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Altered expression of apoptosis-related, circulating cell-free miRNAs in children with familial Mediterranean fever: a cross-sectional study

Emin Murat Karpuzoglu[1] · Rabia Miray Kisla Ekinci[2] · Sibel Balci[2] · Atil Bisgin[3] · Mustafa Yilmaz[2]

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Abstract

Objectives Familial Mediterranean Fever (FMF) is the most common hereditary autoinflammatory disorder characterized by recurrent fever and serositis episodes. Identification of low penetrant or heterozygous *MEFV* mutations in clinically diagnosed FMF patients did raise a concern on whether epigenetic or environmental factors play an additional role in FMF pathogenesis. We aimed to investigate the expression profile of apoptosis-related miRNAs in FMF and their influence on clinical manifestations in the present study.

Method 191 pediatric FMF patients and 31 healthy children included in the study. Expressions of 33 apoptosis-related, circulating cell-free miRNAs were evaluated by a quantitative polymerase chain reaction, statistically calculated within ΔΔCt values and fold changes were evaluated by Welch *T* test, in which $p < 0.05$ were considered to be significant.

Results Nineteen miRNAs, including let-7a-5p, let-7c, let-7 g-5p, miR-15b-5p, miR-16-5p, miR-17-5p, miR-23a-3p, miR-24-3p, miR-25-3p, miR-26a-5p, miR-26b-5p, miR-27a-3p, miR-29c-3p, miR-30a-5p, miR-30d-5p, miR-30e-5p, miR-106b-5p, miR-146a-5p, and miR-195-5p, were found down-regulated; miR-15a-5p, miR-29b-3p, miR-181a-5p, miR-181b-5p, miR-181c-5p, miR-214-3p, and miR-365a-3p were up-regulated in FMF patients. In detail, these miRNAs were similar among FMF patients in terms of genotype, colchicine response, and having an inflammatory attack during analysis.

Conclusion We found that 26 apoptosis-related circulating miRNAs were deregulated in children with FMF. Thus, we speculate that these miRNAs have a role in FMF pathogenesis via apoptotic mechanisms.

Keywords Apoptosis · Children · Familial Mediterranean fever · *MEFV* gene · miRNA

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✉ Emin Murat Karpuzoglu
mkarpuzoglu@gmail.com

Rabia Miray Kisla Ekinci
mir_kisla@hotmail.com

Sibel Balci
drsibelbalci@hotmail.com

Atil Bisgin
abisgin@yahoo.com

Mustafa Yilmaz
drmusyilmaz@hotmail.com

[1] Department of Pediatrics, Faculty of Medicine, Cukurova University, Adana, Turkey

[2] Department of Pediatric Rheumatology, Faculty of Medicine, Cukurova University, Adana, Turkey

[3] Department of Medical Genetics, Faculty of Medicine, Cukurova University, Adana, Turkey

Introduction

Familial Mediterranean Fever (FMF) is a monogenic autoinflammatory disorder worldwide, which usually presents with recurrent fever and serositis episodes since childhood. Fever episodes usually last 12–72 h, resolve spontaneously, and are often accompanied by sterile peritonitis, synovitis, pleuritis, and seldom pericarditis and erysipelas-like erythema [1, 2]. The severity and frequency of FMF attacks can be alleviated by colchicine, which is the standard treatment option in FMF [3, 4]. The most life-threatening complication of FMF is secondary amyloidosis, particularly involving kidneys due to the accumulation of amyloid resultant from excessive inflammation [2, 5].

In addition to the diversity of symptoms, duration, frequency, and intensity of attacks are highly variable between FMF patients. Based on genotype–phenotype studies, it was generally accepted that M694V and M694I mutations are related to severe disease and amyloidosis in distinct

ethnicities [2, 3, 6]. Nevertheless, genotype alone cannot be held responsible for this phenotypic variability.

Following the discovery of Mediterranean FeVer (*MEFV*) gene on the 16th chromosome in 1997, the confirmation of FMF diagnosis became more challenging [7, 8]. Besides the identification of low penetrant mutations, patients with heterozygous and even no *MEFV* mutations have been diagnosed as having FMF [9].

Epigenetics can be roughly described as heritable changes affecting gene expression without any changes in the genome. The major epigenetic mechanisms include DNA methylation, histone modifications, and chromatin remodeling [10]. miRNAs are a main group of small noncoding RNAs and an important component of epigenetic mechanisms. In 1993, Lee et al. first determined lin-4 in Caenorhabditis elegans and suggested that it encodes small RNA products [11]. It is followed by the discovery of let-7, the first human miRNA in 2000, and there are more than 2000 human miRNA annotated in databases currently [12, 13]. The most noteworthy postulated functions of miRNAs are the regulation of epigenetic modifications and maintaining tissue homeostasis. miRNAs are also suggested to be transported by directly gap junctions, extracellular vesicles, exosomes, apoptotic bodies, lipoproteins, and ribonucleoproteins, and play a particular role in cell–cell communication [13, 14]. The role of miRNAs in FMF pathogenesis remains doubtful, because there are only few studies investigating inflammation- and autoimmunity-related miRNAs in FMF [15–21].

The proposed mechanisms of FMF include overactivation of caspase-1, thus excessive IL-1 production due to loss of function of pyrin, a negative regulator of NLRP3 inflammasome assembly [9, 22]. However, recent studies have postulated pyrin as a sensor protein which may be triggered by small GTPases of the Rho family and cytoskeleton changes by interacting with microtubules and actin filaments. Therefore, a gain-of-function mutation in *MEFV* which causes an overactive pyrin was proposed to cause FMF [23, 24]. Another cellular process in which pyrin was suggested to take part is apoptosis [9, 25–27]. Furthermore, it is well accepted that numerous miRNAs, which were defined as having oncogenic and tumor suppressor roles, take part in apoptotic pathways [28]. Therefore, this study was conducted to investigate apoptosis-related miRNAs in FMF and their influence on clinical manifestations.

Materials and methods

Participants

This is a cross-sectional observational study conducted to compare the expressions of certain miRNAs between FMF patients and healthy controls. Patients with FMF, who admitted to our outpatient clinic between March 2017 and July 2017, were included in this study. All patients had been diagnosed as having FMF according to Tel Hashomer diagnostic criteria, in the same department, and were under colchicine treatment at the time of the study [7]. Patients with a follow-up duration of less than 6 months were excluded from the study. Demographic parameters including age, age at disease onset, age at diagnosis, clinical manifestations, duration and dosage of colchicine treatment, treatment responses, and *MEFV* gene-sequencing results were retrospectively collected from medical files of the patients. Patients who lack homozygosity for exon 10 mutations in *MEFV* gene underwent additional genetic analysis for autoinflammatory diseases with recurrent fever, including Cryopyrin-Associated Autoinflammatory Syndromes (CAPS), Tumor Necrosis Factor Receptor Associated Periodic Syndrome, mevalonate kinase deficiency, and Deficiency of Adenosine Deaminase 2 by next-generation sequencing system. Patients with other identifiable mutations in genes related to the aforementioned hereditary autoinflammatory disorders and the presence of recurrent urticarial rash, hearing loss, and skeletal abnormalities suggestive for CAPS were excluded from the study [29]. Colchicine resistance was defined as having one or more attacks each month despite receiving the maximally tolerated dose for at least 6 months [30]. We also grouped the responder patients if the attack frequency decreased at least 50% of baseline and had normal acute-phase reactants (APRs) during attack-free periods as having a favorable response, and if they could not meet these criteria but better than before, the patients were grouped as having a partial response.

APRs, including erythrocyte sedimentation rate, C-reactive protein, and Serum Amyloid-A obtained at study enrollment. The control group consisted of 31 healthy, age and sex-matched participants, admitted to our hospital for well-child preventive care visits. Children with an active infection sign, such as fever, cough, vomiting, diarrhea, and APR elevation, were excluded from control group. Written informed consent was obtained from all participants and their parents prior to the study. The study was approved by the local ethics committee of Cukurova University Medical Faculty (number: 53/2, date: 13.05.2016).

MEFV gene analysis

Leukocyte DNA was isolated from all cases by standard methods. We performed *MEFV* gene analysis by a molecular diagnostics tool, next-generation sequencing platform (MiSeq System, Illumina). The test covers all exons for *MEFV* gene, at least 50 nucleotides upstream and downstream of each exon and 1 kb of both the 5′ promoter regions and the 3′ UTRs.

miRNA analysis

Metanalysis of miRNAs in relation to both disease phenotype and target interactions had been performed by miRbase (searching for known miRNA informations) and miRWalk 2.0 (searching for predicted and validated miRNA interactions); afterward, the apoptosis-related miRNAs were selected. Venous blood samples were obtained from the participants and further centrifuged in 3500 rpm to the supernatant. Total RNA was extracted by utilizing Qiagen RNeasy Mini Kit (Hilden, Germany) according to the manufacturer's instructions. Qiagen miScript II RT kit was used for reverse transcription of RNA to cRNA. MiRNAs were isolated from cRNAs by preamplification with Qiagen miScript Microfluidics PreAmp kit. Assay plate containing miRNAs was diluted with miScrip Microfluidics Universal Primer, Assay Loading Reagent (Fluidigm), and Rnase-free water subsequently. This primer was confronted by Qiagen miScript MicroFluidics PCR kit, which were further analyzed in BioMark (Fluidgm, Germany) for the following 33 miRNAs; let-7a-5p, let-7c, let-7 g-5p, miR-15a-5p, miR-15b-5p, miR-16-5p, miR-17-5p, miR-23a-3p, miR-24-3p, miR-25-3p, miR-26b-5p, miR-26a-5p, miR-27a-3p, miR-29a-3p, miR-29b-3p, miR-29c-3p, miR-30a-5p, miR-30b-5p, miR-30c-5p, miR-30d-5p, miR-30e-5p, miR-98-5p. miR-101-3p, miR-106b-5p, miR-145-5p, miR-146a-5p, miR-181a-5p, miR-181b-5p, miR-181c-5p, miR-195-5p, miR-214-3p, miR-222-3p, and miR-365a-3p.

Statistical analysis

The minimum sample size was calculated as 24, in accordance to the formula including type 1 error rate ($\alpha = 5\%$) and type II fault ($\beta = 0.20$), power ($1-\beta = 80\%$), and predicted means for Group A (FMF patients) and B (healthy volunteer) as 1.5 and 1, respectively. In addition, the standard deviation was considered as 0.8 and sampling ratio (Group A/B) as 6. Categorical variables were presented as numbers and percentages. The distribution of continuous variables was tested by Kolmogorov–Smirnov test for normality, and continuous variables including demographic data were given as median and minimum–maximum (range). miRNA expressions were statistically calculated by Flexsix GE Chipi Fludigm (Biomark) system and obtained Ct values were analyzed according to related software (https://www.qiagen.com/us/shop/genes-and-pathways/data-analysis-center-overview-age/web). The best housekeeping gene was chosen as SNORD68 among the nominate genes by normfinder analysis (https://moma.dk/normfinder-software). ΔCt values of both patients and control group were calculated by subtracting Ct values in the control SNORD68 gene from Ct values in each assay. By subtraction of control Ct values from ΔCt values resulted as ΔΔCt values. Fold regulation results were compared with Welch *T* test. Negative fold regulation value showed decreased expression lower than the control group, whereas positive fold regulations meant increased expression higher than controls. Statistical significance was considered to be $p < 0.005$ in each test.

Results

Demographic and clinical features

This study included 191 patients with FMF, of whom 86 (45%) were female and 105 (55%) were male. Twenty-five patients (13.1%) were during an FMF attack and 166 (86.9%) were on an attack-free period at the time of the study. In FMF study group, the median ages at symptom onset, diagnosis, and study enrollment were 4.42 (range, 1–16.02), 7.13 (1.52–17.02), and 11.89 (range, 3.38–17.8) years, respectively. All of the patients were under colchicine treatment, of whom seven (3.7%) did not respond to colchicine and treated simultaneously with canakinumab. Clinical characteristics and genotypes of the patients are summarized in Table 1. Control group included 31 healthy children, 15 (48.4%) females and 16 (51.2%) males, and the median age of healthy controls was 9.62 (range, 1.2–17.5) years.

miRNA analysis results

The mean plasma miRNA expression levels of both patients with FMF and the control group were elaborately given in Table 2 as ΔCt values. According to the results of the Welch t test, which was performed to compare the ΔΔCt values between groups including unequal sample sizes, we found that 26 of 33 miRNAs differed between FMF patients and controls. Nineteen miRNAs, including let-7a-5p, let-7c, let-7 g-5p, miR-15b-5p, miR-16-5p, miR-17-5p, miR-23a-3p, miR-24-3p, miR-25-3p, miR-26a-5p, miR-26b-5p, miR-27a-3p, miR-29c-3p miR-30a-5p, miR-30d-5p, miR-30e-5p, miR-106b-5p, miR-146a-5p, and miR-195-5p, were found as down-regulated in FMF patients than healthy controls, whereas expression levels of miR-15a-5p, miR-29b-3p, miR-181a-5p, miR-181b-5p, miR-181c-5p, miR-214-3p, and miR-365a-3p were elevated in FMF. The test results were summarized in Table 3. In detail, when we compared the miRNA expression profiles among FMF patients in terms of genotype, including the presence of M694V positivity, exon 10 mutations, and heterozygosity, we did not find any statistically significant difference. Furthermore, miRNA expression levels were found similar between patients with favorable colchicine response and without. Expression of 33 apoptosis-related miRNAs was also similar between patients in an inflammatory attack and 166 patients during attack-free period.

Table 1 Disease characteristics and *MEFV* gene-sequencing results of 191 children with familial Mediterranean fever

Parameters	*n* (%)
Symptoms during an inflammatory attack	
Fever	163 (85.3)
Abdominal pain	175 (91.6)
Arthralgia	120 (62.8)
Arthritis	21 (11)
Nausea	30 (15.7)
Chest pain	8 (4.2)
Erysipelas like erythema	12 (6.3)
Myalgia	4 (6.3)
Diarrhea	11 (5.8)
Constipation	6 (3.1)
Family history of FMF	80 (41.9)
Parental consanguinity	50 (26.2)
Colchicine response	
Favorable response	144 (75.4)
Partial response	40 (20.9)
None responders	7 (3.7)
MEFV genotype	
M694V/M694V	33 (17.3)
M694V/R761H	10 (5.2)
M694V/V726A	5 (2.6)
M694V/M680I	9 (4.7)
M694V/M694I	5 (2.6)
M694V/R202Q	21 (11)
M694V/E148Q	3 (1.6)
M694I/ M694I	1 (0.5)
M694I/ E148Q	4 (2.1)
M680I/M680I	6 (3.1)
M680I/V726A	1 (0.5)
V726A/V726A	1 (0.5)
V726A/M680I	1 (0.5)
V726A/R202Q	6 (3.1)
V726A/E148Q	1 (0.5)
E148Q/E148Q	6 (3.1)
E148Q/E336K	1 (0.5)
E148Q/wt	13 (6.8)
I641F/I641F	1 (0.5)
P369S/wt	3 (1.6)
A744S/wt	2 (1)
R202Q/E148Q	4 (2.1)
R202Q/R202Q	11 (5.8)
R202Q/wt	37 (19.4)
No mutations	7 (3.7)
Total number of patients	191 (100)

FMF familial Mediterranean fever, *MEFV* MEditerranean FeVer

Table 2 Expression levels of apoptosis-related miRNAs according to ΔCt values of among patients with familial Mediterranean fever and healthy controls

miRNAs	ΔCt values of FMF patients ($n=191$)		ΔCt values of control group ($n=31$)	
	Mean	SD	Mean	SD
let-7a-5p	1.96	1.70	3.61	2.33
let-7c	1.36	1.28	2.61	1.95
let-7 g-5p	1.80	1.73	2.88	1.35
miR-15a-5p	1.65	1.37	1.02	1.09
miR-15b-5p	2.50	2.76	5.76	2.68
miR-16-5p	9.14	10.56	37.75	29.54
miR-17-5p	2.72	2.57	8.53	6.41
miR-23a-3p	7.30	7.52	16.30	8.29
miR-24-3p	2.82	2.59	6.36	3.52
miR-25-3p	12.26	12.18	36.53	40.96
miR-26a-5p	3.80	4.81	7.32	3.41
miR-26b-5p	3.39	4.08	6.32	3.08
miR-27a-3p	2.64	2.70	5.58	3.41
miR-29a-3p	1.88	1.63	2.71	2.13
miR-29b-3p	1.72	1.38	0.80	0.98
miR-29c-3p	2.00	1.67	3.04	2.29
miR-30a-5p	2.15	1.88	5.78	5.05
miR-30b-5p	1.54	1.37	1.71	1.05
miR-30c-5p	1.55	1.42	1.91	1.09
miR-30d-5p	1.67	1.59	3.72	3.29
miR-30e-5p	1.75	1.61	4.21	3.66
miR-98-5p	1.39	1.34	1.41	0.91
miR-101-3p	1.61	1.52	2.14	1.94
miR-106b-5p	2.28	2.27	5.82	4.02
miR-145-5p	1.36	1.30	1.62	1.12
miR-146a-5p	2.34	1.87	4.00	2.22
miR-181a-5p	1.71	1.41	0.98	1.06
miR-181b-5p	1.43	1.41	0.43	0.39
miR-181c-5p	1.68	1.45	0.76	1.06
miR-195-5p	8.28	9.21	34.01	27.18
miR-214-3p	1.63	1.47	0.69	0.77
miR-222-3p	1.40	1.34	1.65	1.24
miR-365a-3p	1.70	1.40	0.82	0.98

FMF familial Mediterranean fever, *SD* standard deviation

Discussion

Our study revealed that 26 of 33 apoptosis-related miRNAs' expressions were altered in serum of FMF patients with respect to healthy controls. In detail, nineteen miRNAs, including let-7a-5p, let-7c, let-7 g-5p, miR-15b-5p, miR-16-5p, miR-17-5p, miR-23a-3p, miR-24-3p, miR-25-3p, miR-26a-5p, miR-26b-5p, miR-27a-3p, miR-29c-3p, miR-30a-5p, miR-30d-5p, miR-30e-5p, miR-106b-5p, miR-146a-5p and miR-195-5p were found down-regulated;

Table 3 Fold changes of the apoptosis-related circulating miRNA in patients with familial Mediterranean fever

miRNAs	Fold change	*df*	*p*
miR-181b-5p	1.73	65.91	< 0.001
miR-214-3p	1.24	28.91	< 0.001
miR-181c-5p	1.14	17.75	< 0.001
miR-29b-3p	1.09	20.56	< 0.001
miR-365a-3p	1.04	18.46	< 0.001
miR-181a-5p	0.80	11.33	0.002
miR-15a-5p	0.70	8.42	0.007
miR-98-5p	− 0.02	0.01	0.915
miR-30b-5p	− 0.15	0.64	0.451
miR-222-3p	− 0.23	1.03	0.342
miR-145-5p	− 0.25	1.35	0.289
miR-30c-5p	− 0.30	2.68	0.129
miR-101-3p	− 0.41	2.10	0.181
miR-29a-3p	− 0.53	4.29	0056
miR-29c-3p	− 0.60	5.93	0.026
let-7 g-5p	− 0.68	15.98	< 0.001
miR-146a-5p	− 0.77	15.43	< 0.001
let-7a-5p	− 0.88	14.35	0.001
miR-26b-5p	− 0.89	21.77	< 0.001
miR-26a-5p	− 0.94	24.96	< 0.001
let-7c	− 0.93	11.82	0.002
miR-27a-3p	− 1.08	21.03	< 0.001
miR-30d-5p	− 1.15	11.56	0.002
miR-23a-3p	− 1.16	32.23	< 0.001
miR-24-3p	− 1.17	28.82	< 0.001
miR-15b-5p	− 1.20	39.12	< 0.001
miR-30e-5p	− 1.27	13.65	0.001
miR-106b-5p	− 1.35	22.87	< 0.001
miR-30a-5p	− 1.42	15.62	< 0.001
miR-25-3p	− 1.57	10.72	0.003
miR-17-5p	− 1.65	24.91	< 0.001
miR-16-5p	− 2.05	28.50	< 0.001
miR-195-5p	− 2.04	27.29	< 0.001

df degrees of freedom

miR-15a-5p, miR-29b-3p, miR-181a-5p, miR-181b-5p, miR-181c-5p, miR-214-3p, and miR-365a-3p, were up-regulated. These miRNAs were suggested to target various genes and upregulation of these non-coding miRNAs were linked to oncogenesis and cancer progression; however, except for miR-146a-5p, miR-16-5p, miR-26a-5p, let-7a-5p, and miR-181, they have not been investigated on monogenic autoinflammatory diseases, including FMF.

To the best of our knowledge, the first preliminary miRNA expression study in FMF was conducted in 2016 by Latsoidis et al. Among nine adult FMF patients, they found differently expressed 29 miRNAs, of which miR-4520 was the most promising candidate implicated in biological processes, by further bioinformatic analysis [15]. Subsequently, Koga et al. studied expression profiles of only four miRNAs in nine FMF patients and found extremely low miR-204-3p expression in FMF patients during an attack and even suggested miR-204-3p as a useful biomarker. In the same study, miR-204-3p was also shown to inhibit the secretion of inflammatory cytokines [16].

The most intriguing results came from another study, including 24 FMF patients, grouped by localization of *MEFV* mutations. The expression of circulating miRNAs differed between the groups according to having a typical phenotype with or without an exon 10 mutation [17]. In the same study, the authors proposed that elevated miR-320 expression may be a compensatory mechanism for controlling excessive inflammation caused by exon 10 mutations in FMF [17]. A recent study found 14 miRNAs to be differentially expressed in 12 FMF patients, of which miR-20a-5p, miR-197-3p, let-7d-3p, and miR-574-3p were associated with inflammatory pathways. Patients with homozygote M694V mutations were shown to have up-regulated miR-20a-5p and down-regulated miR-197-3p, for which the authors commented as they may have a role on severe disease phenotype [18].

Hortu et al. performed a more comprehensive workup about miRNAs in 51 pediatric FMF patients, of whom 27.5% were at an FMF attack and 39.2% were colchicine-naïve. Only 15 miRNAs (miR-15a, miR-146a, miR-155, miR-26, miR-21, miR-223, miR-16, miR-181, miR-125a, miR-34a, miR-124a, miR-203, miR-346, miR-132, and miR-23b) were evaluated. Eleven of them, which were previously linked to inflammatory pathways in other studies including rheumatic and autoimmune disorders, were also found as significantly decreased in FMF patients. While patients were grouped according to the mutation type, attack status, and presence of acute-phase reactant elevations, there were no significant differences in these miRNA expressions. The patient group was analyzed and compared within itself, and the expression levels of five miRNAs (miR-132, miR-15a, miR-181a, miR-23b, and miR-26a) in the patients who took colchicine seemed to have increased and levels of 5 miRNAs (miR-146a, miR-15a, miR-16, miR-26a, and miR-34a) in the patients who took colchicine were significantly lower [19]. With respect to this study, we similarly found significantly down-regulated expressions in miR-16-5p, miR-26a-5p, miR-26b-5p, and miR-146a-5p, which suggest their negative regulatory roles on FMF. On the counterpart, we found up-regulated expressions of miR-181a-5p, miR-181b-5p, and miR-181c-5p in FMF patients, which was similar to that study revealing decreased overall expression in FMF patients, but elevated expression in patients under colchicine treatment. In fact, all participants had been received colchicine in our study, and thus, our results may also support colchicine may increase miR-181 expression and thus

control excessive inflammation. Besides, it was previously suggested that colchicine induces cell apoptosis in colon cancer and normal liver cells in a dose-dependent manner [31, 32]. We think that these miRNAs somehow could take part in these apoptotic pathways. Further functional studies should be performed to clarify the effects of colchicine on the expression of these miRNAs. With improving knowledge about the effects of miRNAs, miRNAs-based therapies may be even thinkable in FMF in the near future [33].

Amarilyo et al. observed that four miRNAs (miR-144-3p, miR-21-5p, miR-4454, and miR-451a) were up-regulated and three (miR-107, let-7d-5p, and miR-148b-3p) were down-regulated significantly in FMF patients [20]. Moreover, Demir et al. studied four miRNAs, which were previously found deregulated in FMF patients, and found decreased miRNA-155 and miRNA-204 and increased miRNA-16 and miRNA-451 expressions [21]. Studies investigating miRNAs in FMF were identified through a literature search on MEDLINE/PubMed and Scopus databases, and the main results of the studies are summarized in Table 4.

Apoptosis can be defined as a physiological process and programmed cell death resultant from endogenous or exogenous signals. In the last 2 decades, it has been an area of interest whether apoptosis plays a role in inflammatory and rheumatic diseases, particularly by terminating excessive inflammation [34]. FMF is characterized by recurrent autoinflammation due to dysfunctional pyrin protein, which interacts ASC by its PYD domain and thus activation of caspase-1 [26, 35]. Suggesting the self-limiting nature of the disease, patients were found to have increased neutrophil apoptosis and FasL levels during FMF attacks, compared to healthy controls in a preliminary study [36]. On the other hand, another study reported no difference in neutrophil apoptosis between FMF patients and controls [37]. Similarly, another study found similar serum FasL levels between FMF patients on attack, during remission and control group, which thus yielded no diagnostic aid [38]. In a more recent study, both spontaneous and induced neutrophil apoptosis were found significantly higher in FMF patients during an attack or remission than healthy controls and this finding was supported by the elevation of caspase-3 mRNA. The authors induced apoptosis with lipopolysaccharides, TNF-alpha, MDP, CSK4, ATP, and even colchicine; however, they came up with the question of which endogenous stimuli may trigger the spontaneous neutrophil apoptosis in FMF patients [39].

On the other hand, some miRNAs were prominently altered miRNAs in the present study, which were at least 1.5 times increased (miR-181b-5p) or decreased (miR-16-5p, miR-17-5p, miR-25-3p, and miR-195-5p). Moreover, it was attempted to clarify the role of these miRNAs on apoptosis by functional studies previously. First, miR-181b-5p, which was the most prominently up-regulated miRNA in our study, was suggested to inhibit apoptosis through MEK/ERK/p21 pathway [40]. Besides, decreased expression of miR-17-5p and miR-25 were shown to induce apoptosis by upregulation of target genes including phosphatase and tensin homolog and high mobility group box-1, respectively [41, 42]. Our results also showed decreased serum expressions of miR-17-5p and miR-25-3p, suggesting these miRNAs may be involved in FMF pathogenesis via apoptotic pathways.

In contrary, overexpression of miR-16-5p and miR-195-5p were proposed to activate caspases 3 and 9 and lead to apoptosis in other studies [43, 44]. However, we found significant downregulations of miR-16-5p and miR-195-5p, and upregulation of miR-181b-5p which conflicts with the hypothesis of excessive apoptosis in FMF pathogenesis. Nonetheless, we still speculate that these apoptosis-related miRNAs could have a complex interaction in regulating apoptosis by multiple target genes, be affected by colchicine treatment, and individually activate or control inflammation in FMF patients.

The major limitations of our study were the lack of functional annotation of the expressed miRNAs and the evidence showing the neutrophil apoptosis. Additionally, all patients included in the study were under colchicine treatment, and we cannot be sure that miRNA expression was affected by colchicine treatment or not. Moreover, the etiology could be other autoinflammatory diseases in patients who had insignificant *MEFV* results. Although we performed further genetic analysis, there is still a possibility that the phenotype was caused by mutations in other genes or by novel genes. Thus, further studies are needed to clarify these limitations on this topic in the future.

To our knowledge, this is the first study investigating apoptosis-related miRNAs, widely investigated in the pathogenesis of several cancers before. More intriguingly, we found significant miRNA downregulations and upregulations in FMF patients, regardless of genotype, colchicine response, and having an inflammatory attack during miRNA analysis. We think that the inclusion of genetically heterogeneous FMF patients also makes our study more interesting, since most of the previous studies investigated either M694V homozygote or heterozygote patients. Because, the emerging concerns about FMF pathogenesis, whether epigenetic or environmental factors have an additional effect or not, especially have raised from the presence of a substantial proportion of FMF patients with heterozygote *MEFV* mutations, exon 2 mutations, or no mutations.

In conclusion, our results revealed significant deregulations of 26 apoptosis-related miRNAs regardless of genotype; therefore, we speculate that apoptosis-related miRNAs might be involved in FMF pathogenesis, by affecting apoptotic pathways. After all, we also highlight that there is a thriving need for more work to clarify epigenetics in FMF.

Table 4 Summary of the previous studies investigating miRNAs in patients with familial Mediterranean fever

Previous studies	Latsoidis et al. [15]	Koga et al. [16]	Wada et al. [17]	Akkaya-Ulum et al. [18]	Hortu et al. [19]	Amarilyo et al. [20]	Demir et al. [21]	Our study
miRNA source	Myelomonocytic cells (culture)	Serum	Plasma or serum	Plasma or serum	Plasma or serum	PBMCs	Plasma	Serum
Study Group	9 FMF (A) 8 HC	9 FMF (A) 12 RA 19 HC	24 FMF (A + P) 8 PFAPA	6 FMF (A + P) 6 FMF carriers 6 HC	51 FMF (P) 49 HC	10 FMF (A) 10 HC	30 FMF (A) 30 HC	191 FMF (P) 31 HC
Colchicine status	All patients were 2 days off-medication	nd	nd	All but two patients under C	20 patients were off-medication	All patients	All patients	All patients
MEFV genotype	Heterogeneous M694V hom ($n=1$)	Heterogeneous M694I hom ($n=3$)	Heterogeneous Exon 10 mut ($n=8$) Exon 3 mut ($n=8$) Others ($n=8$)	Homogeneous M694V hom ($n=6$) M694V het ($n=6$)	Heterogeneous M694V hom ($n=16$)	Homogeneous M694V hom ($n=10$)	Heterogeneous M694V hom ($n=7$)	Heterogeneous M694V hom ($n=33$)
Most significant regulation changes in FMF group	miR-4520 (Up)	miR-204-3p (Up)	miR-320 (Up)	miR-20a-5p (Up) and miR-197-3p (Down) in homozygote FMF patients	miR-125a, miR-132, miR-146a, miR-155, miR-15a, miR-16, miR-181, miR-21, miR-223, miR-26a and miR-34a (Down)	miR-144-3p, miR-21-5p, miR-4454, and miR-451a (Up)	miRNA-16 and miRNA-451 (Up)	miR-181b-5p (Up)
				let-7d-3p (Up) and miR-197-3p (Down) in homozygote FMF patients	miR-132, miR-15a, miR-181a, miR-23b, miR-26a and miR-146a, miR-15a, miR-16, miR-26a, miR-34a (Up) in patients receiving colchicine	miR-107, let-7d-5p, and miR-148b-3p (Down)	miRNA-155, miRNA-204 (Down)	miR-16-5p, miR-17-5p, miR-25-3p and miR-195-5p (Down)

A adult, *P* pediatric, up up-regulated, *Down* down-regulated, *het* heterozygous, *hom* homozygous, *mut* mutation, *FMF* familial Mediterranean fever, *HC* healthy controls, *MEFV* Mediterranean fever, *PBMCs* peripheral blood mononuclear cell, *PFAPA* periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis, *RA* rheumatoid arthritis

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Author contributions Dr. Karpuzoglu and Dr. Yilmaz conceptualized and designed the study, drafted the initial manuscript, and reviewed and revised the manuscript. Dr. Kisla Ekinci, Dr. Bisgin, and Dr. Balci collected data carried out the initial analyses, and critically reviewed and revised the manuscript. All authors approved the final manuscript as submitted and agreed to be accountable for all aspects of the work. All co-authors take full responsibility for the integrity of the study.

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Compliance with ethical standards

Conflict of interest Authors declare that they have no conflicts of interest. All co-authors take full responsibility for the integrity of the study.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from parents of the participants included in the study.

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