Inflammation-related differentially expressed common miRNAs in systemic autoinflammatory disorders patients can regulate the clinical course

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ABSTRACT

Objective. *Systemic autoinflammatory diseases (SAIDs) are caused by the malfunctioning of the innate immune system factors. Clinical heterogeneity and undefined pathobiology are common phenomena among SAIDs. In this study, we aimed to assess the involvement of microRNAs in regulating these complex diseases.*

Methods. *The expression pattern of different miRNAs was compared between SAID patients with high auto-inflammatory disease activity index (AIDAI) score and with low AIDAI score, and their role in inflammation-related pathways was investigated. Differentially expressed miRNAs were determined using the Multi Experiment Viewer (MEV) and Transcriptome Analysis Console (TAC) analysis tools using miRNA microarray. Potential targets of miRNAs were enriched for inflammation-related genes and validated using qRT-PCR analysis.*

Results. *Upon performing microarray analysis, 40 differentially expressed miRNAs were identified between mild familial Mediterranean fever (FMF) patients and severe SAID patients. Thereafter, 21 of 40 miRNAs were found to be potentially involved in inflammatory pathways, of which, 8 were further validated through qRT-PCR. The targets of these 8 miRNAs (miR-29b-3p, miR-29c-3p, miR-30e-3p, miR-130b-3p, miR-148a-3p, miR-186-5p, miR-197-3p, and miR-374b-5p) belonged to the inflammation-related genes and pathways.*

Conclusion. *This is the first study to identify miRNAs that might be associated with a more severe disease form of monogenic autoinflammatory diseases. All these miRNAs were associated with cytokine-mediated pathways and might be used for establishing diagnostic and therapeutic methods.*

Introduction

Systemic autoinflammatory diseases (SAIDs) are a group of rare hereditary disease groups that occur in early childhood. SAIDs are diseases of the innate immune system, characterised by sterile inflammatory attacks (1). Among the SAIDs associated with high interleukin-1 (IL-1) production, the most common ones are familial Mediterranean fever (FMF), TNF receptor-associated periodic syndrome (TRAPS), cryopyrin-associated periodic syndromes (CAPS), and mevalonate kinase deficiency (MKD). In approximately half of the SAID patients, the associated gene cannot be defined, and this group is termed as "undefined systemic autoinflammatory diseases (uSAID)" (2). In addition to genetic heterogeneity, clinical heterogeneity introduces challenges in the management of SAIDs (3).

miRNAs are 20-25 nucleotide length small non-coding regulatory RNA molecules, regulating gene expression post-transcriptionally by interference with mRNAs (4). miRNAs contribute to many biological processes such as development, differentiation, and inflammation. They can promote or suppress inflammation by targeting related mRNAs (5). To date 2654 human mature sequences were reported in miRBase, a database using small RNA deep sequencing dataset (6). One mRNA can be regulated with a lot of miRNAs and one miRNA can regulate more than one mRNAs. This unique feature makes them hard to study, so there had been

developed bioinformatic tools to clarify their interactions (7). These bioinformatics tools specialised in subjects such as miRNA sequence, pre-miRNA secondary structure, miRNA gene loci have been developed to answer the questions that researchers who conduct miRNA studies may ask. Besides, data storage platforms were created for analysis (8). On the other hand, the tendency to use one or more databases or bioinformatics tools specific to the miRNA feature to be enlightened is increasing.

Several studies have focused on miRNA profiles in different SAIDs, although most are on FMF. In the comparison of FMF patients and healthy controls, upregulation of miR-20a-5p and downregulation of miR-197-3p in homozygous FMF patients; upregulation of let-7d and miR-574-3p in heterozygous FMF patients were identified (9). Another FMF study identified that miR-144-3p, miR-21-5p, miR-4454, and miR-451a increase and miR-107, let-7d-5p, and miR-148b-3p decrease in homozygous FMF patients (10). Blood miRNA profiling study of TRAPS patients showed that miR-134, miR-17-5p, miR-498, miR-451a, miR-572, miR-92a-3p expression levels were downregulated in TRAPS patients compared with healthy controls (11). In another study, it was concluded that miR-223, which was reduced in CAPS patients, targets NLRP3 as a result of cell culture studies and plays a role in suppressing inflammation (12). However, these studies have not addressed the association of these common miRNAs with the severity and pathogenic pathways. In recent years, especially studies conducted in cancer, showed that miRNAs can affect the clinical course of patients and have important contributions in the follow-up of the disease prognosis and the treatments (13). We lack such studies in SAIDs.

In this study, we have attempted to define that differentially expressed miRNAs may cause a more severe disease course by comparing mild and severe patients with IL-1 related autoinflammatory syndromes. The common inflammation-related miRNA signatures, potential clinical course regulator, were identified for the first time in a group of SAID patients including severe FMF, CAPS, and undefined SAIDs patients while comparing them with mild FMF patients.

Table I. Demographics and clinical data of patients, n (%) or median (minimum-maximum).

	Total (n=19)	Mild SAID (n=7)	Severe SAID (n=12)
Age (year)	11.4 (2-17)	11 (6-16)	11.58 (2-17)
Gender			
Female	11 (57.9)	4 (57.1)	7 (58.3)
Male	8 (42.1)	3 (42.9)	5 (41.7)
MEFV mutations			
M694V/M694V	9	4	4
M694V/ M680I	3	2	1
M680I/ M680I	1	1	1
NLRP3 mutations	2	-	2
MVK mutations	2	-	2
No mutation	2	-	2
CRP (mg/dL)	0.30 (0.1-0.76)	0.28 (0.13-0.76)	0.32 (0.1-0.74)
AIDAI Scores	52.6 (0-99)	1.29 (0-4)	82.6 (65-99)

Material and methods

Patients

FMF patients met the childhood criteria and the recently suggested Eurofever/PRINTO for FMF (14, 15). CAPS and MVKD patients met the Eurofever/PRINTO classification criteria for hereditary recurrent fevers (15). The mild disease was defined as those having AIDAI score <5, whereas all the other patients had AIDAI scores at least >50. Mutation analysis was carried out for MEFV in all of them. The FMF patients included in this study all had mutations in exon 10. 2 patients had mutations in the NLRP3 gene and 2 patients in the MVK gene. 2 patients did not have mutations in MKD, CAPS, or TRAPS associated genes (Table I).

Total blood miRNA isolation

Blood samples were collected to PAXgene Blood RNA tubes (Qiagen, Valencia, California, USA) from patients who were involved in the study. MicroRNA isolation was performed by using PreAnalytiX miRNA Kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions. The purity and quantities of isolated RNAs were determined in NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA), and a 2.5% agarose gel electrophoresis technique was used for the quality control of the samples.

miRNA microarray analysis

Differential expression of miRNAs in patient groups was determined by GeneChip miRNA 4.0 Array (Affymetrix by Thermo Fisher Scientific, Santa Clara, CA, USA), analysis according to the manufacturer's instructions. 600 ng total RNA was loaded to the array plate for microarray analysis. Signal intensities (presence/absence values) and signal histograms of all chips were evaluated as quality control criteria for the miRNA microarray. The raw data were analysed with the Multi Experiment Viewer (MeV v4.9.0; The Institute for Genomic Research) and Affymetrix Transcriptome Analysis Console Version 2 (TAC 2.0) in order to determine miRNAs between groups with a similar trend. In addition to TAC 2.0 analysis, Significance Analysis of Microarrays (SAM) analysis in MeV programme was applied. miRNAs that are found as differentially expressed at least 2-fold change in both MeV-SAM analysis and TAC 2.0 analysis and statistically significant as FDR=0 for MeV-SAM analysis; and $p<0.05$ for TAC-ANOVA analysis were identified.

Pathway analysis and selection of target miRNAs

Genes likely to bind miRNAs were identified using the miRWalk 2.0 database. Lists were created from genes determined by at least 6 out of 11 programmes for each miRNA by using this database. Gene lists were loaded into the DAVID (The Database for Annotation,

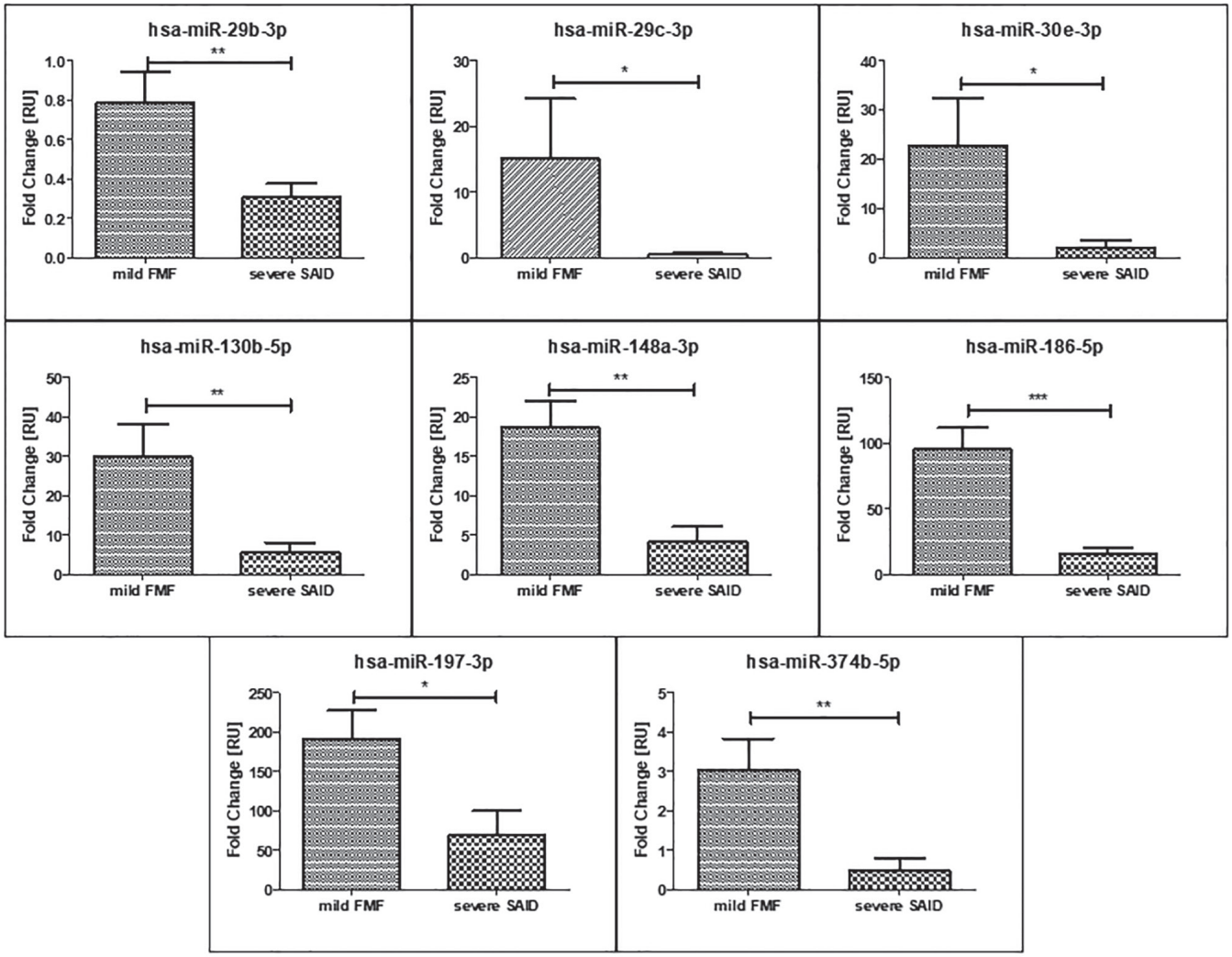

Fig. 1. qRT-PCR validation of 24 differentially expressed miRNAs associated with inflammatory pathways. The relative expression of validated 8 miRNAs was shown and their expression normalised to RNU48 snRNA, by using the 2-ΔΔCT method. Statistical significance was determined using the Student t-test, *p<0.5, **p<0.01, ***p<0.001.

Table II. The list of differentially expressed miRNAs with a fold change ≥2 and p-value <0.05 in the severe SAID group compared to mild FMF patients subjects as a result of analysis of two different programmes. *p<0.05; **p<0.01; ***p<0.001; FDR: false discovery rate.

	Multi experiment viewer (MeV)		Transcriptome analysis console (TAC)	
	Fold change	q value (%)	Fold change	ANOVA p değeri
hsa-miR-186-5p	-66.67	0.0	-88.3	$7x10^{-6}$***
hsa-miR-29c-3p	-22.22	0.0	-13.66	$8x10^{-6}$***
hsa-miR-374b-5p	-55.56	0.0	-80.00	$2x10^{-5}$***
hsa-miR-30e-3p	-23.81	0.0	-52.90	$6x10^{-5}$***
hsa-miR-130b-5p	-27.03	0.0	-18.56	$1x10^{-4}$***
hsa-miR-148a-3p	-22.73	0.0	-19.76	$4x10^{-4}$***
hsa-miR-197-3p	-4.49	0.0	-4.23	$4x10^{-4}$***
hsa-miR-29b-3p	-34.48	0.0	-29.44	$3x10^{-5}$***

Visualization and Integrated Discovery) v. 6.7 programme, and genes were classified in terms of pathways. The first classification was made according to the analysis results of the KEGG (Kyoto Encyclopedia of Genes and Genomes) programme and Geneontology-Panther programme. miRNAs which involve in inflammation-related pathways (interleukin signalling pathway, inflammation induced by chemokine and cytokine signalling pathway, toll receptor signalling pathway, apoptosis signalling pathway, TGF-beta signalling pathway) were selected. Microarray validation studies were continued with the candidate miRNAs.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) for validation experiments

For the validation of microarray results, the cDNA was synthesised with TaqMan® Advanced miRNA cDNA synthesis kit (Thermo Scientific, Waltham, MA, USA). Amplification of miRNAs was performed by using the TaqMan® Advanced miRNA Assays (Thermo Scientific, Waltham, MA, USA). RNU48 expression was used as a normaliser. The reactions were carried

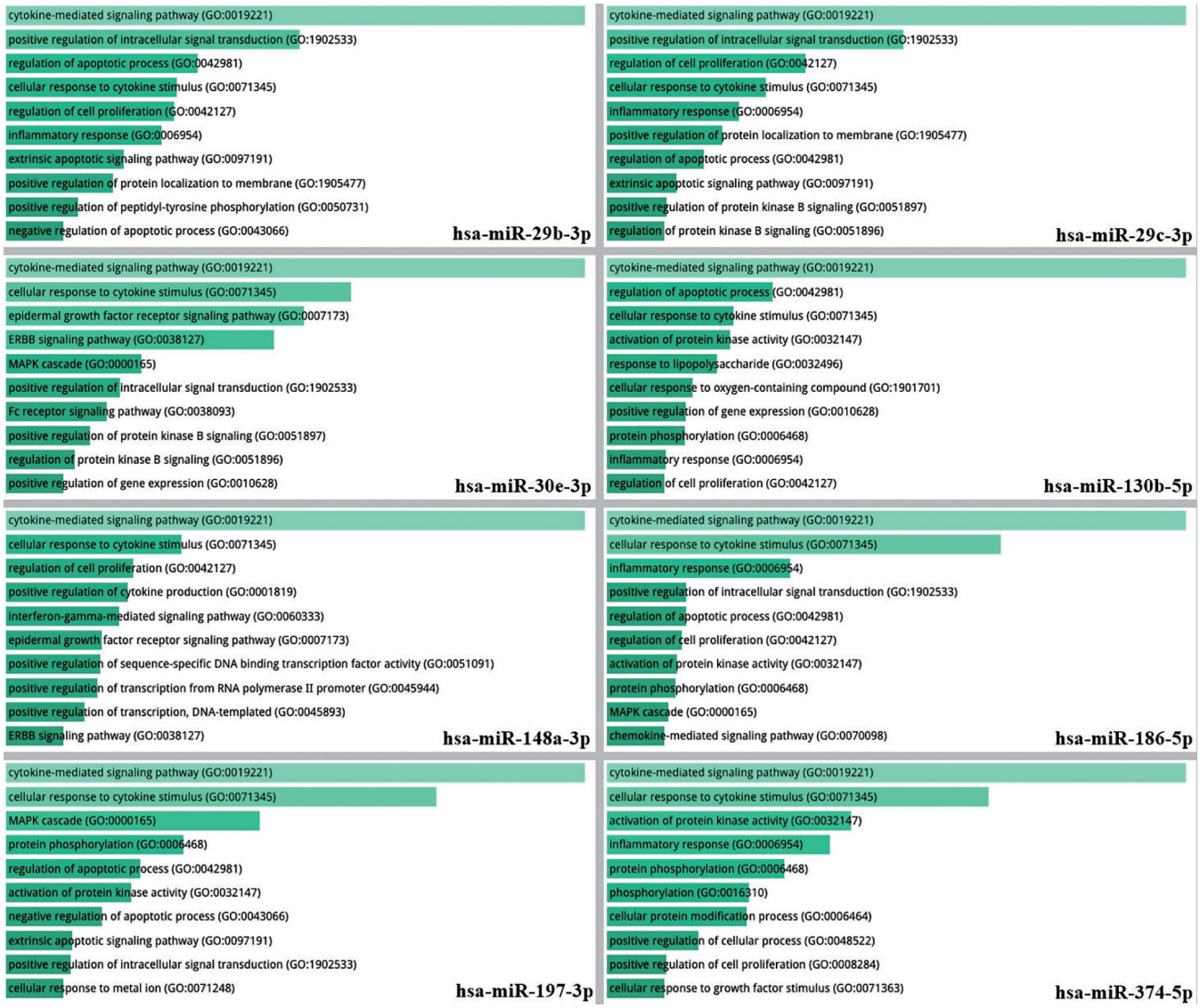

Fig. 2. Gene ontology (GO) term enrichment analysis of the inflammation-related target genes of miR-29b-3p, miR-29c-3p, miR-30e-3p, miR-130b-3p, miR-148a-3p, miR-186-5p, miR-197-3p and miR-374b-5p within the 'Biological Process' category. Bar graphs are sorted by *p*-value ($p<0.05$). The longer bars and lighter coloured bars mean that the term/gene-set is more significant.

out on the Bio-Rad IQ5 Real-time PCR Detection Systems (Bio-Rad, Hercules, CA, USA).

Bioinformatic analysis of target genes, enrichment and pathway analysis and visualisation

Advanced bioinformatics analyses were performed with selected 8 miRNAs. The gene lists were enriched in terms of inflammation-related 1071 genes (16). Gene Ontology (GO) enrichment analysis was performed using all inflammation-related pathway genes of each candidate miRNA in terms of the biological processes by the Enrichr platform. Putative target genes, components of innate immunity, of 8 miRNAs were enriched in terms of innate immunity-related genes reported in innate-cured database (17). Experimentally validated target genes of miRNAs were filtered by 3' UTR luciferase activity assay using miRTarBase database (18). To visualise inflammation-related potential target genes, Cytoscape platform was used (19).

Statistical analysis

The DAVID database was used to study the biological mechanism and gene ontology of the selected genes miRNAs. Gene Ontology (GO) database was used to perform the pathway enrichment analysis and the significant genes (*p*-value <0.05) were selected. The biological processes involved in selected miRNAs were listed, and the bars (*p*-value <0.01) were reported as important processes. For the miRNA microarray analysis, the Significance Analysis of Microarrays (SAM), and ANOVA test were used to determine the differentially expressed miRNAs in MeV and TAC programmes, respectively. For the validation studies by qRT-PCR experiments, fold changes in the expression level of miRNAs in groups were evaluated by student-t-test with the *p*-value of <0.05 being considered as significant. Statistical analyses were performed using the GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA).

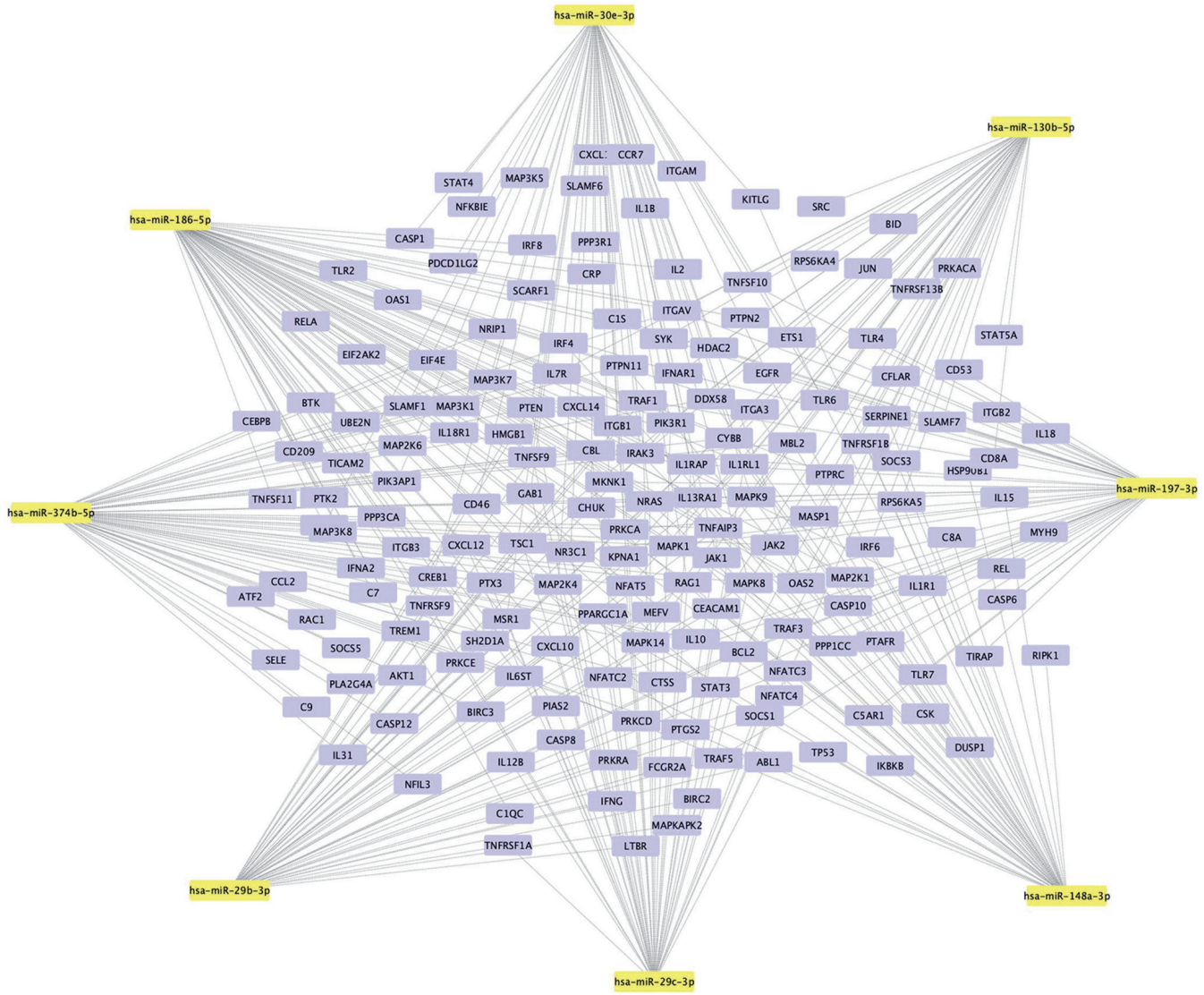

Fig. 3. Representation of enrichment analysis of innate immunity related target genes of miR-29b-3p, miR-29c-3p, miR-30e-3p, miR-130b-3p, miR-148a-3p, miR-186-5p, miR-197-3p and miR-374b-5p. Yellow boxes represent validated miRNAs and blue boxes represent putative target genes. Interaction map was formed by Cytoscape 7.0.

Ethics approval

The study was approved by the Hacettepe University Non-Interventional Clinical Researches Ethics Board (GO 15/1744-19). Written consent was obtained from all parents and children.

Results

Study population

The mean age of the patients was 11.4 and there were 11 girls. There were 13 patients with homozygous exon 10 mutation: 6 patients had an AIDAI score >50 and were classified as those with severe disease. Seven patients had an AIDAI score <5 and were classified as a mild disease. There were six patients with other IL-1 related SAID, who all had severe disease with AIDAI score >50 (Table I).

miRNA microarray analysis revealed 40 differentially expressed miRNAs in severe SAID groups

miRNA microarray analysis was performed for the identification of differentially expressed miRNAs that are associated with inflammation in severe SAID patients. As a result of the comparison of mild FMF patients, 40 differentially expressed miRNAs were found in severe SAID patients with a threshold of fold change ≥2.0, FDR=0, and $p<0.05$ according to both the MeV and TAC 2.0 programmes (Supplementary Table S1).

Target genes of 21 candidate miRNAs are associated with inflammation-related pathways

The potential target genes of selected miRNAs were determined by using miRWalk 2.0, a database of both predicted and experimentally validated miRNA target genes. Target genes of candidate miRNAs were subjected to DAVID- KEGG pathway analysis and revealed that 21 out of 40 selected miRNAs, differentially expressed in severe SAID groups by microarray analysis, were associated with the top five inflammatory pathways (interleukin signalling pathway, inflammation induced by chemokine and cytokine signalling pathway, Toll receptor signalling path-

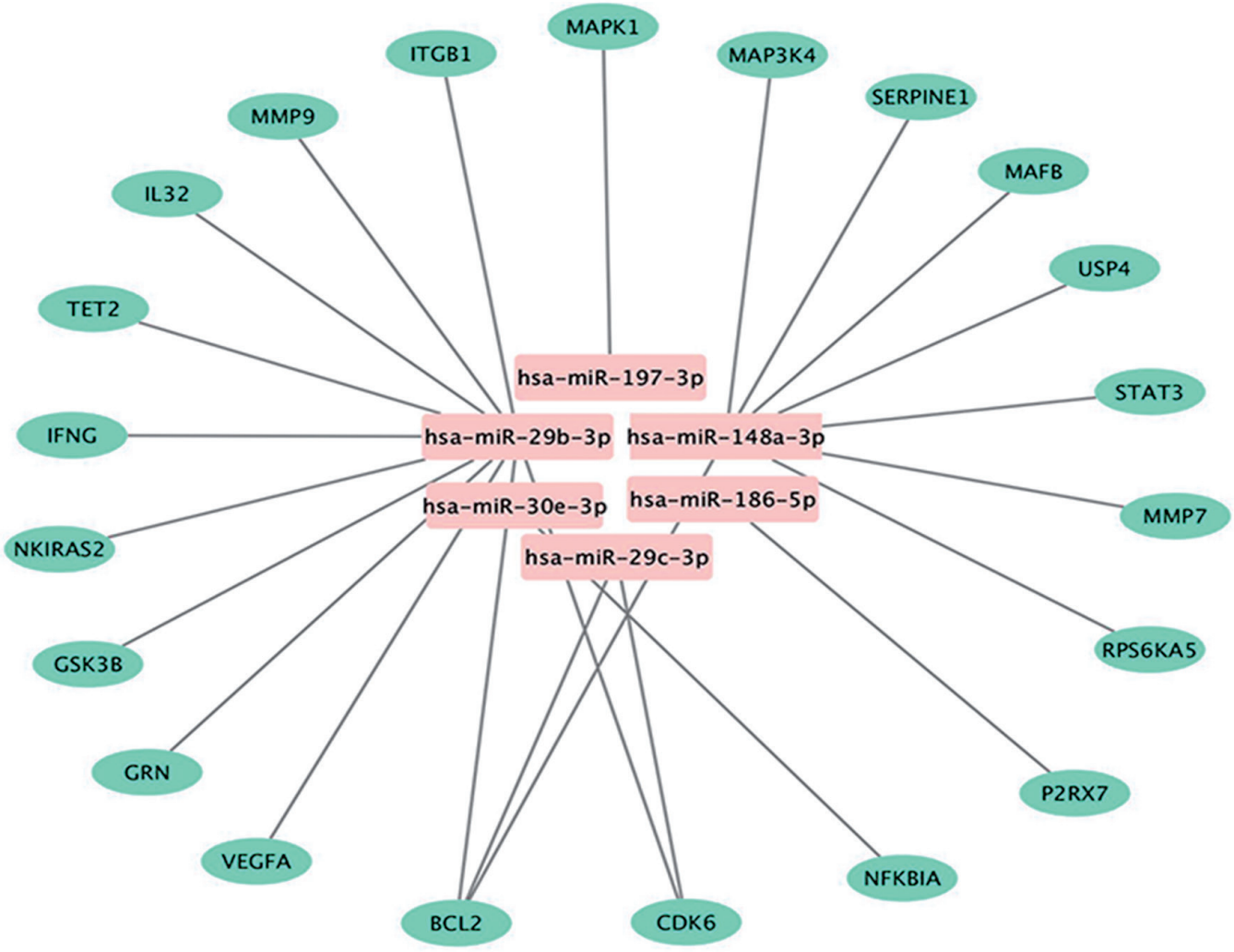

Fig. 4. Experimentally validated innate immunity-related target genes of 6 validated miRNAs (miR-29b-3p, miR-29c-3p, miR-30e-3p, miR-148a-3p, miR-186-5p, miR-197-3p). Target genes reported as a result of the 3'UTR luciferase activity assay, one of the most reliable techniques in miRNA studies, were listed. Red boxes represent validated miRNAs and yellow boxes represent experimentally validated target genes. The interaction map was formed by Cytoscape 7.0. (Experimentally validated target genes of miRNAs were obtained from the miRTarBase database and experimentally validated, and inflammation-related target genes were not reported for miR-130b-3p and miR-374b-5p.)

way, apoptosis signalling pathway, TGF-beta signalling pathway) (Suppl. Table S2).

Validation of miRNA expression of selected miRNAs in patient samples by qRT-PCR analysis

Out of these 21 differentially expressed miRNAs, 8 miRNAs, miR-29b-3p, miR-29c-3p, miR-30e-3p, miR-130b-3p, miR-148a-3p, miR-186-5p, miR-197-3p and miR-374b-5p were validated. Experimental validation of the expression of these 8 miRNAs by qRT-PCR analysis revealed that all of them were significantly downregulated in severe SAID patients when compared with mild FMF patients (Fig. 1). A similar result was observed in miRNA microarray analysis (Table II). The fold changes of these eight miRNAs determined in mild FMF vs severe FMF comparisons are also shown in Supplementary Table S3. In addition to 8 miRNAs identified in the comparison of mild FMF and severe SAID, four miRNAs (miR-326, miR-335-5p, miR-7-1-3p, miR-22-5p) were detected and validated when comparing just FMF patients among themselves (Suppl. Fig. S1).

Thus, eight miRNAs were chosen as the priority candidates and computational prediction of their targets and gene enrichment analysis were conducted for these miRNAs. Therefore, these are associated with a more severe disease course defined by high AIDAI scores.

Determination of deep biological processes of validated 8 miRNAs

Target genes of validated miRNAs were sorted with previously reported VNFLAMMATORY gene list and the putative inflammatory target genes of candidate miRNAs were subjected to GO enrichment analysis in terms of the biological process domain (Fig. 2). Among the pathways, the first rank biological pathway of all validated miRNAs was the cytokine-mediated signalling pathway. Cellular response to cytokine stimulus and inflammatory response were other most prominent pathways involved by miR-29b-3p, miR-29c-3p, miR-130b-3p, miR-197-3p, and miR-374b-5p. Another important mechanism for inflammation,

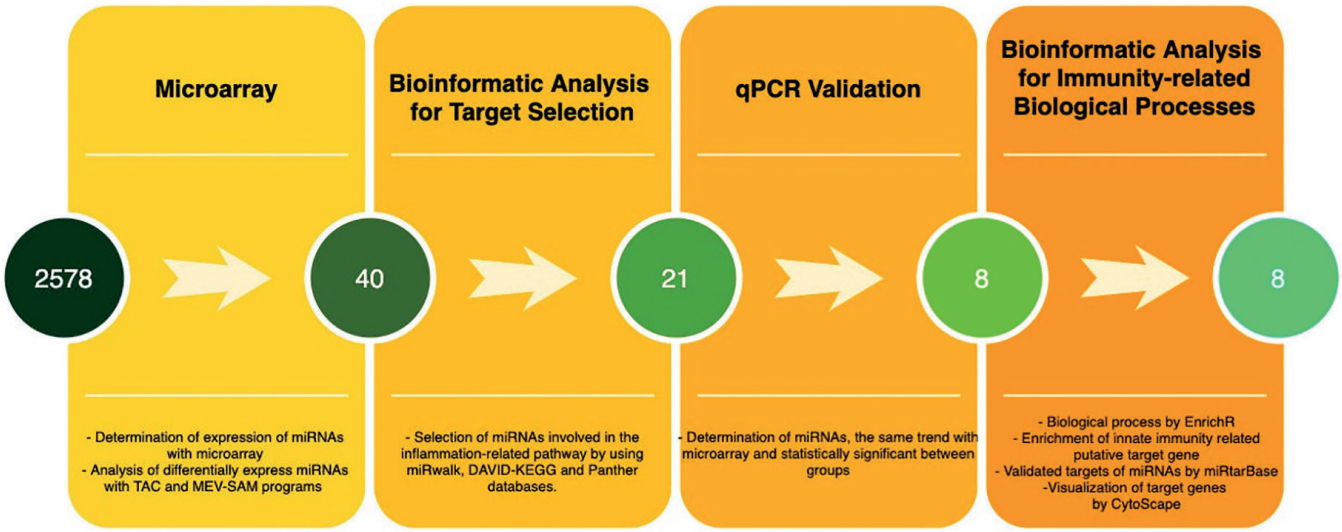

Fig. 5. Workflow of determination of miRNAs associated with inflammation, regulates clinical course in severe SAID groups.

regulation of apoptotic process also observed in miR-29b-3p, miR-29c-3p, miR-130b-3p, miR-186-5p, miR-197-3p and miR-374b-5p. Therefore, these 8 miRNAs might have a potential role in the regulation of inflammation.

Crosstalk of validated miRNAs over putative target genes, functional in innate immunity

The result of the enrichment analysis of innate-immunity related target genes of miR-29b-3p, miR-29c-3p, miR-30e-3p, miR-130b-3p, miR-148a-3p, miR-186-5p, miR-197-3p and miR-374b-5p is represented in Figure 3. Five genes associated with autoinflammation phenotypes (MEFV, RIPK1, IL10, TNFRSF1A, TNFAIP3) in the Infevers database (The registry of Hereditary Auto-inflammatory Disorders Mutations) were found as target genes (20). IL-1β, IL18, and CASP1, major innate immune system elements and inflammasome components were also found to be regulated by these miRNAs. The interaction map was revealed that these 8 miRNAs have potential in the regulation of inflammation by affecting inflammation-related genes. Differential expression of miR-197-3p which targets the IL1R1 gene in FMF patients was reported (21). Also, differential expression of miR-30e-3p which targets the IL-1β gene in SAID patients was observed (22) (not shown in the figure). Experimentally validated target genes of 6 out of 8 miRNAs previously reported are represented in Figure 4. Most of the target gene studies of miRNAs were conducted in different disease groups such as cancer, not SAIDs. Even so, it is clear that the genes identified in these diseases, observed in secondary inflammation, will contribute to the understanding of SAID pathogenesis.

The main concept and results of the study are summarised as a pipeline representation in Figure 5. These 8 miRNAs might have a potential role in the pathogenesis of SAIDs and regulation of clinical courses of patients, so they were chosen as strong candidates for promising studies.

Discussion

This is the first study defining eight miRNAs that may be associated with a more severe disease course in monogenic autoinflammatory diseases. We have compared 7 mild FMF patients with pathogenic exon 10 mutations with patients with a severe phenotype, both with FMF and other IL1 related diseases. These miRNAs were all associated with cytokine-mediated pathways.

Due to excessive innate immune responses, uncontrolled systemic inflammation is observed in systemic autoinflammatory diseases. These are an expanding group of monogenic or multifactorial disorders. Advances in genetic testing have made great contributions to the identification of the responsible genes and variants in this disease group (1). However, we still need to understand the causes of clinical heterogeneity. For example, some SAID patients with the same pathogenic mutations may display a severe phenotype whereas some may be almost asymptomatic. Duplication of disease-causing genes, which have been shown in the MEFV gene recently, can be a possible explanation (23). Again, some SAIDs may manifest comorbidities and even other chronic manifestations of inflammation. This heterogeneity affects the management of these patients. Due to the clinical and genetic heterogeneity observed in this group, additional determinants and potential biomarkers are needed in addition to genetic testing in the management of the clinical processes of these patients (2).

In recent years, it has become very important to define key pathways and regulatory factors for diseases with common features. Identifying shared common pathways that may be responsible for disease development is crucial for determining the clinical course of patients and defining common treatment goals. Recent studies show that miRNAs, a member of the non-coding RNA class, have an important role among the molecules investigated for this purpose. The reason for this great potential lies in the natural properties of miRNAs (13). miRNAs mostly regulate the inflammation process by changing the expression levels of cytokines, transcription factors, and interleukin-related genes. The

investigations that have started with cancer research now continue in other disease groups. Hence, there is an increase in the available miRNA data in research.

Data on miRNA-target gene studies are often collected in cancer-related research, and there are very few studies on inflammatory diseases. The FMF-related study showed a lower miR-204–3p expression in the serum of FMF patients during an attack, inhibiting inflammatory cytokine release via the phosphoinositide 3-kinase γ pathway by targeting the PIK3CG gene (24). It was found that miR-223, decreased in patients with CAPS, suppressed inflammation by targeting NLRP3 (12). In our study, we observed miRNA expression level differences in patients with different clinical courses. Several studies are showing that miRNAs identified in this study are involved in inflammation-related processes in different diseases and various cell types.

Different miRNAs were detected in studies conducted to examine the microRNA profile in FMF patients with different mutations in the literature (3, 25). As a result of the analyses we performed in our paediatric patient population, four miRNAs whose expression changed just in FMF patients with different clinical courses could not be detected in these studies. In a study investigating the effect of miRNAs on disease severity in FMF, miR-197-3p downregulation and miR-20a-5p upregulation were detected in severe FMF patients. Decreased expression of miR-197-3p was also detected in our severe patient group (Suppl. Table S3).

The eight miRNAs defined in our study were associated with cytokine-related pathways along with others. MiR-29b-3p defined in the study has been previously shown to protect against endotoxin-induced apoptosis and inflammatory response were detected in cardiomyocytes (26). There has been evidence that miR-30e-3p regulates processes such as autophagy, cell invasion, and migration (27, 28). Our third miRNA, miR-29c-3p contributes to the inhibition of NLRP3 inflammasome in microglial cells in Parkinson's patients (29). The regulatory roles of miR-130b-3p in various inflammation-related diseases (early lupus nephritis, obesity, atherosclerosis) have been described (30-32). In the study of human aortic valve cells, miR-148a-3p has been identified as a novel suppressor of the NF-κB signalling pathway and inflammatory gene expression (33). Changes in miR-186-5p expression level were reported that alters the expression of inflammatory markers in prostate cancer cells (34). Differential expression of miR-197-3p which targets the IL1R1 gene in FMF patients was reported (9, 21). Also, in another study conducted in hepatocellular carcinoma, they found that miR-197-3p regulates cell invasion (35). Our last miRNA, miR-374b-5p, suppresses migration and invasion of bladder cancer cells, and it regulated the type-I interferon response during JEV infection in microglial cells (36, 37).

The first limitation of the represented study is the lack of mild non-FMF patients. Hence, we did not have CAPS, MKD, or other autoinflammatory disease patients with low AIDAI scores, so we did not include them in the mild SAID group. The second limitation of the study is that the study population share the same ethnic group, just Turkish. Although the limitations of the study, the results of our study and those in the relevant literature suggest that the unique miRNA signature of severe SAID patients may contribute to the disease severity in this disease group.

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