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hsa-miR-149-5p Diminish MAPK and PI3K/Akt Signalling Pathways through Down-regulation of *ERbB3* in SLE Patients

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Abstract

Introduction: In Systemic Lupus, miRNAs construct a substantial layer of post-transcriptional gene expression regulation. Availability of sensitive and specific methods for their detection makes them candidates for potential biomarker discovery. Here we compared miRNAs particular to SLE patients with healthy controls.

Methods: Total RNA and miRNAs were isolated from blood and serum of 16 SLE patients and 8 healthy controls for microarray assays. Potential target genes were predicted and interrogated with mRNA profiling data. BLAST alignment analysis was done between differentially expressed miRNAs and predicted target genes. Microarray results were confirmed by QRT-PCR.

Results: 10 miRNAs were differently expressed in SLE patients of which, *has-miR-149-5p* was up-regulated about 8.5 fold. Among predicted targets only *ERbB3* approved by mRNA profiling and found to be down-regulated approximately by two fold. BLAST alignment analysis of mature sequence of *has-miR-149-5p* and *ERbB3* sequence revealed that 16 of 18 nucleotides belong to *hsa-miR-149-5p*, matched with nucleotides in minus chain of target gene (89%). Two nucleotides "mismatches" did not interfere with target mRNA degradation.

Conclusion: We conclude *hsa-miR-149-5p* degrades *ERbB3* gene's primary transcript before splicing meaning that up-regulation of *hsa-miR-149-5p* activates direct/indirect apoptosis by stopping *ERbB3* translation and could be a biomarker candidate for lupus activity.

Keywords: *hsa-miR-149-5p*; *ERbB3*; Systemic Lupus Erythematosus; biomarker

Introduction

MiRNAs are small, approximately 20 nucleotides in length, non-protein-coding single strand RNAs. Their ability to regulate gene expression post-transcriptionally by binding to target mRNAs with low specificity leading to inhibition of gene expression through targeted mRNA degradation, mRNA cleavage, or translational arrest is the hallmark of miRNAs [1-3]. So miRNAs manifest temporal tissue-specific gene expression patterns [4]. MiRNAs are transcribed by RNA polymerase II and related transcription factors. After passing through a lot of control layers, eventually biogenesis cycle of miRNAs has been completed and they are converted to functional mature molecules [5].

Systemic Lupus Erythematosus (SLE) represents the prototype of human autoimmune diseases. The etiology of this autoimmunity disorder remains unknown but autoantibody production and immune complex formation that lead to intense inflammation and multiple organ damage were determined in it [6]. SLE has a prevalence of ~40 cases per 100,000 individuals with onset typically occurring in women of childbearing age. Increasing evidence has shown that aberrant expression of miRNAs is involved in the pathogenesis of the lupus [7-10].

According to the significant role of miRNAs in regulation of signaling pathways and functions of the immune system elements, such as cytokines, in this study we aimed to investigate one or a group of miRNAs particular to SLE patients compared with healthy control group. This approach will provide novel insights into the effects of miRNAs on disease activity and contribute to development of biomarkers for the diagnosis and treatment assessment.

Clinical Significance

Growing evidence demonstrated that miRNAs can function as regulatory elements on signaling pathways and play significant roles in immune responsing cells. Serum miRNAs would be as early promise as non-invasive biomarkers for disease activity, different organs involvement and treatment monitoring assessments in complex autoimmune diseases such as Systemic Lupus Erythematosus (SLE).

Material and Methods

Study participants and sample processing

In this study a total of 24 subjects, including 8 healthy volunteers, 16 SLE patients were participated. The mean age for healthy control group was 38.1 ± 10.5 and for SLE patients was 39.5 ± 12.05. After obtaining the Ethics approval from the Clinical Research Ethics Committee of the Istanbul Faculty of Medicine and written informed consents, 5 mL blood was collected from each participant. After twice centrifugation at 1900 x g for 10 min at 4°C and at 16000 x g for 10 min at 4°C, their serum supernatant were collected and aliquoted in 1.5 mL tubes then stored at -20°C. Peripheral Blood Mononuclear Cells (PBMC) were isolated according to Ficoll-Hypaque density gradient method [11,12].

miRNA and total RNA extraction

Total RNA 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. Both

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miRNA and total RNA samples 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, RIN numbers equal to or higher than 7.5-8 were acceptable. Aliquots of the both miRNA and total RNA samples were used for all the experiments in both the microarray and RT-qPCR measurements.

Microarray analysis

Samples were labelled with Cy3 and then heat denatured and hybridized to Agilent 8 x 60 K miRNA microarrays V19 comprised of 2006 probes targeting a comprehensive selection of human miRNAs alongside with virus miRNAs and control probes from Sanger miRBase at 37°C for 20 h. After hybridization and washing processes, slides were scanned in Agilent Microarray Scanner (Agilent Technologies). For further analysis Gene Spring software was used and the common deregulated miRNAs presented in serum samples were listed in Table 1.

For mRNA microarray assay Agilent Sure Print G3 Human Gene Expression Microarray v2 (G4851B, Agilent Technologies) was used in which 200 mg of total RNA per each sample was labelled and hybridized according to the manufacturer's instructions. Image acquisition and feature extraction were as described for miRNA microarrays.

Target Prediction

In order to reject the potential false positive rates resulted from the single use of the each software, only the targets that were approved and predicted by microRNAorg, TargetScan and PITA prediction tools at the same time, were considered [13-16]. Only cytokine related genes and related pathways were taken into the account since the main goal of the study was to examine the effects of cytokines on the etiology of the SLE. The KEGG pathway database package presented 229 pathways were used in this study [17,18].

BLAST Alignment Analysis between Differentially Expressed miRNAs and Predicted Potential Target Genes'Sequences

Each candidate miRNA identified by microarray assay used as a separate query (mature miRNA sequences were used). The BLAST (Basic Local Alignment Search Tool) [http://www.ncbi.nlm.nih.gov/BLAST] program was used to find matches in predicted target genes' transcripts sequences at the National Center for Biotechnology Information (NCBI). The outputs from this database search were compared and the best matches from searches were selected based on the length of the match, percentage of identity of match, lack of gaps or deletions and inclusion of the seed sequence.

Validation of mature candidate miRNAs and *ERBB3* isoforms by qRT-PCR

In order to verify the miRNA microarray results, the mature miRNAs were assayed by qRT-PCR. RT-primers purchased from TAG Copenhagen (Denmark) and designed as described for miR-specific RT-qPCR primers [19-24]. Following cDNA synthesis, QRT-PCR primers designing was done using "miRprimer" software [24]. 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 $2^{-(CtmiRNA- CtU6)}$.

In the case of *ERBB3* isoforms (s and 1), the total RNA was reverse transcribed to cDNA and transcript levels of them were measured in a Stratagene Mx3000P (MX3000P, Stratagene, USA). The QRT-PCR primer pairs designed using Primer-BLAST [25] are listed in Table 2. Amplification was done using FastStart SYBR Green Master Kit (Roche, Germany) according to the manufacturer's instruction. The real-time PCR reactions were performed for each sample at 95°C/5 min as an initial polymerase activation step; and then 45 amplification cycles at 94°C/15 sec and 59°C/30 sec. The relative expression of mRNAs was calculated by ΔΔCT method.

Statistical tests such as Student t test (2 groups) or ANOVA were used for group-wise comparisons and all of them were performed 2-sided. All statistical analyses was performed using "Statistical Package for the Social Sciences v20.0" (SPSS Inc, Chicago, IL, USA). The significance level of ($p < 0.05$) was mentioned.

Results

Identification of SLE-Specific miRNAs in microarray analysis

Comparing SLE patients group (N=16) with control group (N=8), a total of 10 miRNAs were detected by miRNA microarray among which, *has-miR-149-5p* was up-regulated miRNA compared with control, representing approximately an 8.5-fold changes. These 10 deregulated miRNAs are demonstrated in Table 3.

Prediction of potential target genes for hsa-miR 149-5p

Based on prediction by microRNAorg, TargetScan and PITA prediction softwares, *hsa-miR 149-5p* was expected to target 142 genes in total.

To explore the role that aberrantly expressed miRNAs play in the

Gene_name	Forward primer 5´_ 3´	Reverse primer 5´_ 3´
U6*	GCTTCGGCAGCACATATACTAAAAT	CGCTTCACGAATTTGCGTGTCAT
hsa-miR-149-5p	GCAGGGGAGTGAAGACAC	GGTCCAGTTTTTTTTTTTTTTTCTC
hsa-miR-933	GCAGGGGAGAGGTCTC	GTCCAGTTTTTTTTTTTTTTTAGGGA

*indicates reference gene

Table 1: QRT-PCR primer sequences for assayed miRNAs.

Gene_name	Forward primer 5´_ 3´	Reverse primer 5´_ 3´	Anneal Temp (°C)
*GAPDH**	ATGGGGAAGGTGAAGGTCG	GGGGTCATTGATGGCAACAATA	58
ERBB3 (transcript variant s)	CCTGCAGTGGATTCGAGAAGT	ACCTTTAGACAGCGGCCAAG	59
ERBB3 (transcript variant 1)	TTGACTGGAGGGACATCGTG	AATGAGAAGCCCCGGTTGTAG	59

*indicates reference gene

Table 2: QRT-PCR primer sequences for isoforms of ERBB3.

Systematic_name	FC	Reg	*P* value	Active_seq	Chr	Comparison	mirbase accession number
hsa-miR-149-5p	8.62	up	0.0257	GGGAGTGAAGACACGGAG	2	SLE *vs.* C	MIMAT0000450
hsa-miR-1825	11.17	up	0.0406	GGAGAGGAGGGCAC	20	SLE *vs.* C	MIMAT0006765
hsa-miR-3675-3p	9.90	up	0.0033	TTGGGGGAGTTCCTTA	1	SLE *vs.* C	MIMAT0018099
hsa-miR-4313	5.91	up	0.0019	GGGTTTGGGGCCA	15	SLE *vs.* C	MIMAT0016865
hsa-miR-574-5p	16	up	0.0062	ACACACTCACACACACAC	4	SLE *vs.* C	MIMAT0004795
hsa-miR-933	5.75	up	0.0113	GGGAGAGGTCTCCCT	2	SLE *vs.* C	MIMAT0004976
hsa-miR-1304-3p	3.68	up	0.0472	GGGGTTCGAGGCT	11	SLE *vs.* C	MIMAT0022720
hsa-miR-2116-3p	5.59	up	0.0376	GGGAGTTCTTGGCATG	15	SLE *vs.* C	MIMAT0011161
hsa-miR-3195	6.75	up	0.0364	AACCCGGGCCCG	20	SLE *vs.* C	MIMAT0015079
hsa-miR-365a-3p	8.75	up	0.021	ATAAGGATTTTTAGGGGCATTA	16	SLE *vs.* C	MIMAT0000710

Table 3: List of ten SLE deregulated miRNAs identified by miRNA microarray analysis. FC: Fold Change; Reg: Regulation; SLE: Systemic Lupus Erythematosus; C: Control.

Systematic_name	*P* value	Potential Molecular Targets	Pathway
hsa-miR-149-5p	0.00000004	*ERBB3*, AKT3, BIM	ErbB-Signaling-Pathway
	0.00000004	REPS2, FOXO4, SP1	EGF-EGFR-Signaling-Pathway
	0.00355071	IL-13	Cytokines-and-Inflammatory-Response
	0.00355071	SP1	JAK-STAT, IL-17-Signaling-Pathway
	0.00355071	TAB3	IL-1-Signaling-Pathway, TNF-alpha-Signaling-Pathway
	0.00355071	AKT3	Toll-like-Receptor-Signaling-Pathway
	0.00355071	Torc2	AMPK-Signaling-Pathway
	0.00355071	NFATC3	B-Cell-Receptor-Signaling-Pathway
	0.00355071	BBC3	TP53-Network

Table 4: The potential gene targets for the differentially expressed hsa-miR 149-5p in SLE patients compared to healthy controls. P value ≤ 0.05 indicates the difference is significant.

Name	Fold Change	Fold Change *P* value	Pathway	Pathway *P* value
ERBB3	2.05	≤0.05	ErbB-Signaling-Pathway	0.00361
	2.07			

Table 5: Validation of the targets which were predicted for miRNAs with differential expression pattern in SLE patients compare to healthy controls. P vale ≤ 0.05 indicates the difference is significant.

cytokine related pathways, we only assigned putative targets of these miRNAs, into wikipathways. Table 4 displays these target genes, and the pathways they are involved. The *p*-value was adjusted as ≤ 0.05.

Validation of Bioinformatics Prediction Using a Simultaneous mRNA/miRNA Expression Profiling Approach

To increase the accuracy of bioinformatics' predictions, mRNA expression profiling was done simultaneously. Among the targets which were predicted for hsa-miR 149-5p with differential expression pattern in SLE patients compared to healthy controls only one (6.6 %) *ERBB3* (two polymorphic forms) were found to be down-regulated approximately by two fold as shown in Table 5.

This finding is logic since the expression level of miRNAs and mRNAs should be inversely correlated based on the fact that miRNAs regulate gene expression by inhibiting translation or inducing deadenylation of mRNAs followed by their degradation.

Analysis of *ERbB3* gene and *has-miR-149-5p* alignment

To further investigate the role of *hsa-miR-149-5p* in *ERbB3* gene regulation, similarity matches were screened by comparing the nucleotide sequence of *hsa-miR-149-5p* with the *ERbB3* gene sequence in the "Homo sapiens" genome registered in the NCBI database. The randomness of the regions showing homology according to the obtained alignment was statistically evaluated by BLAST. The expected value (e), which is exponentially related to the similarity value (S) according to the "Gumble Extreme Value Distribution" (EVD) formula, was calculated from the maximum likelihood of nucleotide probabilities. Bit scores are determined by taking logarithms of S value so that errors can be minimized [26]. Since *E* values less than 0.1 or 0.05 are typically taken to represent biological significance and indicate that the alignment has not occurred by chance, *E* values smaller than 0.05 were selected from the output of the BLAST search [27]. The similarity of sequences over 50 is considered significant for the bit score [27,28]. So, among the results the one in which the *E* score was (0.003), with the bit score of (26.3 '13') and with the highest "Query coverage" value (72.2%) was selected.

Base pairing region of *has-miR-149-5p* with its target mRNA, *ErbB3*, was located between nucleotides at position 9144-9156 on mRNA and was belonged to the intronic region as shown in Figure 1.

Figure 1: Matching analysis of *hsa-miR-149-5p* with the sequence between 9144 and 9156 positions of the *ErbB3* gene in the genome of "Homo sapiens" registered in the NCBI database.

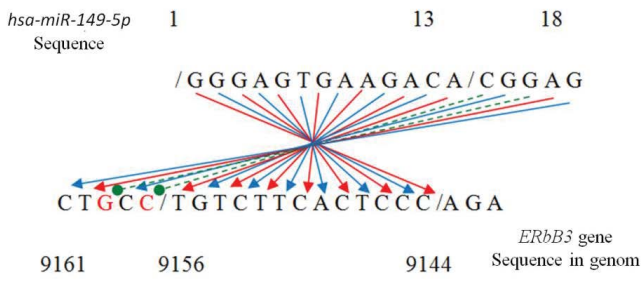

Figure 2: The *hsa-miR-149-5p* and *ErbB3* gene sequence matching. 13 nucleotides out of the 18 nucleotides belonging to *hsa-miR-149-5p* bind to the target gene's minus (-) chain by 100%. Among 18 nucleotides, only at positions 9157 and 9159 (2 nt) matching will not occur.

Findings from this study are of great importance in that the miRNA encoded by *hsa-miR-149-5p* breaks down the target mRNA prior to the clipping phase. As shown in Figure 2, 16 of the 18 nucleotides belonging to *hsa-miR-149-5p* match with the nucleotides in the minus (-) chain of the target gene (89%). It has also been determined that C nucleotide at position 9157 and nucleotide G at position 9159 "mismatches" do not interfere with target mRNA degradation.

Validation step by quantitative real-time PCR

However a few differences in the fold changes detected between the miRNA microarray and qRT-PCR results, qPCR results confirmed a similar expression pattern of selected miRNAs. when SLE patients were compared with healthy control group, the fold changes of *hsa-miR-149-5p* and *hsa-miR-933* in microarray test were +8.62 and +5.75 (up-regulation) respectively, while in qRT-PCR test they were +7.15 (*P value*=0.004) and +4.36 (*P value*=0.007). Also, qRT-PCR validated a similar expression patterns for both isoforms of *ERBB3* transcript (*P value* <0.05).

Discussion

As mentioned above, *hsa-miR-149-5p* was up-regulated by approximately 8.5 fold changes. Our study did not reproduce microarray analysis results reported by Te et al. In which *hsa-miR-149** was found to be down-regulated in EBV transformed cells from African-American SLE patients with nephritis but was not changed in PBMCs of same individuals. Interestingly enough, in the same study, up-regulation of *hsa-miR-149** in either SLE European-American individuals and in SLE-affected monozygotic twins samples derived cell lines was reported [29].

Among the predicted target genes obtained from the bioinformatics studies for *hsa-miR-149-5p*, *ERbB3* gene expression level was also confirmed experimentally by mRNA microarray analysis and validated by QRT-PCR assay.

About 90% similarities, according to NCBI sequence match analysis results, indicate that *hsa-miR-149-5p* regulates *ERbB3* gene's primary transcript by degrading it before splicing. In this study it is revealed that the translation of mRNA isoforms (s and 1) produced by the primary transcript of *ERbB3* gene [30] is inhibited by degradation through *hsa-miR-149-5p*.

In this study one of the target mRNA candidates for *hsa-miR-149-5p* belongs to the *ERbB3* gene. *ErbB3*, Epidermal Growth Factor Receptor (EGFR), is a member of the tyrosine kinases family. *ErbB3* is different from other family members due to lack of real kinase activity [31]. Thus, *ErbB3* is only active as a heterodimer complex and shows the highest affinity for ErbB2; the most efficient complex for the activation of the downstream pathways is the *ErbB2/ErbB3* heterodimer [32]. The downstream of the ErbB2/ErbB3 heterodimer, PI3K/Akt pathway is induced and plays an important role in the proliferation, survival and invasion of cancer cells [33,34]. *ErbB2/ErbB3* heterodimer have been shown to play a role in the growth of human lung adenocarcinoma cells via PI3K/Akt pathway activation [35,36]. The *ERbB3* signal activates the PI3K/AKT/FRAP1/RPS6 pathway leading to Caspase 3 (CASP3) mediated apoptosis and thus contributes to tumor growth [37]. In other words, it allows cancer cells to immortalize by suppressing apoptosis. Many studies revealed that dysregulated apoptosis machinery account for predisposing to lupus in SLE mice models [38-40]. In a recent study by Bischoff et al. using a vector containing the 3'UTR of *ERbB3* cloned downstream of the luciferase cDNA, it was shown that *miR-149* directly targets *ERbB3.* They showed that down-regulation of *ERbB3* receptor alongside with multiple down-stream signalling molecules reduces the growth factor Heregulin (HRG) responses [41]. Based on the above information and findings it can be postulated that up-regulation of *hsa-miR-149-5p* activates direct or indirect apoptosis by stopping the *ERbB3* transcripts translation.

On the other hand, in some previous studies it has been shown that due to diminished phosphorylation of PKC_{δ}, the phosphorylation decreases also in ERK, MEK and RAF of MAPK pathway in T-cells of patients with active-lupus patients [42,43]. Similar results were also found in microarray results. According to the fact that ERK-MAPK pathway regulates the DNA methyltransferase1 (Dnmt1) level in these cells [44], it was suggested that aberrant activation of Dnmt1 can contribute to overexpression of LFA-1 making T-cells autoreactive [42,45,46]. But the mechanism by which PKC_{δ} activation is impaired has remained unknown. Based on our findings, we suggest that decreased *ERbB3* expression mediated by *hsa-miR-149-5p* may be fundamental to part of these abnormalities through abrogation of *ERbB3* (EGFR) mediated tyrosine phosphorylation of PKC_{δ}. As a result, impaired T-cell ERK-MAPK pathway (PKC_{δ} _ Ras _ Raf _ MEK _ ERK) seems to be responsible for decreasing Dnmt1 level, inducing auto reactivity in T-cells.

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 de adenylation 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.

Conclusion

In this study for each case (SLE or Healthy) miRNA and mRNA expression profiling were done separately and without creating of miRNA and mRNA pools. This issue has improved our study's strengths and accuracy.

Based on above findings, we postulate that this *hsa-miR-149-5p* could be attractive as biomarker candidate for disease activity assessment of lupus and *hsa-miR-149-5p* and *ERbB2/ERbB3* signalling pathway may be serving as molecular targets or pathways that can be specifically targeted for therapy. Before accepting the *hsa-miR-149-5p* as a molecular target, the information provided in this study should be extended to further studies with larger sample sizes.

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Declaration of Interest Statement

The authors whose names are listed above certify that they have no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from Gene Spring 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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