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Differential Expression of micro RNAs and their Association with the Inflammatory Markers in Familial Mediterranean Fever Patients

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Familial Mediterranean fever (FMF) is an autoinflammatory genetic disease resulted from the mutation of pyrin, which contributes to the formation of inflammasome complex. Therefore, activation of cytokines is one of the hallmarks of FMF pathogenesis. This study aimed to investigate the role of miRNAs as regulatory biomarkers for inflammation in patients with FMF. 50 FMF patients and 25 healthy subjects were included in this study. Q RT-PCR was used to determine plasma expressions of miR-181a and miR-125a, while IFN-γ and IL-17 were estimated using ELISA technique. Our results indicated that, the expression of miR-181a was significantly decreased ($p = 0.006$) while miR-125a expression was insignificantly reduced ($p = 0.101$) also IL-17 levels were significantly higher ($p = 0.003$) and plasma IFN-γ levels were insignificantly increased ($p = 0.322$) in FMF patients than control group. Correlation analysis revealed a positive correlation between miR-181a expression and lymphocyte percentages ($p = 0.048$), while a significant negative association was observed between miR-125a and C-reactive protein (CRP) ($p = 0.005$) in FMF patients. However, there were no associations between miR-125a and miR-181a with IFN-γ and IL-17 in FMF patients. miR-181a and miR-125a could be used as regulatory biomarkers for inflammation in FMF patients.

Keywords: microRNAs, Inflammation, FMF, IFN-γ, IL-17.

Familial Mediterranean Fever (FMF) is an autoinflammatory disease commonly found among Eastern Mediterranean population. FMF occurred due to mutations in the MEFV (MEditerranean FeVer) gene[1, 2]. Incorrect coding resulted from MEFV mutations disturbs function of pyrin protein, and leads to uncontrolled inflammation. Several studies examining genotype-phenotype correlation in FMF patients with different clinical findings and therapeutic approaches showed that FMF is the paradigm of all the monogenic autoinflammatory disease. Pyrin is implicated in the formation of inflammasome complex. Pyrin impairment leads to autoinflammatory disease, resulting in aberrant production of interleukin (IL)-1β and IL-18. Consequently, cytokine activation is involved in the pathogenesis of FMF[3]. Neutrophils play a major role in the inflammatory processes during the

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attacks of FMF. There are data showing persistent inflammation in attack-free MF patients as indicated by elevated levels of certain proinflammatory cytokines.[4-6]. Interleukin (IL)-17 can modulate certain neutrophil functions by stimulating their maturation and migration. Elevated IL-17 leads to massive peripheral neutrophilia associated with increased levels of granulocyte colony stimulating factor (G-CSF) and enhanced granulopoiesis[7]. It can recruit neutrophils into the peritoneal cavity by neutrophil-specific chemokines released from the peritoneal mesothelium[8]. Amplification of persistent inflammatory responses may be the primary function of IL-17, as it can activate many cell types as well as stimulate the secretion of several inflammatory cytokines including TNF-α, IL-6, IL-8, IFN-γ, and chemokines[9,10]. Treatment with colchicine has been found to reduce these cytokines. Epigenetic pathways may be responsible for the variability of the clinical presentation of FMF such as miRNAs which could be a part of these pathways[11].

MicroRNAs (miRNAs) are small evolutionarily preserved non-coding RNA molecules (16-24 nucleotides) that disturb expression of their target mRNAs and have a role in biological processes as in cell growth, differentiation, and death. Diverse subsets of CD4+ T cell like Th1, Th2, Th17, and T regulatory cells, have several functions in immune activation and tolerance. They are demonstrated to respond to dynamic micro-environmental indices and be involved in regulation of T cell development, survival and functions. Thus, miRNAs are implicated in the immune physiological condition, on the one aspect, and have a role in controlling the immune tolerance, on the opposite. The cytokines are among the main proteins that miRNAs target; these cytokines serve as vital upstream signals and primary functional outputs[12].

It was suggested that, miRNAs can be used as a biomarker in various diseases. Plasma expression of miRNAs varies in many autoimmune and auto-inflammatory conditions. Consequently, miRNAs could have a regulatory function in the development and activation of inflammation and could be useful for diagnosing and monitoring the inflammation-related disorders[3]. Furthermore, it has also been found that miRNAs are involved in the development of several immune cells, including development and proliferation of T and B lymphocytes, neutrophils, and regulate the release and activation of inflammatory mediators[11].

MiRNA-181 (miR-181) is preferentially expressed in many organs, especially in the bone marrow and spleen in significant levels. Due to its up-regulation in the spleen, miR-181 has been considered for its potential requirement and role in T-cell development and survival[13]. Recent evidences have reported the implication of miR-181 members in the differentiation and functions of immune cells, such as differentiation and activation of B and T lymphocytes[14]. Additionally, gene ontology investigation of predicted targets of miR-181a and -b has detected an over-representation of immune pathways involving signaling of T-cell receptor and transforming growth factor beta (TGF)-β[15].

MiRNA-125a (miR-125a) regulates expressions of several cytokines implicated in naive CD4 + T-cell differentiation in humans. The chronic expression of miR-125a decreased levels of cytokines like IL-10 receptor α, IL-2 receptor β, and IFN-γ. Introducing miR-125 into naive CD4+ T-cells resulted in reducing expressions of molecules presented on the surface of Th1 and Th2 memory cells and led to elevation of naive CD45RA+, CD45RO-, elucidating miR-125 function in the preservation of tolerance and keeping naive T-cell status. In addition, miR-125 may reduce the effector function and activation of T-cells, which is shown by decreased levels of intracellular IFN-γ and IL-13. MiR-125 down-regulation is accompanied by effector memory CD4+ T-cell phenotype[14].

In the current study, we object to investigate the potential involvement of microRNAs in the regulation of inflammation in FMF patients. Also, to examine the expression patterns of miR-125a and miR-181a and plasma levels of the inflammatory cytokines (IFN-γ and IL-17) in FMF patients compared to healthy controls. In addition, we evaluate the correlation between these miRNAs and the clinical and laboratory manifestations of FMF patients, and then estimate their association with IFN-γ and IL-17 expression in FMF patients.

Patients and Methods

Ethics

Ethics Committee of the National

Research Centre (NRC), Giza, Egypt, approved this study and written informed consents were obtained from the parent/guardian of all children at enrolment and before any study procedure.

Study Subjects

In this case–control study, 75 subjects were included, with age ranging from 3 to 16 years. There were 50 patients with FMF and 25 apparently normal controls.

Patients were recruited from the Clinical Genetics Department, Medical Research Center of Excellence, National Research Centre, Giza, Egypt. Patients with FMF were diagnosed according to the Tel Hashomer Diagnostic Criteria[16], and were on colchicine treatment at the time of the study. Clinical Characteristics and treatments of FMF patients are shown in table 1.

RNA extraction and Reverse Transcription

MiRNAs were isolated and extracted from plasma of all subjects of the study groups using miRNeasy Mini kit (Qiagen, Germany) and by following the manufacturer's instructions. cDNA was synthesized using TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems) and using specific primers by following the manufacturer's instructions. Reverse transcription was performed under the following thermal conditions: starting at 16 °C for 30 min followed by 42 °C for 30 min and finally at 85 °C for 5 min and the resulting cDNA was kept at "80 °C until use.

Real time PCR quantification

A real-time quantitative PCR (qRT-PCR) was done using TaqMan® MicroRNA Assay kit and TaqMan® Universal Master Mix (Applied Biosystems) to quantify the expression levels in triplicate of mature miR-181a and miR-125a using 7500 fast real-time PCR system by following the manufacturer's instructions. RNU48 was used as a reference gene (housekeeping gene). A single plex reaction was used in this study. The expression levels of target miRs were normalized to RNU48 and relative quantification (Rq) of miRNA expression was calculated using RQ formula ($2^{-\Delta\Delta CT}$). ΔCt was determined by subtracting the Ct values for RUN48 from the Ct values for the target miR. Q RT-PCR was carried out with cycling conditions of: 50°C for 2 min, 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and1 min at 60°C[17].

Determination of IFN-γ and Interleukin 17

Plasma IFN-γ and IL-17 levels of study patients and healthy subjects were assessed in duplicate by the "commercially-available solid-phase sandwich ELISA kit" (Elabscience, Elabscience Biotechnology Co., Ltd) following the protocol provided by the manufacturer.

Data analysis and statistics

Data were collected, revised, verified then analyzed using SPSS version 19.0 software (SPSS Inc., Chicago, Illinois, USA). Comparison between expression levels of miRs and cytokines was performed using non-parametric Mann-Whitney U test. Spearman rank correlation to test the association of miR expression levels with laboratory data and inflammatory cytokines of patients. *P* value of <0.05 was reflected in statistical significance. Quantitative data were described using mean and SD or median and range.

RESULTS

Demographic and clinical data

Demographic and clinical characteristics as well as laboratory findings of 50 patients with FMF are presented in Table (1).

Evaluation of plasma miR-181a and miR-125a expression

Our findings delineated that, miR-181a expression was significantly down-regulated in FMF patients in comparison with healthy controls. In FMF patients, miR-181a expression level was 5.85-fold lower than healthy controls (Table 2).

In addition, our results demonstrated the under-expression of miR-125a in patients with FMF in comparison with control group. 2.27-fold down-regulation of miR-125a expression was detected in FMF patients in comparison with control group (Table 2).

Plasma levels of inflammatory cytokines

Our results showed that, the levels of IFN-γ were higher in FMF patients (Median, Range: 108, 33-263) than that of the control group (Median, Range: 81, 35-191) (*P*=0.322). Moreover, IL-17 expression was significantly elevated in FMF patients (Median, Range: 11, 3.77-92.74) compared with healthy controls (Median, Range: 2.9, 0.34-8.41) (*P*=0.003) (Figure 1).

The correlation of the plasma miR-181a and miR-125a expression with the laboratory parameters and cytokines levels in patients group

Correlation analysis showed that, miR-181a expression have a significant positive correlation with lymphocyte percentages in FMF patients while no correlation was detected with the other laboratory data (white blood cells, neutrophils, CRP and ESR) of FMF patients. Furthermore, our data indicated that miR-125a expression have a significant negative correlation with CRP while no correlations were found between miR-125a and the other laboratory data of FMF patients (Table 3).

In addition, our data indicated that there are no associations between miR-181a and miR-125a with the inflammatory cytokines (IFN-γ and IL-17) in FMF patients (Table 3).

DISCUSSION

FMF is an inherited autoinflammatory disease caused by the pyrin mutation; this mutation participates in inflammasome complex formation.

Table 1. Clinical and laboratory findings among FMF group

Characteristic		FMF patients (n=50)
Gender, male/female %		52/48%
Age, median (years) (Range)		9.5 (3-16)
Consanguinity %		42%
Family history %		66%
Inflammatory attack, positive/negative %		58/42%
Colchicine Responders/ Non-Responders %		78/22%
Medications (colchicine*) %		100%
MEFV genotype, % of patients		
Homozygous	M680I	14%
	M694I	8%
	M694I	46%
Heterozygous	V726A	10%
	E148Q	18%
	R761H	4%
Laboratory findings		
Hemoglobin(g/dl), Mean ± SD (Range)		13 ± 1(11.3-15.7)
Platelets ($\times 10^3/mm^3$), Mean ± SD (Range)		280±97 (50-450)
WBCs ($\times 10^3/mm^3$), Median (Range)		6.8 (4.7-22.9)
Lymphocytes%, Mean ± SD (Range)		47.5±10.3 (29-64)
Neutrophils%, Mean ± SD (Range)		43±10 (28-63)
CRP (mg/L), Median (Range), (normal ≤ 5)		16 (1.4-45)
ESR (mm/hr), Median (Range)		24 (5-56)

* The dose of colchicine ranged from 0.5-1.5 mg/day

Table 2. Expression of miR-181a and miR-125a in FMF patients

	N	Rq Median (Min-Max)	*P* value
miR-181a	50	0.171 (0.01-1.2)	0.006*
miR-125a	50	0.44 (0.04-2.08)	0.101

Rq: The Relative Quantification

* Significant at $P< 0.01$ compared with controls using Mann-Whitney test.

Consequently, activated cytokines contribute to the pathogenesis and activation of FMF[18].

MiRNAs are small endogenous RNAs which regulate gene expression post-transcriptionally by binding to their targets. Recent studies have shown aberrant miRNA expression in various diseases such as the autoinflammatory diseases which indicated their potential effects in the pathogenesis of these diseases[3, 19]. In current study, we aimed to assess the plasma expression of some candidate miRNAs associated with autoimmune pathogenesis and inflammation.

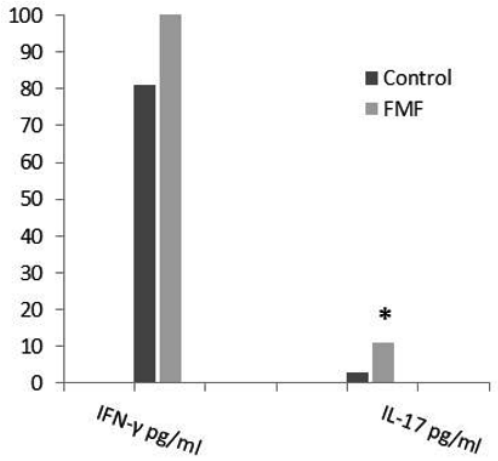

Fig. 1. Plasma levels of IFN-γ and IL-17 in FMF patients and healthy subjects. Bars show the results as the median.
* Significant at P< 0.05 * compared with controls using Mann-Whitney test.

Our findings showed a significant down-regulation in the expression of miR-181a (-5.85 folds) of FMF patients compared with healthy subjects. Similar findings have been reported by a study of Hortu *et al.* (2019), in which there is a reduction in expression of miR-181a in FMF cases relative to healthy controls while it was observed to have elevated when compared with patients not receiving colchicine therapy[11].

On the other hand, Karpuzoglu *et al*. (2020) revealed that miRNAs expression levels were altered in the serum of patients with FMF when compared with the control group. In detail, miR-181a, miR-181b, miR-181c, and miR-365a were deregulated. These miRNAs were suggested to target different genes, and upregulation of these non-coding miRNAs was associated with oncogenesis and autoinflammatory diseases, including FMF[20]. Also, Hortu *et al*. (2019) worked on miRNAs more comprehensively in 51 pediatric FMF patients. They demonstrated that only 15 miRNAs including miR-181a and miR-125a had aberrant expressions[11].

Moreover, a study by Lashine *et al*. (2011) showed miR-181a under-expression in juvenile SLE patients. However, they described no difference between the healthy controls and FMF patients in that study[21].

In addition, our results demonstrated the under-expression of miR-125a (-2.27-fold) in FMF patients compared to the control group. This finding is consistent with the demonstration of Hortu *et al*. (2019) that the expression pattern of 11 miRNAs, including miR-125a and miR-181a, in the pediatric

Table 3. Correlation of miR-181a and miR-125a expression levels with the laboratory parameters and cytokines in FMF group

	miR-181a expression		miR-125a expression	
	R	*P* value	R	*P* value
White blood cells (WBCs)	0.486	0.154	-0.394	0.26
Lymphocytes	0.636	0.048*	0.419	0.228
Neutrophils	-0.433	0.211	-0.262	0.464
CRP	-0.872	0.054	-0.975	0.005*
ESR	-0.564	0.322	-0.667	0.219
IFN-γ	0.261	0.467	0.212	0.556
IL-17	0.552	0.098	0.382	0.276

R: Spearman's correlation coefficien
*: significant correlation at the 0.05 level.

FMF patients were markedly lower than those of the healthy control[11]. Others demonstrated that miR-125a expression down-regulated in FMF patients relative to the healthy control group[22].

It has been found that both miR-181a and miR-181c play important roles in premature stages of T cell development. miR-181a, which has a function in differentiation and activation of T-cells, is present in enough amounts during maturation of T-cells before CD4+ and CD8+ stages and is diminished in the later stages. TCR signaling after transcription is regulated by miR-181a, in which process up-regulation of miR-181a leads to elevation of TCR signaling in T-cells and vice versa[14]. That matches our study in which a significant positive association between miR-181a expression level and lymphocyte percentages in FMF group. Similar results have been reported by Li et al. (2007) and Schaffert *et al*. (2015) who have shown that high levels of miR-181a and -b in various stages of T cell development causes induction of positive and negative choice via promoting TCR sensitivity and signaling strength in human and mice, respectively[23, 24].

Several studies[25, 26] demonstrated that miR-125a has a marked role as an anti-inflammatory agent in controlling the autoimmune diseases; this is in agreement with our results which indicated a significant negative association between miR-125a expression level and CRP of FMF group. This is in accordance with Murata et al[25] who found that miR-125a was negatively correlated with some indices of disease activity including CRP in Rheumatoid arthritis. Also, Sun et al[26] reported that miR-125a was negatively associated with CRP, ESR, IL-17, and TNF-γ in Crohn's disease patients.

In this study, we found that IL-17 expression was significantly up-regulated in FMF patients in comparison with healthy controls. In an earlier study performed by Koga et al. (2016), IL-17 and IL-18 levels in the serum of FMF patients were markedly increases in comparison with those of healthy controls while they were comparable in FMF patients in attack and remission[18]. Moreover, FMF patients have been reported by Koga *et al*. (2018) to show an elevated level of serum inflammatory cytokines like IL-1β, IL-6, IL-17, and IL-18. They recently revealed the specific cytokine network amongst FMF patients through the use of a multi-suspension cytokine array[19]. A previous study reported that the serum concentration of IL-17 is significantly increased during FMF attacks[27].

In this study, we found that the IFN-γ serum levels were higher in FMF patients than that of the control group. That is in agreement with Köklü *et al*. (2005) who showed that median IFN-γ plasma levels in FMF patients both with and without attack were significantly higher than the healthy controls ($P < 0.05$). In addition, higher IFN-γ plasma levels were observed in patients with acute FMF attacks in comparison with patients in attack-free periods ($P < 0.05$). IFN-γ plasma levels were comparable in colchicine treated and untreated patients[28].

Furthermore, our data indicated no associations between miR-125a or miR-181a with the inflammatory cytokines (IFN-γ and IL-17) in FMF patients. But it was found several other miRNAs that could be involved in the regulation of IL-23/IL-17 axis by indirect mechanisms in autoimmune diseases[29]. Other studies demonstrated that miR-125a and miR-181a were negatively correlated with IL-17 levels in patients with the active inflammatory disease[26, 30, 31]. While it has been found that miR-181a expression is inversely associated with the severity of inflammation, it is still unclear which pathway this expression gets along[11].

In conclusion, miR-181a and miR-125a could be used as regulatory biomarkers for inflammation in FMF patients. Further functional researches may be helpful to elucidate and clarify the role of miRNAs, especially in the regulation of inflammation in FMF. MiRNAs might have a promising therapeutic role in auto-inflammatory diseases as FMF.

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Conflict of interest

The authors declare that they have no conflict of interest.

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