target genes. MiRNA and mRNA microarray results were confirmed by QRT-PCR. Finally, the amounts of cytokines were measured by multiplex ELISA method. ***Results:*** The results of study showed that among differentially expressed miRNAs in SLE patients with renal involvement compared to those without renal involvement, *hsa-miR-766-3p*, may play pivotal roles in PI3K-AKT-mTOR pathway. In addition according to the obtained data it is suggested that blood-borne proinflammatory cytokines such as IL-4, IL-6 and TNF-α alongside with disease stage and severity may contribute to this differential expression of these miRNA which may be leading to insulin resistance. Finally, *hsa-miR-621,* which was differentially expressed in hypertensive SLE patients without renal involvement and a positive ANA test with its predicted target gene

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"Kallikrein-related peptidase 9" may play a role in the pathophysiology of hypertension in SLE. ***Conclusions:*** We reported some human miRNAs which were differentially expressed in SLE patients according to disease activity and renal involvement. Larger studies are necessary to confirm our findings and detect further biomarkers.

Introduction

Systemic Lupus Erythematosus (SLE) is an autoimmune disease characterized by the production of autoantibodies which affects many organs including joints, skin, kidneys and nervous system. The disease occurs nine times more often in women than men. The cause of SLE is unknown; however, some genetic susceptibilities [1-3] and environmental factors [4-8] have been shown to be involved in its pathogenesis.

The clinical signs and symptoms of SLE may be mild or severe [9-11]. Its presentation may be active or chronic [12, 13]. Active and severe cases of SLE are almost associated with high titers of antinuclear antibodies (ANA) [14], low levels of serum complements [15, 16] and alternations in serum cytokine levels [17-19].

Recently the potential of miRNAs that are small, non-coding RNA molecules have been extensively studied as diagnostic and prognostic biomarkers in autoimmune diseases [20-26]. In this study we aimed, first; to elucidate whether specific miRNA profile are seen in SLE patients with renal involvement as well as in active SLE disease by performing microarray analysis of miRNAs expression in serum samples of SLE patients, Second; to identify cytokines genes' expression profiling performing microarray analysis of mRNAs expression in PBMCs of them, then comparing between the profiles with related amounts in the serum and finally to study target-gene-mediated functional roles of miRNAs, which have been correlated to disease development and progression.

Materials and Methods

Ethics approval and consent to participate

Prior to the start of the study ethical approval was obtained from the Clinical Research Ethics Committee of the Istanbul Faculty of Medicine. Informed consents were obtained from those eligible participants for whom, the purpose of the study were verbally and clearly explained.

Ethical approval for the study was obtained from the Clinical Research Ethics Committee of the Istanbul Faculty of Medicine, Turkey (No: 1325). All study subjects provided written informed consent for the collection of samples and subsequent analysis. This study was conducted according to the principles and guidelines expressed in the declaration of Helsinki.

Participants' consent and baseline characteristics

All patients fulfilled the American College of Rheumatology (ACR) diagnostic criteria of SLE [9, 10]. The study consisted of 16 patients, including 9 SLE patients with clinical renal involvement, and 7 SLE patients without renal involvement who were referred from Department of Rheumatology of Cerrahpasa School of Medicine (Istanbul/ Turkey). Characteristics of patients are shown in Table 1. Clinical and demographic data such as age, gender, blood pressure as well as laboratory data such as the amount of proteinuria in 24 hours, serum creatinine levels type and titer of antinuclear antibodies (ANA-anti DNA), serum complement levels (C3- C4) were collected at the time of consent. Renal involvement was defined as increase in proteinuria (>150 mg/24 hours) or increase in serum creatinine (>1.4 mg/dL) or both. Hypertension was defined if BP was higher than 140 mmHg systolic, higher than 90 mmHg diastolic or both. Active lupus was defined as elevated ANA, anti-DNA antibodies and hypocomplementaemia (C3 or C4 or both).

Table 1. Characteristics of 16 patients with SLE. Hypertension was defined if BP was higher than 140 mmHg systolic, higher than 90 mmHg diastolic or both. Levels >150 mg/24 hours was mentioned for abnormal proteinuria and levels >1.4 mg/dL was mentioned for elevated serum creatinine. Normal ranges for C3 and C4 were (0.89 - 1.87 g/l) and (0.16 - 0.38 g/l) respectively. For ANA, titers <1/80 and for anti-DNA<100 U/mL accepted as Negative and Normal amounts. F = Female, M = Male, (+) = Yes, (-) = No, L = Low, N = Normal, H = High

Case Number	Sex/Age	Hypertension	Elevated Serum Creatinine	Abnormal Proteinuria/24h	C3 Levels	C4 Levels	ANA	anti-DNA
1	F/45	+	-	-	L	L	+	+
2	F/59	+	-	-	N	L	+	-
3	F/46	+	-	-	L	L	+	+
4	F/28	-	-	-	N	N	+	-
5	F/44	+	-	+	L	L	+	+
6	F/60	+	-	+	N	N	-	-
7	F/39	-	-	+	L	L	+	+
8	M/29	-	-	+	N	N	+	-
9	F/23	-	+	+	N	N	-	-
10	M/39	-	-	+	L	L	+	+
11	F/57	-	-	+	N	N	-	-
12	F/26	-	-	-	N	L	+	+
13	F/40	+	-	-	L	L	+	+
14	F/53	-	-	+	H	N	+	-
15	F/29	-	-	+	L	L	+	+
16	F/55	+	-	-	N	H	-	-

Blood and Serum sample collection

Blood samples were collected into EDTA tubes while serum samples were collected into a tube with a clot activator and serum gel separator which then centrifuged at 1900 x g for 10 minutes at 4C°. All supernatants were pooled and centrifuged once again at 16000 x g for 10 min without brake at 4C°. Serum samples were aliquoted in 1.5 ml tubes and stored at -20 °C. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation at 400 g for 40 min at room temperature. The cells at the interface were washed twice with phosphate buffered saline (PBS) [27, 28].

Total RNA and miRNA isolation

Total RNA was isolated from PBMCs using "RNeasy Mini Kit" (Qiagen) and miRNA isolation from serum samples was done using “mirVana PARIS" (Ambion); according to the manufacturer's instruction. In this study both miRNA and total RNA samples were analyzed by bioanalyzer using “RNA 6000 Nano kit” and “Small RNA Kit” (Agilent) respectively, according to the manufacturer's instructions. In the case of total RNA, the study was continued with samples with RIN number of 7.5-8 or higher. Aliquots of the both miRNA and total RNA samples were used for all the experiments in both the microarray and RT-qPCR measurements.

Microarray Experiment

Agilent Human miRNA Microarray v19 (G4872A, Agilent Technologies) was used to identify free miRNAs presented at relatively high level in serum samples. Each slide contained probes generated from 2006 human miRNAs from Sanger database v19. Per each sample 100ng total RNA was hybridized to the microarrays. The serum miRNA expression profiles of lupus patients with renal involvement were compared with those without renal involvement. MicroRNA labeling, hybridization and washing were carried out according to the manufacturer’s instructions. Hybridized microarrays were scanned with a microarray scanner (Agilent, SureScan) and features were extracted using the “Agilent Feature Extraction” (AFE) image analysis tool.

For gene expression analysis the Agilent SurePrint G3 Human Gene Expression Microarray v2 (G4851B, Agilent Technologies) was used to identify genes up or down regulated in PBMCs. 200 ng of total

Table 2. QRT-PCR primer sequences for assayed miRNAs. * indicates reference gene

Gene_name	Forward primer 5´_ 3´	Reverse primer 5´_ 3´
U6*	GCTTCGGCAGCACATATACTAAAAT	CGCTTCACGAATTTGCGTGTCAT
hsa-miR-5571-5p	TCCGAGGAAACTCTTAACG	AGGGTTTTTTTTTTTTTTTGACCTG
hsa-miR-766-3p	GACACCCCGACCTCA	AGTCGTTTTTTTTTTTTTTTGACCT
hsa-miR-4731-3p	CAACCCCCGGTGAAC	TGTGATTTTTTTTTTTTTTTGACCTGG
hsa-miR-5697	CTTTGATGAACTGACGCGA	TCCTTTTTTTTTTTTTTTTGACCTGG
hsa-miR-621	TTCGCGACAACGATCG	GAATGGATTTTTTTTTTTTTTTGACCTG

Table 3. QRT-PCR primer sequences for assayed mRNAs. * indicates reference gene

Gene_name	Forward primer 5´_ 3´	Reverse primer 5´_ 3´	Anneal Temp (°C)
GAPDH*	ATGGGGAAGGTGAAGGTCG	GGGGTCATTGATGGCAACAATA	58
ADORA3	TGATCCTGCACTGTCCTCTCTG	GTTCAGCTTGACCACGCAGAT	59
IRF5	GACATCCCCAGTGACAAGCA	AGAACACCTTGCACTGACACA	58
IRS2	CAAGGAAGACCAACCATGGAG	AGGAGCAGAGACACCTGCAAC	58
SOCS6	CCCGAGGATGAGAGTCAGGTAG	TGGAGGTAGCAATGGTGAGAGTG	58

RNA per each sample was hybridized to the microarrays. The mRNA expression profiles of lupus patients with renal involvement were compared with those without renal involvement. RNA labeling, hybridization and washing were carried out according to the manufacturer's instructions. Image acquisition and feature extraction were as described for miRNA microarrays.

For both types of the microarray experiments data pre-processing and differential expression analysis was done using the GeneSpring software v12.6 (Agilent).

In both microarray experiments, hybridization was carried out independently in triplicate.

Validation of mature miRNAs and candidate mRNAs by qRT-PCR

To verify the miRNA microarray results we assayed for the mature miRNAs using qRT-PCR. RT-primers (5'-CAGGTCCAGTTTTTTTTTTTTTTTVN, where V stands for A, C, G and N stands for A, C, G, T) were purchased from TAG Copenhagen (Denmark) that was designed according to the logics previously described for miR-specific RT-qPCR primers [29-34]. cDNA synthesis was done according to the manufacturer's protocol. QRT- PCR primers were designed using "miRprimer" software [34] (Table 2). Amplification was done using FastStart SYBR Green Master Kit (Roche, Germany) according to the manufacturer's protocol. Reactions were accomplished for each sample at 95°C/10 min, followed by 40 cycles of 95°C/15 sec and 60°C/1 min in a Stratagene Mx3000P (MX3000P, Stratagene, USA). The relative amount of each miRNA to reference gene (U6 RNA) measured by the equation $2^{-(Ct_{miRNA} - Ct_{U6})}$.

In the case of mRNAs, the total RNA was reverse transcribed to cDNA and transcript levels of randomly selected genes (two from up-regulated genes and two from down-regulated genes) were measured in a Stratagene Mx3000P (MX3000P, Stratagene, USA). The QRT-PCR primer pairs of selected genes are listed in Table 3. Amplification was done using FastStart SYBR Green Master Kit (Roche, Germany) according to the manufacturer's protocol. The real-time PCR reactions were accomplished for each sample at 95°C/5min as an initial polymerase activation step; and then 45 amplification cycles at 94°C/15 sec and 58°C/30 sec. The relative expression of mRNAs was calculated by ΔΔCT method.

Multiplex ELISA

In order to detect multiple cytokines simultaneously multiplex ELISA method was used (BioRad). Each sample measurement was done triplicate. For each cytokine standard curve was generated by using the reference cytokine concentrations supplied in this kit. All experiment steps were performed according to manufacturer's instruction. After final washing step to remove the unbound Streptavidin-PE, the plate set was placed in the previously calibrated Bio-Plex 200 reader instrument (Bio-Rad), in which red laser (635nm) detected the spectral properties of the beads while the green laser simultaneously inducing the amount of fluorescence associated with phycoerythrin. Then, using the "Bio-Plex Manager" software (Bio-

Table 4. Comparison of miRNA level between patients with and without renal involvement. FC>2 and P vale ≤ .05 indicates the difference is significant. FC = Fold Change, Reg = Regulation, R = Renal Involvement, NR = No Renal Involvement

Systematic_name	FC	Reg	*P* value	Active_seq	Chr	Comparison	mirbase accession
hsa-miR-5571-5p	29.61	down	0.0205	GGGAGGCTCCTTTGA	22	R *vs.* NR	MIMAT0022257
hsa-miR-766-3p	20.76	down	0.0369	GCTGAGGCTGTGGGGCT	X	R *vs.* NR	MIMAT0003888

Table 5. Comparison of miRNA levels according to presence or absence of C3 hypocomplementemia in patients with/ without renal involvement. FC >2 and P vale ≤ .05 indicates the difference is significant. FC = Fold Change, Reg = Regulation, R = Renal Involvement, NR = No Renal Involvement, $C3^+$ = C3 hypocomplementemia, $C3^-$ = Normal C3

Systematic_name	FC	Reg	*P* value	Active_seq	Chr	Comparison	mirbase accession
hsa-miR-5571-5p	6.32	down	0.0079	GGGAGGCTCCTTTGA	22	NR-$C3^+$ *vs.* NR-$C3^-$	MIMAT0022257
hsa-miR-766-3p	2.17	down	0.0189	GCTGAGGCTGTGGGGCT	X	R-$C3^+$ *vs.* R-$C3^-$	MIMAT0003888

Rad) data was interpreted in the form of Median Fluorescence Intensity (MFI) and the concentration (pg / ml). Samples which were out of the acceptable range and the standard curve were removed from the analysis. All statistical analyzes was performed using "Statistical Package for the Social Sciences v17.0" (SPSS Inc, Chicago, IL, USA). The significance level of (p <0.05) was mentioned.

Statistical analysis

Statistical tests such as Student t test (2 groups), ANOVA, or Kruskal-Wallis test (n group) were used for group-wise comparisons and all of them were performed 2-sided. All statistical analyzes was performed using "Statistical Package for the Social Sciences v17.0" (SPSS Inc, Chicago, IL, USA). The significance level of (p <0.05) was mentioned.

Results

Comparison of miRNA levels between SLE patients with and without renal involvement

SLE patients were sub grouped depending on whether or not they had renal involvement. There were 2 miRNAs, which were significantly down-regulated in serum of SLE patients with renal involvement (N=9) compared to those without renal involvement (N=7) (Table 4).

Comparison of miRNA levels according to presence or absence of hypocomplementemia in SLE patients with/without renal involvement

In this part of study, coincidence of renal involvement and hypocomplementemia (C3 or/ and C4) were taken into the consideration for differential analysis. For this purpose a two-way ANOVA was performed. This procedure identifies genes with altered miRNA expression in cases with/without renal involvement in combination with C3 or C4 hypocomplementemia.

First round of analysis contains miRNAs whose expression was dependent on renal involvement and C3 level. A total of 2 deregulated miRNAs were identified, of which, *has-miR-5571-5p* was down-regulated by 6.32 fold changes in non-renal SLE patients with C3 hypocomplementemia (N=3) compared to non-renal cases without C3 hypocomplementemia (N=4) (p ≤0.05) and *has-miR-766-3p* was down-regulated by 2.17 fold changes in renal SLE patients with C3 hypocomplementemia (N=4) compared to renal cases without C3 hypocomplementemia (N=5) (p ≤0.05) (Table 5).

Table 6. Comparison of miRNA levels according to presence or absence of C4 hypocomplementemia in patients with/ without renal involvement. FC>2 and P vale ≤ .05 indicates the difference is significant. FC = Fold Change, Reg = Regulation, R = Renal Involvement, NR = No Renal Involvement, $C4^+$ = C4 hypocomplementemia, $C4^-$ = Normal C4

Systematic_name	FC	Reg	*P* value	Active_seq	Chr	Comparison	mirbase accession
hsa-miR-5571-5p	4.56	down	0.0039	GGGAGGCTCCTTTGA	22	NR-$C4^+$ *vs.* NR-$C4^-$	MIMAT0022257
hsa-miR-4731-3p	9.15	down	0.028	AGTGTTGGGGGCCA	17	NR-$C4^+$ *vs.* NR-$C4^-$	MIMAT0019854
hsa-miR-766-3p	2.63	down	0.0125	GCTGAGGCTGTGGGGCT	X	R-$C4^+$ *vs.* R-$C4^-$	MIMAT0003888

Table 7. Comparison of miRNA levels according to presence or absence of ANA in patients with/ without renal involvement. FC>2 and P vale ≤ .05 indicates the difference is significant. FC = Fold Change, Reg = Regulation, R = Renal Involvement, NR = No Renal Involvement, ANA^+ = Antinuclear antibodies positive, ANA^- = Antinuclear antibodies negative

Systematic_name	FC	Reg	*P* value	Active_seq	Chr	Copmarision	mirbase accession
hsa-miR-5697	32.87	down	0.00	CCTTTATCATGAAACTACTTG	1	NR-ANA^+ *vs* NR-ANA^-	MIMAT0022490
hsa-miR-621	26.38	down	0.00	AGGTAAGCGCTGTTGC	13	NR-ANA^+ *vs* NR-ANA^-	MIMAT0003290

At the second part of these analyses, miRNAs expression that was dependent on renal involvement and C4 level were investigated. A total of 3 deregulated miRNAs were detected. Among cases without renal involvement there were 2 miRNAs which were down regulated in cases with C4 hypocomplementemia (N= 5) compared to those without C4 hypocomplementemia (N= 2) (Table 6). Also in the case of patients with renal involvement *has-miR-766-3p* was down regulated in cases with C4 hypocomplementemia (N= 4) compared to those without C4 hypocomplementemia (N= 5).

Comparison of miRNA levels according to presence or absence of ANA

In this part of analysis factors of renal involvement and presence or absence of ANA were assessed simultaneously, no significant expressed miRNA was detected in SLE patients with renal involvement and positive ANA (N= 6) compared with those of negative ANA (N= 3). Two miRNAs with significantly down-regulated expression level were identified in non-renal cases with positive ANA (N= 6) compared to those with negative ANA (N= 1) (Table 7).

Comparison of miRNA levels according to presence or absence of anti-DNA

When SLE patients were studied according to anti-DNA test results solely or in combination with renal involvement, no significant expressed miRNA was detected.

Target prediction and pathway analysis

The target genes for each differentially expressed miRNA were predicated by microRNAorg, TargetScan and PITA prediction tools. Given that the prediction softwares often suffer from high false positive rates [35-38], only target genes predicted by all three independent tools were taken in to the account.

Since the goal of this study was to focus on cytokine related genes and to better define the concept of cytokines imbalances in SLE patients, only these genes and related pathways were taken into the consideration.

Because of alterations of steroid metabolism in patients with SLE could be important in the pathogenesis of this disease, the predicted targets of their metabolism related pathways, were mentioned too.

Table 8. The potential gene targets for the differentially expressed miRNAs in SLE patients according to renal involvement, presence or absence of hypocomplementemia and ANA status. *P* value ≤ .05 indicates the difference is significant. 1: *P* value of 0.00001. 2: *P* value of 0.0033

Systematic_name	*P* value	Potential Molecular Targets	Pathway
hsa-miR-766-3p [R vs NR R-C3$^+$ vs R-C3$^-$ R-C4$^+$ vs R-C4$^-$]	0.00001-0.0033	PIK3R1	B-Cell-Receptor-Signaling-Pathway[1], IL-4-Signaling-Pathway[1], Interferon-type-I[1], IL-11-Signaling-Pathway[2], IL-9-Signaling-Pathway[2], Regulation of Toll-like-Receptor-Signaling-Pathway[2], IL-3-Signaling-Pathway[2], IL-2-Signaling-Pathway[2], IL-7-Signaling-Pathway[2], TCR-Signaling-Pathway[2], IL-1-Signaling-Pathway[2], Kit-Receptor-Signaling-Pathway[2], IL-6-Signaling-Pathway[2], IL-5-Signaling-Pathway[2].
	0.00001	ETS1	B-Cell-Receptor-Signaling-Pathway
	0.00001-0.0033	IRS2	IL-4-Signaling-Pathway[1], Interferon-type-I[1], EPO-Receptor-Signaling-Pathway[2],
	0.0033	TFE3	TGF-Beta-Signaling-Pathway
	0.0033	NSMAF	TNF-alpha-Signaling-Pathway
	0.0033	ENDRA	G Protein-Coupled Receptor-mediated Signaling-Pathway (GPCRs)[2]
	0.0033	PIK3R1	Androgen-Receptor- Signaling-Pathway
hsa-miR-621 [NR-ANA$^+$ *vs.* NR-ANA$^-$]	0.0008	MAF	Transcriptional-Activation by NRF2
	0.0008	CAB39	Endochondral-Ossification
	0.0008	MO25	AMPK-Signaling-Pathway
	0.0008	ETV5	Androgen-Receptor- Signaling-Pathway

The KEGG pathway database package presented 229 pathways were used in this study [39-40].

Potential target genes of miRNAs which were differentially expressed in patients according to renal involvement, presence or absence of hypocomplementemia and ANA status

Based on prediction of above softwares, targets were found only for 2 out of 5 miRNAs. For *hsa-miR-766-3p* and *hsa-miR-621* there were found 135 and 33 target genes respectively in total. Table 8 displays these miRNAs, their target genes, and the pathways in which target genes are involved. The *p*-value was adjusted as ≤ 0.05.

These findings confirm that a single miRNA could influence thousands of potential targets and the same gene could be also targeted by multiple miRNAs.

Comparison of mRNA levels between SLE patients with and without renal involvement

There were 119 mRNAs which were differentially expressed in PBMCs of SLE patients with renal involvement compared with those without renal involvement. Of 119 mRNAs, 39 were up-regulated while the remaining 80 were down-regulated. Figure 1 depicted hierarchical clustering of the 16 SLE patients divided into two groups.

Both, up and down-regulated mRNAs were assigned into wiki pathways, to find out if there is any alteration cytokines related genes or not (Table 9 and Table 10), display the results of pathways analysis related to up and down-regulated mRNAs respectively.

Also GO ontology analyses were done for both up and down-regulated gene group individually in order to determine the biological functions of these genes. The majority of up-regulated genes were found to have purinergic receptor activity and cell surface receptor binding activity (Figure 1).

It is known that purinergic receptors (*ADORA3*) are involved in several cellular functions such as proliferation and cytokine secretion (Table 9). Genes have arginine decarbox-

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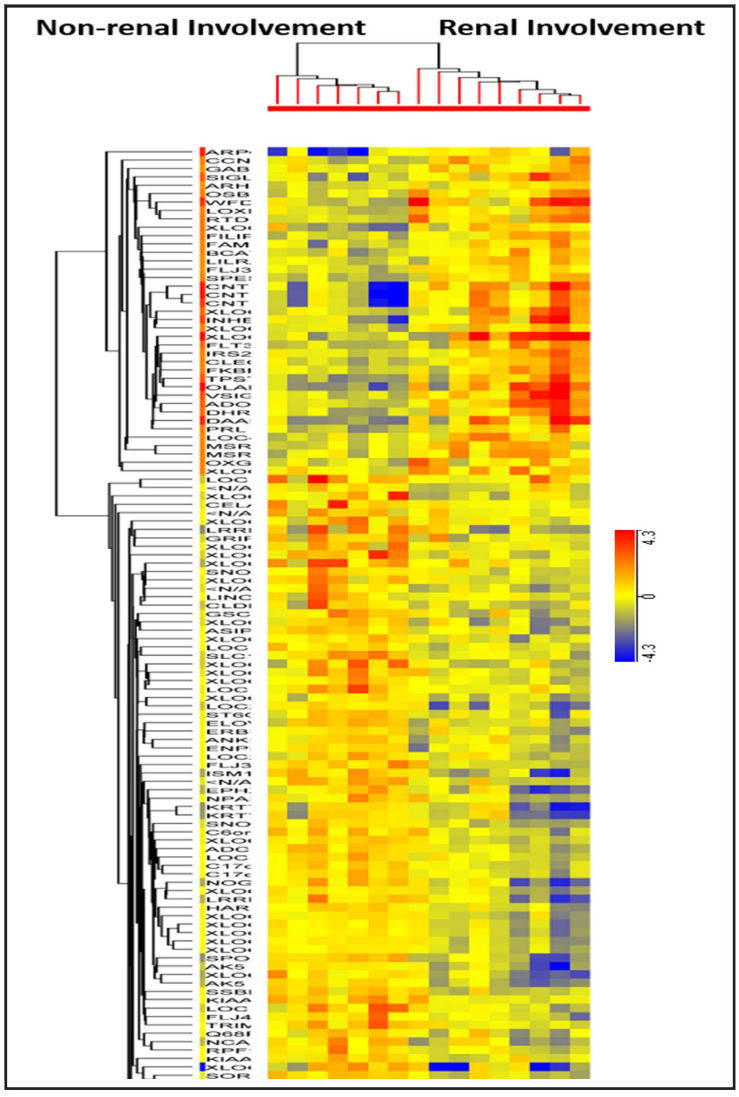

Fig. 1. Genes differentially expressed in PBMCs between SLE patients with/without renal involvement. Genes were shown by the ratio of hybridization intensity between these two groups and those which are highly expressed are highlighted in red while those which are little expressed highlighted in blue.

ylase activity as well as those with alpha-N-acetyl-galactosaminide alpha 2; 6-sialyltransferase activity belonged to the group of genes down-regulated in cases with renal involvement. And eventually another dazzling finding was that (*ERBB2*) gene which has a role in negative regulation of immature T-cell proliferation in thymus was placed between those with down-regulated expression pattern (Table 10).

According to these analyses there were 5 mRNAs which were differentially expressed in PBMCs of SLE patients without renal involvement and with C3 hypocomplementemia (N=3) compared with those without C3 hypocomple-

Table 9. Pathway analysis related to up-regulated mRNAs in patients with renal involvement compared to those without renal involvement. *P* vale ≤ .05 indicates the difference is significant. 1: *P* value of 0.0526. 2: *P* value of 0.0546

Name	Fold Change	Fold Change *P* value	Pathway	Pathway *P* value
IRS2	2.47	0.00836	IL-4-Signaling-Pathway[1], Interferon-type-I-Signaling-Pathway[2]	0.0526[1], 0.0546[2]
ADORA3	4.05	0.00363	GPCRs-Class-A-Rhodopsin-like	0.23

Table 10. Pathway analysis related to down-regulated mRNAs in patients with renal involvement compared to those without renal involvement. *P* vale ≤ .05 indicates the difference is significant. 1: *P* value of 0.21. 2: *P* value of 0.078

Name	Fold Change	Fold Change *P* value	Pathway	Pathway *P* value
NOG	3.85	0.0074	TGF-Beta-Signaling-Pathway	0.783
ERBB2	2.15	0.0083	EGF-EGFR-Signaling-Pathway[1], ErbB-Signaling-Pathway[2]	0.21[1], 0.078[2]

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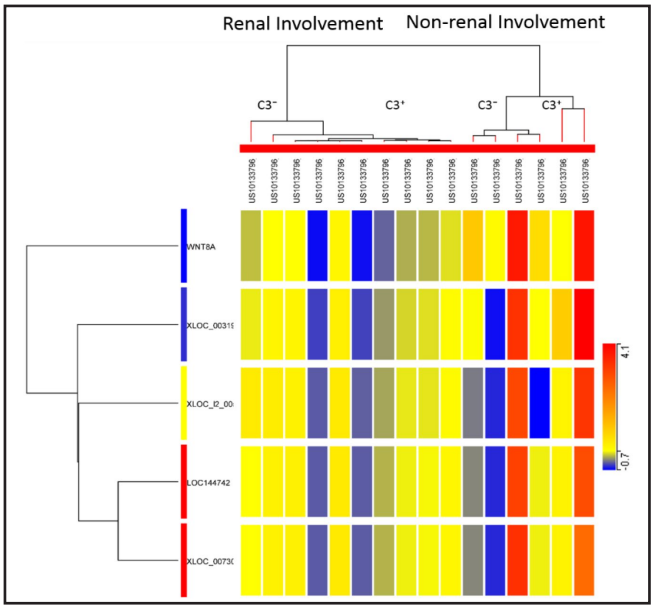

Fig. 2. Genes differentially expressed in PBMCs of patients without renal involvement but with C3 hypocomplementemia compare to those with normal levels of C3. Genes were shown by the ratio of hybridization intensity between cases and highly expressed ones are highlighted in red while lowly expressed ones are highlighted in blue.

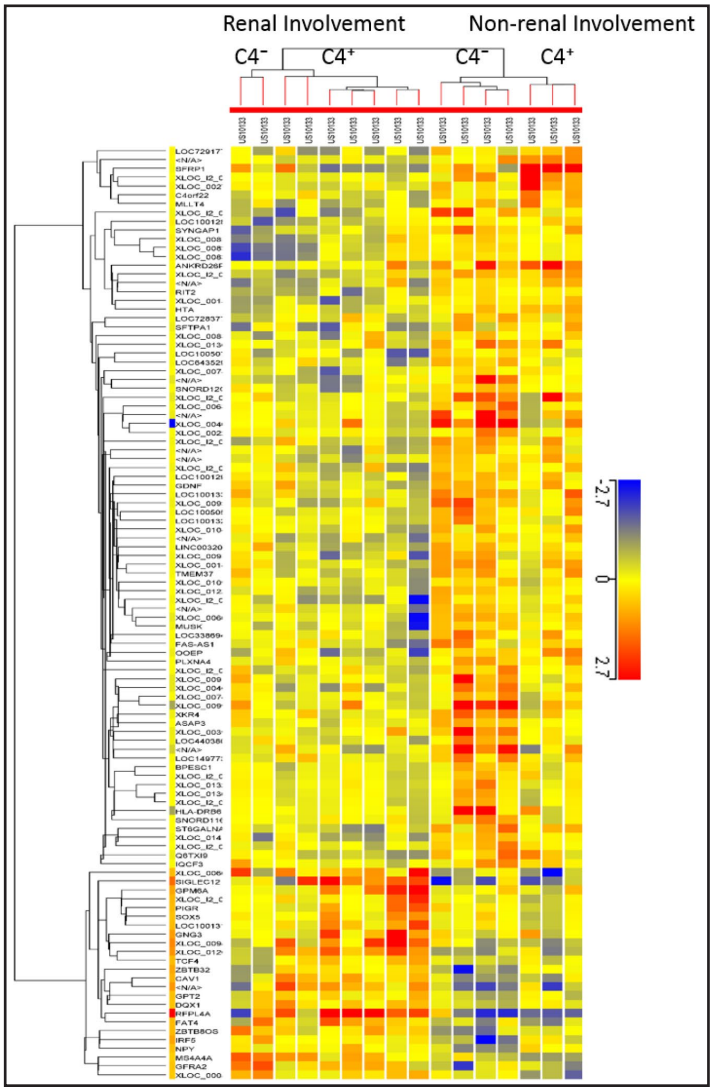

Fig. 3. Genes differentially expressed in PBMCs of patients without renal involvement but with C4 hypocomplementemia compare to those with normal levels of C4. Genes were shown by the ratio of hybridization intensity between cases and highly expressed ones are highlighted in red while lowly expressed ones are highlighted in blue.

mentemia (N=4). All 5 mRNAs were down-regulated significantly (Figure 2) depicted hierarchical clustering of these 5 deregulated mRNAs.

These down-regulated mRNAs were assigned into wiki pathways to find out if there is any alteration in cytokines related genes or not, but no results were found in this area of the investment.

According to GO ontology analysis all of these genes were found to play roles in Wnt receptor signaling pathway as well as calcium modulating pathway.

When coincidence of renal involvement and C4 hy-

Table 11. Pathway analysis related to down-regulated mRNAs in none-renal SLE patients with C4 hypocomplementemia compare to those with normal C4 levels. *P* vale ≤ .05 indicates the difference is significant

Name	Fold Change	Fold Change *P* value	Pathway	Pathway *P* value
IRF5	4.13	0.0086	Apoptosis (Interferon regulatory factors)	0.046
			Toll-like-Receptor-Signaling-Pathway	0.053

Table 12. Interesting Go analysis out-come which was obtained from analysis of up-regulated mRNAs in renal SLE patients with C4 hypocomplementemia compare to those with normal C4 levels. *P* vale ≤ .05 indicates the difference is significant

Name	Fold Change	Fold Change *P* value	Biological Process
GFRA2	2.99	0.0085	Cellular-Response-to-Cytokine-Stimulus

Table 13. Pathway analysis related to down-regulated mRNAs in none-renal SLE patients with positive ANA test result compare to those with negative ANA test result. *P* vale ≤ .05 indicates the difference is significant

Name	Fold Change	Fold Change *P* value	Pathway	Pathway *P* value
SOCS6	16	0.0042	IL-3-Signaling-Pathway	0.0835

Table 14. Pathway analysis related to up-regulated mRNAs in none-renal SLE patients with positive ANA test result compare to those with negative ANA test result. *P* vale ≤ .05 indicates the difference is significant

Name	Fold Change	Fold Change *P* value	Pathway	Pathway *P* value
HLA-B	11.90	0.0286	Type-II-Interferon-Signaling-Pathway	0.0012

pocomplementemia were taken into consideration, in patients without renal involvement, 22 mRNAs were found down-regulated and 66 mRNAs were up-regulated in cases with C4 hypocomplementemia (N=5) compared to those with normal C4 levels (N=2). Figure 3 depicted the hierarchical clustering of these mRNAs.

Pathway analyses were done for both up and down-regulated groups. For down-regulated ones two cytokine related pathway were detected (Table 11). At the same time GO ontology analysis showed that these genes have alanine-oxo-acid transaminase, glial cell-derived neurotrophic factor receptor, transcription co-repressor, as well as nitric-oxide synthase binding activities. For up-regulated ones there were no cytokine related pathway and according to GO ontology analysis main part of these genes have sialyl transferase activities as well as ARF-GTpase activator activity.

When a comparison in patients with renal involvement done according presence or absence of C4 hypocomplementemia, 21 down-regulated and 11 up-regulated mRNAs were detected in cases with C4 hypocomplementemia (N=4) compared to those with normal levels of C4 (N=5). Pathway analyses were done for both up and down-regulated groups. But there was no cytokine related pathways between results. According to GO ontology analysis most of down-regulated mRNAs have binding, catalytic or enzyme regulator activities. Intriguingly, when GO ontology analysis was done for up-regulated mRNAs, GFRA2 was detected which play role in cellular response to cytokine stimulus but during web surfing no additional information was obtained (Table 12). Most of the up-regulated mRNAs have glial cell derived neurotrophic factor receptor activity.

Comparison of mRNA levels according to presence or absence of ANA

Here co-occurrence of renal involvement and positive ANA result were studied. A total of 142 mRNAs with different expression levels were detected in patients without renal involvement and positive ANA (N=6) compared to those with negative ANA (N=1). From these numbers, 138 were down-regulated for which only one pathway related to cytokines was detected (Table 13). For up-regulated group also only one pathway related to cytokines was detected (Table 14). According to GO ontology analysis most of the genes down-regulated

have molecular functions such as action transporter activity, ferric iron transporter activity, aryldialkylphosphate activity as well as potassium channel activity, while on the other hand, for the genes up-regulated only MHC class I receptor activity related to *HLA-B* was detected.

Validation of bioinformatics prediction using a simultaneous mRNA/miRNA expression profiling approach

According to previous reports, the proportion of correct predictions can be as low as 10% [35-38]. To increase the accuracy of bioinformatics' predictions, mRNA expression profiling was done simultaneously. Based on the fact that miRNAs regulate gene expression by inhibiting translation or inducing deadenylation of mRNAs followed by their degradation, it was reasoned that the expression level of miRNAs and mRNAs should be inversely correlated if one regulates the other. Indeed, for up-regulated miRNA, predicted target genes should be down-regulated and vice versa. For this reason the mRNA profiling data were interrogated for the target genes.

Among the targets which were predicted for miRNAs with differential expression pattern in SLE patients with renal involvement compared to patients without renal involvement, only one *IRS2* were found to be up-regulated approximately by 2.5 fold in cases with renal involvement.

Validation study

QRT-PCR results obtained here validated the microarray data however, a few discrepancies in the fold changes detected between the miRNA microarray and qRT-PCR results. For example, when SLE patients with renal involvement were compared with those without renal involvement, the fold changes of *hsa-miR-5571-5p* and *hsa-miR-766-3p* in microarray test were -29.61 and -20.76 (down-regulation) respectively; while in qRT-PCR test they were -27.38 (P_{value}=.008) and -19.49 (P_{value}=.003). Finally, qRT-PCR confirmed a similar expression pattern of four candidate transcripts randomly selected for validation step: Two up-regulated ($ADORA_3$ and IRS_2) and Two down-regulated (IRF_5 and $SOCS_6$).

Multiplex "ELISA"

In this part of the study, serum cytokine levels of patients with lupus nephropathy and those without renal involvement were compared. In all cases with renal involvement average values for serum levels of G-CSF (60 *vs.* 34), IL-6 (66 *vs.* 5.7), IL7 (16 *vs.* 10), IL-8(33 *vs.* 25), IL-10 (10 *vs.* 3), IL12 (33 *vs.* 10), GM-CSF (39 *vs.* 16), IL-13(15 *vs.* 6), IFN-γ (292 *vs.* 144), TNF-α (74 *vs.* 20) and MIP-1β (142 *vs.* 98) were high. According to T-statistic *P* values range are considered on borderline of statistical significance (0.05 <p <0.1).

Discussion

MiRNAs regulate a variety of cellular functions and play roles in physiological and pathological processes of many human diseases such as cancer, cardiovascular disease and immune system related diseases. They can also play role as negative regulators of gene expression in different stages of kidney diseases [41]. Some studies revealed the potential contribution of miRNAs in the regulation of autoimmune genes and signaling pathways in the pathogenesis of SLE [42]. Chen et al. showed that under normal conditions, circulating miRNAs are derived from circulating blood cells [43].

In our study by comparing circulating miRNA profile of SLE patients with renal involvement and those of without renal involvement, significant decreases in the expression levels of *hsa-miR-766-3p* (encoded on X chromosome) and *has-mir-5571-5p* (encoded on 22nd chromosome) were found (29.61 and 20.76 fold respectively). Over expression of *hsa-miR-766-3p* was also found in $CD4^+$ T cells of active lupus patients in a study conducted by Hewagama et al. shown [44].

Regarding miRNA target predictions, we used three independent programs (micrornaorg, TargetScan and PITA) and these bioinformatics facilities predicted *Irs2*, *Pik3r1*, *Ets1*, *Tfe3*, *Nsmaf* and *Endra* as possible target genes for *hsa-miR-766-3p* (*P* = .0033 - .00001) (Table 8). In study of Hewagama et al., using theTargetScan, DAVID and ConceptGen programs "suppressor of cytokine signaling1" (SOCS1) and "c-Cbl, Cbl proto-oncogene" (CBL) were estimated as potential target genes for *hsa-miR-766-3p* [44]. Interestingly in another study again by Hewagama et al., using TargetScan program only, "suppressor of cytokine signaling1" (SOCS1), "suppressor of cytokine signaling 3" (SOCS3) and "programmed cell death 1" (PDCD1) were estimated to be potential target genes [45]. Different results obtained from all these studies' different bioinformatics programs are because they do not predict completely accurate data.

In this study, to prove the accuracy of the bioinformatics prediction, mRNA expression profiling was also made, and the data obtained was questioned for the predicted target genes. Since the goal of this study was to focus on cytokine related genes and to better define the concept of cytokines imbalances in SLE patients, only these genes and related pathways were taken into the consideration. Except for *Irs2*, rest of data obtained from gene expression profiling was not matched with the predictions. *Irs2* target gene has increased about 2.47 folds in patients with renal involvement compared to those without renal involvement. This data suggest that *Irs2* gene is regulated by *hsa-miR-766-3p*.

These results do not contradict the accuracy of targets which have been predicted for detected miRNAs in this study, because unavailability of these targets in gene expression profiling data can be explained by the emphasis on the fact that miRNAs regulate gene expression at the post-transcriptional level not only by deadenylation and mRNA decay but also through translational repression. So functional experimental studies are required to verify and establish the association between aberrantly expressed miRNAs and SLE patients with/without renal involvement.

It is asserted that in various tissues, IRS proteins regulate specific signal pathways [46]. IRS2 "Insulin receptor substrate 2" is a cytoplasmic signaling molecule mediating insulin, "insulin like growth factor" (IGF), and other cytokines to show their effects. Insulin and IGF1 would be reachable to PI3K "Phosphatidylinositol-4, 5-bisphosphate 3-kinase" pathway, through tyrosine phosphorylation of IRS proteins [46]. Thus IRS2, considered a key regulatory point for PI3K-Akt cascade where IRS2 signal is required for suppression of apoptosis. In normal conditions insulin receptor following stimulation by Insulin or IL-4, is phosphorylated by tyrosine kinase. PI3kinase activation is mediated by binding of PI3KR "phosphoinositide-3-kinase, regulatory subunit" to phosphorylated IRS proteins. Stress-induced cytokines such as TNF-α and IL-6 promote the phosphorylation of serine residues of the IRS1/IRS2 proteins and inactivate them. As a result all pathways related to them would be inactivated [47]. These cytokines increase inhibition of IRS-mediated kinase activation; which can lead to disruption of insulin-dependent glucose uptake and may cause insulin resistance [48, 49]. In a study conducted in 2013, the expression of *IRS2* in renal epithelial cells and up regulation of it in patients with diabetic nephropathy was shown [50]. Insulin resistance and its mediated type 2 diabetes and finally diabetic nephropathy have been reported in SLE [51, 52].

When the findings of this study and data obtained from above literatures considered together we can hypothesize that in patients with renal involvement high levels of TNF-α and IL-6 inhibit IRS2; thereby on the one hand lead to indirectly inactivation of Akt and on the other hand lead to insulin resistance resulting in the insulin-dependent glucose uptake. However the body reduces the expression of *hsa-miR-766-3p* to overcome these effects and in this way by eliminates the repression of IRS2 and its expression increases. TNF-α and IL-6 however was increased in our SLE patients with renal involvement, but this increase was not statistically significant. The borderline elevation of these cytokines was probably due to administration of immunosuppressive drugs to our patients which has also been reported by Gehi et al. [53].

The second miRNA that was found to be down regulated in renal SLE patients was *hsa-miR-5571-5p.* To our Knowledge *hsa-miR-5571-5p* has not been reported previously in other studies associated with SLE or renal diseases. In 2012, this miRNA was reported in a study focused on Sjögren's syndrome pathogenesis but this data could not been verified by Taqman PCR assays [54].

Hypocomplementemia (low C4 /low C3) is a marker of SLE activity. When simultaneity of renal involvement and hypocomplementemia (C3 and / or C4) taken into consideration significant decreases in the expression levels of both *hsa-miR*-5571-5p and *hsa-miR-766-3p* were detected. However, this decline was more moderate. These changes can be explained either by inactivation of SLE by immunosuppressive therapy or can be due to high CG% components of miRNAs and possible binding of them to target mRNAs associated with complement pathway.

The third miRNA detected among SLE patients with renal involvement, was *hsa-miR-4731-3p* that its expression level decreased 9.15 fold in cases with C4 hypocomplementemia (P = 0.028). Any information about this miRNA was not obtained by Web browsing.

In SLE patients without renal involvement, when cases with positive ANA test results were compared with those with negative ANA test results, 32.87 fold decreases in expression level of *has-miR-5697* was detected. Despite being highly significant, *hsa-miR-5697* only has been reported as a biomarker in non-metastatic prostate cancer in a study conducted in 2009 [55]. Also in hypertensive SLE patients without renal involvement and with positive ANA test results, *has-miR-621* was sharply down regulated (26.38 fold) compared with normotensive cases [56]. According to TargetScan and PicTar target prediction programs, *has-miR-621* is associated with "Kallikrein-related peptidase 9" (*klk9*) gene (P = 0.0008) [57]. Hypertension is one of the most important symptoms of SLE and lupus nephropathy. In hypertensive lupus patients before commencement of hypertensive nephropathy, KLK9 appears in the urine [58]. This study suggests that *has-miR-621* and KLK9 may play important role in the pathophysiology of hypertension in SLE [56]. However, larger studies with increased number of patients are needed to confirm this finding.

In this study for each sample miRNA and mRNA expression profiling were done separately and without creating of samples' miRNA and mRNA pools.

Smaller sample size and clinical heterogeneity of SLE patients are limitations of our study. So results must be confirmed in larger number and clinically homogenous patients.

Conclusions

We reported some human miRNAs which were differentially expressed in SLE patients according to disease activity and renal involvement. Larger studies are necessary to confirm our findings and detect further biomarkers.

Disclosure Statement

The authors declare that they have no competing interests.

Acknowledgments

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The data that support the findings of this study are available from GeneSpring software v12.6 (Agilent) but restrictions apply to availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of Agilent.

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