Alteration of the microRNA expression profile in familial Mediterranean fever patients

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Received on May 13, 2017; accepted in revised form on September 26, 2017.

Clin Exp Rheumatol 2017; 35 (Suppl. 108): S90-S94.

Key words: inflammation, epigenetics, microRNAs, familial Mediterranean fever, array, inflammatory pathways.

Ethics: Written consent was acquired from the patients and controls involved in the study and approved by national ethics committee (23.01.2013, no. GO13/54-07).

Funding: This work was supported by Technical and Scientific Research Council of Turkey (TUBITAK), grant no: 214S106 and Hacettepe University, Scientific Research Projects Coordination Unit, grant no: 013D05101005, and the Turkish Society of Rheumatology, Turkey.

Competing interests: none declared.

ABSTRACT

Objective. *Phenotypic heterogeneity in familial Mediterranean fever (FMF) disease indicated that FMF is not a simple monogenic disease. Therefore it has been suggested that epigenetic factors can be one of the reason for the variations. We undertook this study to test potential involvement of miRNAs in the pathogenesis of FMF.*

Methods. *miRNA array was performed on whole blood RNA samples from 6 healthy controls (-/-), 6 FMF patients (M694V/M694V), 6 carriers who displayed the disease phenotype (M694V/-) and 6 healthy carriers (M694V/-). The raw data was analysed by Multi Experiment Viewer (MeV) and candidate miRNAs were determined according to fold change (more than 2.0 or less than -2.0). The validation of differentially expressed miRNAs was done by qRT-PCR. Then we performed pathway analyses with using bioinformatics tools.*

Results. *14 miRNAs were found to be significant among groups through the analysis with MeV. miR-20a-5p, miR-197-3p, let-7d-3p and miR-574-3p were found to be associated with inflammatory pathway related genes according to DAVID analysis. MiR-20a-5p (FDR: 0,00, FCH: 5.55) was significantly up regulated whereas miR-197-3p (FDR: 0,00, FCH: -2.27) was down regulated in homozygotes patients. Both let-7d-3p (FDR: 0.00, FCH: 28.75) and miR-574-3p (FDR: 0.00, FCH: 3.95) were up regulated in heterozygote patients group.*

Conclusion. *We showed that there are several differentially expressed miRNAs both in homozygote and heterozygote FMF patients compared to controls and healthy carriers. Thus we suggest that these miRNAs, related with inflammatory pathways may be responsible for the expression of the disease in FMF.*

Introduction

Familial Mediterranean fever (FMF) is the most common autoinflammatory disease among Eastern Mediterranean (1). It is known as rare disease, but has a high prevalence in Turkish population. FMF is caused by mutations in the *MEFV* (MEditerranean FeVer) gene (2). Several studies examining genotype-phenotype correlation in FMF patients with different clinical findings and therapeutic approaches showed that FMF may not be a simple monogenic disease. Different modifiers like epigenetic factors can be the reason for phenotypic seen in FMF. Recent data claimed that epigenetic dysregulation is effective in the pathogenesis of certain inflammation and autoimmunity (3).

Among epigenetic changes, microRNAs (miRNAs) play role as gene expression regulators and have emerged as crucial players in various biological processes such as cellular proliferation and differentiation, and apoptosis (3). Thus miRNAs may be responsible for post-transcriptional and/or translational changes in inflammatory protein products and hence may have a role in the expression of rheumatic diseases. It has been shown that, miR-155 and miR-146a are induced by LPS (Lipopolysaccharide), TNF-α (Tumour necrosis factor-alpha) and IL-1β (Interleukin-1 beta) in a NF-κB-dependent manner (Nuclear factor-kappaB) (4, 5). The increased expression of miR-146a in several cell types has been consistently reported to have a role in the pathogenesis of rheumatoid arthritis (6). MiR-155, miR-146a and miR-21 are the common miRNAs related with multiple sclerosis, systemic lupus erythematosus and bacterial infections (7). Recently two different groups showed different miRNAs related with FMF. Wada *et al*. showed that expression pat-

Table I. Demographic and clinical characteristics of patients and healthy subjects.

		MEFV mutation	Signs and symptoms	Treatment
Homozygote patients	Patient 1.LZ	M694V/M694V	fever, peritonitis, sacroiliitis, arthritis,	Colcihicine, Anakinra, Certolizumab
	Patient 2.DNU	M694V/M694V	fever, peritonitis, arthritis	Colcihicine, Anakinra
	Patient 3.CI	M694V/M694V	fever, peritonitis, arthritis	Colchicine
	Patient 4.FK	M694V/M694V	fever, peritonitis, arthritis	Colcihicine, Etanercept
	Patient 5.TB	M694V/M694V	fever, peritonitis	Canakinumab
	Patient 6.MK	M694V/M694V	fever, peritonitis, plevritis	Canakinumab
Heterozygote patients	Patient 1.YEK	M694V/-	fever, peritonitis	Colchicine
	Patient 2.RDZ	M694V/-	fever, peritonitis	Colchicine
	Patient 3.BŞ	M694V/-	fever, peritonitis, plevritis	Colchicine
	Patient 4.EA	M694V/-	fever, peritonitis	Colchicine
	Patient 5.MÇ	M694V/-	fever, peritonitis	Colchicine
	Patient 6.YA	M694V/-	fever, peritonitis	Colchicine

terns of circulating miRNAs in patients between FMF episodes differ among FMF subgroups based on *MEFV* mutations, which may use as useful biomarkers (8). Latsoudis *et al*. studied miR-4520a and its target RHEB, the main activator of mTOR signalling. They suggested that miR-4520a expression levels are significantly deregulated in FMF patients and highly dependent on the presence of pyrin mutations, especially of the M694V type (9).

This study aimed to explore the potential involvement of miRNAs in the pathogenesis of FMF and whether they could offer an explanation to the phenotypic heterogeneity seen in disease. For this purpose we studied homozygote patients with severe disease phenotype. We also hypothesised that epigenetic factors may be operative in the disease expression in heterozygotes. For this aim we compared heterozygotes with disease expression and those who were healthy carriers.

Materials and methods

Patients

Blood samples were collected in PAXgene Blood RNA (Qiagen, Valencia, California, USA) tubes from 6 healthy controls (-/-), 6 patients (M694V/M694V), 6 carriers who displayed the disease phenotype (M694V/-) and 6 healthy carriers (M694V/-) from Hacettepe University Hospital, Ankara, Turkey. All patients displayed a typical phenotype with attacks of 12–72 hour duration. C-reactive protein (CRP) levels and complete blood count (CBC) of patients were also checked.

Table II. MicroRNA profile of total blood of control, carrier and patient group using GeneChip miRNA 2.0 Array (Affymetrix). Statistical analysis was performed using SAM. FCH > 2.0; FDR < 0.05. FCH: fold change; FDR: false discovery rate.

		FCH	FDR
Homozygote patients (M694V/M694V) *vs*. controls (-/-)	hsa-miR-20a-5p	5.554	0.000
	hsa-miR-29b	3.299	0.000
	hsa-miR-19b	2.641	0.000
	hsa-miR-30e	2.118	0.000
	hsa-miR-197-3p	0.437	0.000
	hsa-miR-1308	0.425	0.000
Heterozygote patients (M694V/-) *vs*. healthy carriers (M694V/-)	hsa-let-7d*	28.745	0.000
	hsa-miR-1226	16.096	0.000
	hsa-miR-125a-5p	10.412	0.000
	hsa-miR-574-3p	3.953	0.000
	hsa-miR-197	3.711	0.000
	hsa-miR-4298	3.326	0.000
	hsa-miR-941	3.041	0.000
	hsa-mir-1826	0.176	0.000

Since the homozygote patients had a severe disease phenotype, 5 of them were also receiving biologic therapies. The median ages were 28 for homozygote patients, 26 for heterozygote patients, 33 for heterozygote carriers, 34 for healthy controls. Female/Male ratio was 11/13 (Table I). Written consent was acquired from the patients and controls involved in the study and approved by national ethics committee (23.01.2013 date, GO13/54-07 number). Mutation analysis was performed on *MEFV* gene by DNA sequencing for every individual.

RNA isolation and microarray

RNA was isolated from total blood with PreAnalytiX miRNA Kit (Qiagen, Valencia, California, USA), given the fact that neutrophils and monocytes are the main cell types important in FMF pathology and the possibility of probing miRNAs in circulation. The quality and quantity of RNAs were determined by spectrophotometric measurements at 260 and 280 nm with a NanoDrop ND 1000 (Thermo Scientific, Waltham, MA, USA).

The expression levels of miRNAs were determined with GeneChip miRNA 2.0 Array (Affymetrix) according to the manufacturer's recommendations. The raw data was analysed by Multi Experiment Viewer (MeV) and candidate miRNAs were determined according to fold change (more than 2.0 or less than -2.0), false discovery rate, and abundance in neutrophils and monocytes.

Real time quantitative PCR analysis

The validation of differentially expressed miRNAs was done by qRT-PCR (Quantitative reverse transcription polymerase chain reaction). The number of the patients was increased

for the validation step (n=10 for each group) (Supplementary Table I).

Reverse transcription was carried out using TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). PCR amplification was done using an ABI Prism 7500 sequence detector (Applied Biosystems, Foster City, CA, USA).

All reactions were performed with TaqMan® MicroRNA Assays (Applied Biosystems, Foster City, CA, USA) primers by using TaqMan® Universal PCR Master Mix, no AmpErase® UNG (Applied Biosystems, Foster City, CA, USA) in Biorad iQ5 (Bio-rad, Hercules, CA, USA). For quantification, $2^{-\Delta\Delta CT}$ method was used. Each sample was tested in triplicate and all quantifications were normalised using RNU48 as referans gene.

Bioinformatics analysis

miRNA target genes were determined in miRWalk, the database on predicted and validated miRNA targets. This database combines many prediction programs including DIANA-mT, PICTAR4, PICTAR5, miRanda, miRDB, PITA, miRWalk, RNA22, RNAhybrid, TargetScan. We chose the target genes that were determined by at least six of these programs. Then the target gene list was clustered in DAVID (The Database for Annotation, Visualization and Integrated Discovery) v6.7 through BioCarta and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway maps.

Results

Fourteen miRNAs expression levels altered significantly among the groups

Many miRNAs were found as differentially expressed significantly among groups through the analysis with Multi Experiment Viewer (MeV) (Table II). Among these 14 candidate miRNAs, miR-20a-5p, miR-197-3p, let-7d-3p and miR-574-3p were found to be associated with inflammatory pathway related genes according to DAVID analysis (Supplementary Fig. 1-7). MiR-20a-5p (FDR: 0.00, FCH: 5.55) was significantly up regulated whereas miR-197-3p (FDR: 0.00, FCH: -2.27) was down regulated in homozygotes patients. Both let-7d-3p (FDR: 0.00, FCH: 28.75) and miR-574-3p (FDR: 0.00, FCH: 3.95) were up regulated in heterozygote patients group.

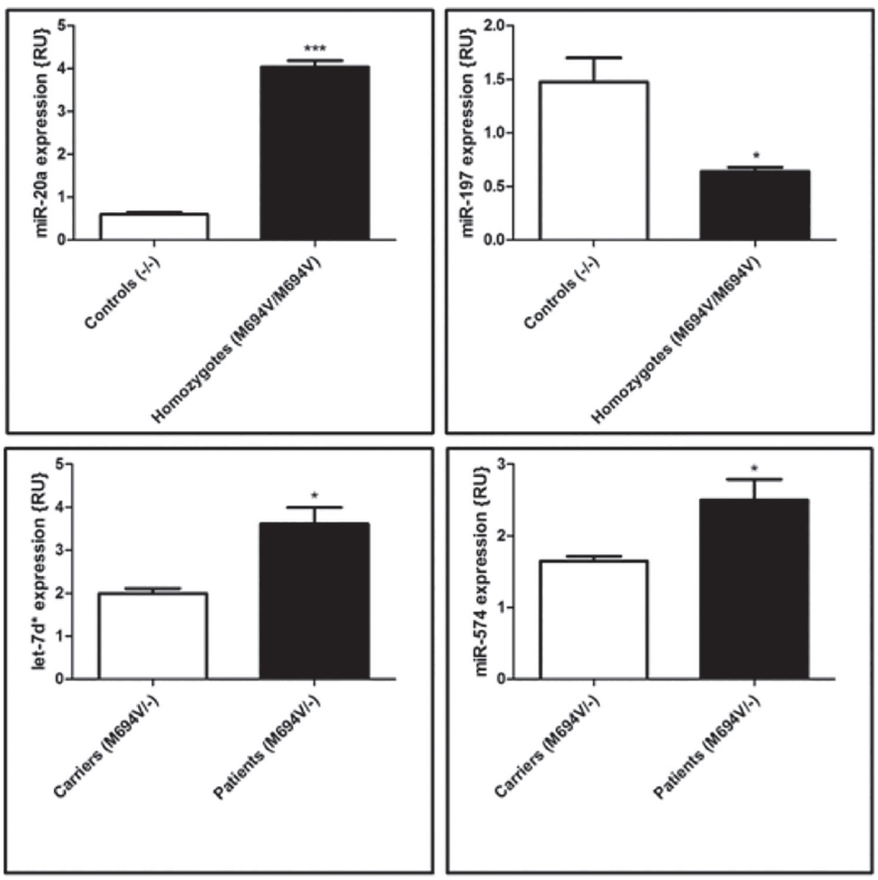

Fig. 1. Validation of differentially expressed miRNAs by qRT-PCR. RNAs from control, carrier and patient groups were used and the expression of the indicated miRNAs was measured using qRT-PCR. MiRNA expression was normalised to U48 RNA. *p<0.05; **p<0.01; ***p<0.001.

Table III. CRP (C-reactive protein) and ESR (erythrocyte sedimentation rate) levels of patients.

		CRP mg/dL	ESR mm/hour
Homozygote patients	Patient 1. LZ	2.73	27
	Patient 2. DNU	11.1	37
	Patient 3. CI	1.59	6
	Patient 4. FK	13.2	15
	Patient 5. TB	0.162	6
	Patient 6. MK	1.6	40
Heterozygote patients	Patient 1. YEK	0.21	10
	Patient 2. RDZ	0.388	2
	Patient 3. BŞ	0.161	2
	Patient 4. EA	0.12	2
	Patient 5. MÇ	8.66	15
	Patient 6. YA	0.2	3

Four selected miRNAs were validated successfully

Results of the miRNA expression profiling were validated in patients. qRT-PCR analysis confirmed the results of the miRNA profiling for 4 miRNAs. From two of them; miR-20a showed induction and the other one; miR-197 showed reduction in the homozygote group compared to controls. Another two miRNAs; miR-574 and let-7d increased in heterozygote patients compared to carriers (Fig. 1). All these miRNAs found to play possible role

in TGF-beta (Transforming growth factor beta) signal, TLR (Toll-like receptor), and NLR (NOD-like receptor) signalling pathway, apoptosis and actin cytoskeleton regulation, they were chosen as candidate miRNAs for the remaining experiments.

Discussion

FMF is characterised by clinical and laboratory signs of inflammation. We suggested that two miRNAs (miR-20a and miR-197) are effective in the inflammation of homozygote FMF patients with severe disease phenotype. We also have studied the heterozygote *MEFV* mutation carriers who display the disease phenotype and compared them to healthy carriers, to understand whether epigenetic mechanisms were effective in the presentation of the disease in this specific group of patients. We have shown that there were marked expression of two specific miRNAs (miR-574 and let-7d*) in heterozyogtes that display the disease phenotype, that were not present in those who were "healthy". Complete blood count (CBC) samples were also taken at the same time for standardisation of the number of the cells in patients. Especially the percentage of the inflammatory cells was checked. Also patient's clinical status was carefully examined. All samples have similar percentage of neutrophil cells (Supplementary Table II and III). During the blood sample evaluation we aim the get to less disease activity with effective agents including anakinra and canakinumab (Table I).

Treatments of FMF patients were tailored according to frequency, duration and severity of FMF attacks. As shown in Table II, some of patients were on parenteral treatments such as anakinra and canakinumab. To eliminate the possible confounding effects of FMF attacks on miRNA levels, blood samples of both patient groups were taken at attack-free periods. However, some patients had increased acute phase reactants (Table III, Supplementary Table IV). The possible association of acute phase reactants with miRNA levels during FMF attacks should be assessed in further studies.

Abnormal expression of several miRNAs has been reported in different kinds of samples in immune system related disorders. The ones circulated inside exosomes and stable in plasma or serum maybe suitable for clinical use as potential biomarkers. In FMF, detection of such a biomarker may be used for explaining the severity of the disease for complex cases. Recently two groups were focused on biomarkers studies for FMF disease. Wada *et al*. and Latsoudis *et al*. showed some miRNAs levels differs among patients dependent on their mutations in *MEFV* gene (8, 9). They are more likely to identify possible biomarkers for disease, as the study groups are from patients who have mutations in exon 10 or exon 3. But our study mainly focused on the miRNAs related with inflammation seen in patients for explaining pathophysiology of the disease with epigenetics. In this study, we showed for the first time that there are several differentially expressed miRNAs both in homozygote and heterozygote FMF patients compared to controls and healthy carriers apart from their mutation types, which seems to be important in disease pathogenesis. Our experimental and bioinformatics analysis results may contribute to explain phenotypic heterogeneity seen in patients.

The differential expression of miR-197 in homozygous FMF patients and symptomatic carriers can be explained by different treatments that those patients have. All homozygote patients are using drugs other than colchicine such as anakinra, certolizumab, etanercept and canakinumab, however, heterozygote patients are being treated by daily use of colchicine (Table I). Actually these differences according to phenotype make miR-197 a more important target for FMF disease. However functional studies are needed to explain its exact effect on the autoinflammation mechanism.

One of the candidate miRNAs in homozygotes vs. control group, miR-20a has been shown to be negative regulator of inflammation in rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS) by modulating expression of apoptosis signal-regulating kinase (ASK) 1, a key component of the toll-like receptors 4 pathway, upstream of p38 mitogen-activated protein kinase (10). In another study, signal-regulatory protein α (SIRPα) that modulates leukocyte inflammatory responses, was found to be one of the target genes of miR-20a and its belonging cluster miR-17-92 (11). The other miRNA, miR-197 was found to have an important role in the reactivation of liver inflammation by targeting IL-18 during HBV (Hepatitis b virus) infection (12) and regulate IL-6/STAT3 (Interleukin 6/ Signal transducer and activator of transcription 3) inflammatory signalling pathway in hepatocellular carcinoma (HCC) (13).

It has been known for a long time that some carriers display the disease although this is an autosomal recessive disease. Many studies have addressed possible explanations such as a second candidate gene, allelic gene expression analysis and complex heredity models were theorised (14). Since there was no substantial explanation we hypothesised that epigenetic control mechanisms such as DNA methylation, histone modifications, chromatin remodeling and non-coding RNAs (miRNAs, etc.) can be important for explaining molecular pathophysiology of FMF. MiR-574, in heterozygotes *versus* healthy carrier group, was indicated as a possible modulator of inflammation (raised IL-8, reduced miR-146a) with type 2 diabetes (T2D) patients (15). There is no study that shows the relation between inflammation and let-7d* as yet.

Bioinformatics analysis revealed that some of these miRNAs target mRNAs clustered in inflammatory pathways such as cytokine secretion, apoptosis, and cell migration. Functional validation efforts of differentially expressed miRNAs, through identifying its target genes experimentally and manipulating miRNA levels (overexpression or knock-down of miRNA) in specific cells are underway.

The descriptive results of this study revealed that miRNAs can be one of the epigenetic factors that play role in FMF pathogenesis which will in turn represent a model for its possible application to the other inherited autoinflammatory disorders.

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