Evaluation of the *MEFV* gene expression in Egyptian patients with familial Mediterranean fever

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Background
Familial Mediterranean fever (FMF) is the most common autosomal recessive autoinflammatory disease worldwide, mainly affecting patients of Mediterranean origin. It is the first hereditary periodic fever syndrome whose *MEFV* gene has been identified. Correlation between the mRNA level of the *MEFV* gene and FMF pathogenesis is elusive.

Aim
This study aims to investigate the relationship between common mutations in *MEFV* gene and the mRNA expression in Egyptian patients with FMF, as well as the phenotype/genotype correlation.

Patients and methods
This study included 65 patients with FMF, who were chosen based on clinical criteria as well as 26 healthy controls. Mutation detection was performed by direct Sanger sequencing and restriction fragment length polymorphism. Quantitative real-time PCR was employed to assess the relative expression level of the *MEFV* gene.

Results
The data revealed significantly lower *MEFV* gene expression in patients compared with healthy controls (P = 0.001). *MEFV* mRNA expression level was lower in patients with identified mutations compared with those with undetected mutations. However, the difference was statistically insignificant. Additionally, we found that the *MEFV* gene expression level was not associated with age, consanguinity, disease severity, or serum amyloid A levels.

Conclusion
The study confirmed the relation between reduced *MEFV* expression level and the FMF disease, which emphasizes the role of gene expression in the pathogenesis of this disease.

Keywords:
autoinflammatory disease, familial Mediterranean fever, gene expression, *MEFV* gene, mutations

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Background

Familial Mediterranean fever (FMF) (OMIM: 249100) is the most common autosomal recessive monogenic autoinflammatory disease. FMF commonly occurs among people of the Mediterranean Basin, with the highest prevalence reported among Sephardic Jews, Armenians, Turks, and Arabs (Yıldız *et al.*, 2020). FMF is characterized by recurrent serositis (e.g. peritonitis, pleuritis, synovitis) as well as recurrent fever. Individual and ethnic differences may be observed in both the course and frequency of the clinical symptoms (Sarı *et al.*, 2014). Symptoms mostly resolve within 3–4 days, with the intervals between the attacks being relatively symptom free. However, myalgia and arthritis may have a prolonged course (Onen, 2006). Amyloidosis, the most significant complication of FMF, is responsible for long-term morbidity and mortality related to FMF (Özen *et al.*, 2017).

MEFV is the causative gene of FMF disease (Consortium IFMF, 1997). *MEFV* gene is composed of 10 exons and is located on chromosome 16. The *MEFV* gene codes for pyrin or marenostrin protein (781 amino acids) (Ben-Chetrit and Touitou, 2009) which has a direct and/or indirect significant role in inflammasome regulation. Any qualitative or quantitative alteration of pyrin expression may have a vital effect on the balance between proinfammatory and antiinflammatory signaling pathways that may later result in uncontrolled inflammation (Grandemange *et al.*, 2011). The inflammasome complex and the inflammatory pathway have been reported to be involved in the pathogenesis of FMF. Gene-expression profiling can reveal transcriptome profiles, elucidate disease phenotypes, and bring to light new diagnostic and predictive biomarkers

(Schnappauf *et al.*, 2019). The mRNA expression level of the *MEFV* gene was found to be altered in patients carrying *MEFV* mutations independent of the mutation type, being more affected during the attacks (Notarnicola *et al.*, 2002; Üstek *et al.*, 2007; Kirectepe *et al.*, 2011), although some studies found no significant difference (Booty *et al.*, 2009). Therefore, this study aims to investigate the relationship between *MEFV* gene mutations and the mRNA abundance in Egyptian FMF patients and their phenotype/genotype correlation.

Patients and methods

A total of 65 patients with FMF disease were recruited for this study with the vast majority came from Delta region in Egypt. In adult patients (n = 8, mean = 33.7 years), FMF disease was diagnosed based on Tel Hashomer clinical criteria (Samli *et al.*, 2006). In children patients (n = 57, mean = 8.7 years), FMF disease was diagnosed according to Yalçınkaya *et al.* (2009). Moreover, 26 healthy controls with matched age and sex were included. The International Severity Score for FMF was used in both children and adults (Demirkaya *et al.*, 2016). Patients and controls were recruited from the Clinical Genetics Department, National Research Centre. Laboratory tests for complete blood count, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), kidney functions, liver functions, serum amyloid A (SAA), and urine analysis were also executed. *Helicobacter pylori* infection was assessed by the stool antigen test. Abdominal and pelvic ultrasonography was performed for all cases. Regular colchicine therapy was started to all the patients once the diagnosis was confirmed. Ethical approval was obtained from the Ethics Committee of the National Research Centre. Written informed consent was obtained from all patients or their guardians and were included in the study.

Mutation analysis

The DNA was extracted from 2 ml of peripheral blood into EDTA-anticoagulated tubes, using the QIAamp DNA Blood Isolation Kit (Qiagen, Hilden, Germany)following the manufacturer's protocol. The DNA concentration was determined using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, USA). Two DNA fragments of the *MEFV* gene (exons 2 and 10) were amplified by PCR using the following primers: exon 2 forward: 5′ GTGGGACAGCTTCATCATTTTG 3′ , exon 2 reverse: 5′ CCTTCTCTCTGCGTTTGCTC 3′ , exon 10 forward: 5′ TTACTGGGAGGTGGAG GTTG 3′ and exon 10 reverse: 5′ GAGGAGCTGTGT TCTTCCCTC3′ (Simsek *et al.*, 2011). PCR amplification was performed under the following conditions: one cycle of 5 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 55–58°C, and 45 s at 72°C; and one cycle of 5 min at 72°C, followed by a 4°C hold. Amplification was carried out in a thermal cycler (Biometra, Göttingen, Germany). Only exon 10 of the *MEFV* gene was screened by direct Sanger sequencing of the PCR-amplified fragments, and sequences were analyzed by Sequence Scape 2.5 software (Applied Biosystems, Foster City, California, USA), whereas exon 2 was amplified for restriction fragment length polymorphism analysis by using Ava1 restriction enzyme (Fermentas, Lithuania, Waltham, Massachusetts, USA).

Gene expression

The total RNA was extracted from whole leukocytes using the Pure Link RNA Mini Kit (Ambion, Foster City, California, USA) according to manufacturer's instructions. cDNA synthesis was performed using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Wilmington, USA) according to manufacturer's instructions. To determine the *MEFV* mRNA expression level for patients with FMF (n = 65) and the healthy control group (n = 26), qPCR was performed using thermal cycler 480II (Roche Diagnostics, Mannheim, Germany). *MEFV* and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) amplifications were achieved independently. For the amplification of the *MEFV* gene, the following designed primer was used: *MEFV* forward: 5′ TGGAAACAAGTGGGAGAGGC 3′, *MEFV* reverse: 5′ CTCCGTGGGCACAGTAACTA 3′. According to Etem *et al.* (2015), the following primers were used for the *GAPDH* gene; forward: 5′ AGGTCATCCCTGAGCTGAACG 3′ and *GAPDH* reverse: 5′ GGTGTCGCTGTTGAAGTCAGA 3′, so two reactions were performed for each patient. Each reaction contained 10 µl of Maxima SYBR-Green, 1 µl of forward primer for either the *GAPDH* or *MEFV* gene, 1 µl of reverse primer for either the *GAPDH* or *MEFV* gene, 1 µl of cDNA (3000 ng), and 7 µl water nuclease-free. The reactions were performed using the following thermal cycles: 10 min at 95°C, 40 cycles of 15 s at 95°C, and 30 s at 55°C. All measurements were performed in triplicate. Data were collected and analyzed with the software accompanying the thermal cycler 480II equipment using a $2^{-\Delta Ct}$ method for quantification of the relative mRNA expression levels (Schmittgen and Livak, 2008).

Statistical analysis

The Statistical Package for Social Science (IBM SPSS, Chicago, USA), version 20 was used for

statistical analysis. The normality assumption was tested with the Shapiro–Wilk test. Mann–Whitney *U* test was used to compare gene expression. The confidence interval was set to 95%. The accepted margin of error was set to 5%. A two-sided *P* value less than 0.05 was considered a statistically significant result.

Results

Demographic, clinical, and laboratory data of the studied patients are presented in Table 1. Regarding genotype distribution, we found that the heterozygous M694I variant was the most common (30.8%), whereas the homozygous M680I, homozygous V726A, and heterozygous R761H mutations were the least common (1.5% each). The genotype–phenotype correlation demonstrated an association between M694I variants and disease severity, although it was insignificant (P = 0.072). Nevertheless, the proportion of mutant variants was greater in patients who had moderate or severe symptoms. The presence of mutations in *MEFV* per se was found to be positively correlated with disease severity (P = 0.009) (Table 2). Regarding allele frequencies, there was a significant correlation between the M694I mutant allele and different severity grades; the mutant allele was more common in patients who had moderate or severe symptoms (P = 0.028) (Table 3).

Table 1 Demographic, clinical, and laboratory data of the studied patients

	Overall (*n*=65)
Current age (years)	
Median (Q1, Q3)	9.7 (6.3, 13.2)
Age of onset (years)	
Median (Q1, Q3)	5.0 (2.6, 8.0)
Sex [*n* (%)]	
Males	35 (53.8)
Females	30 (46.2)
Positive consanguinity	28 (43.1)
Positive family history	33 (50.8)
Severity [*n* (%)]	
Mild	13 (20)
Moderate	31 (47.7)
Severe	21 (32.3)
Clinical presentation [*n* (%)]	
Abdominal pain	65 (100)
Fever	62 (95.4)
Arthritis	51 (78.5)
Red rash	15 (23.1)
Laboratory abnormalities	
Anemia	59 (90.8)
Elevated ESR	55 (84.6)
Elevated CRP	50 (76.9)
Increased serum amyloid A	14 (21.5)
Helicobacter pylori positive	3 (4.6)
Abdominal and pelvic ultrasonography [*n* (%)]	
Hepatomegaly	10 (15.4)
Splenomegaly	6 (9.2)
Hepatosplenomegaly	4 (6.2)

CRP, C-reactive protein; ESR, erythrocyte sedimentation rate.

The data also showed that *MEFV* gene expression was significantly lower in patients compared with controls (P = 0.001) (Table 4). *MEFV* mRNA expression level was lower in patients with identified mutations compared with those with undetected mutations. However, the difference was statistically insignificant (P = 0.165) (Table 5). *MEFV* gene expression was independent of age, consanguinity, disease severity, or SAA levels.

Discussion

FMF is an autoinflammatory autosomal recessive disease abundant among individuals of Mediterranean descent. In Arab countries, FMF is considered a public health concern owing to high consanguinity in this population (El-Shanti *et al.*, 2006). Positive consanguinity was present in 43.1% of the current studied cases. Similarly, Inal *et al.* (2009) reported 40% of patients with positive consanguinity, whereas Dundar *et al.* (2012) noted a lower percentage (16%). The ethnic traditions and the familial susceptibility of FMF in different populations may be the cause of these differences.

In this study, abdominal pain was the most frequently reported complaint, followed by fever, arthritis, and red rash, with frequencies of 100, 95.4, 78.5, and 23.1%, respectively. Previous studies reported similar results but with less frequency than we found in our study (Cekin *et al.*, 2017; Mansour *et al.*, 2019). Meanwhile, in a varied population of Arabs, Sephardic Jews, French, Turks, Armenians, and others, the main clinical manifestations reported by patients were fever, abdominal signs, thoracic signs, joint signs, erysipelas-like erythema, splenomegaly, and amyloidosis (Grateau *et al.*, 2000). Abdominal ultrasound in this study revealed hepatomegaly in 15.4% of cases, splenomegaly in 9.2%, and hepatosplenomegaly in 6.2%. Nevertheless, different abdominal ultrasound findings were published in the study of Ishak *et al.* (2006). Variation in the predominant gene mutation in different populations may lead to altered clinical symptoms of patients with FMF (Özen *et al.*, 2017).

The degree of anemia was assessed based on WHO-recommended hemoglobin cutoff points for children aged 0.5–4.9 years (<110 g/l), 5–11 years (<115 g/l), and 12–15 years (<120 g/l), and for women (<120 g/l) and men (<130 g/l) (WHO, 1968). In our study, laboratory investigations revealed that 90.8% of cases were anemic. In addition, inflammatory

Table 2 Comparison between disease severity grades and mutations in the studied patients

	Severity [*n* (%)]			Total (*n*=65)	*P**
	Mild (*n*=13)	Moderate (*n*=31)	Severe (*n*=21)		
rs28940580 (M680I)					0.437
GG (wild-type)	12 (92.3)	24 (77.4)	18 (85.7)	54 (83.1)	
GA	1 (7.7)	7 (22.6)	2 (9.5)	10 (15.4)	
AA	0	0	1 (4.8)	1 (1.5)	
rs28940578 (M694I)					0.072
GG (wild-type)	12 (92.3)	21 (67.7)	10 (47.6)	43 (66.2)	
GA	1 (7.7)	9 (29.0)	10 (47.6)	20 (30.8)	
AA	0	1 (3.2)	1 (4.8)	2 (3.1)	
rs61752717 (M694V)					0.687
AA (wild-type)	13 (100.0)	29 (93.5)	21 (100.0)	63 (96.9)	
AG	0	2 (6.5)	0	2 (3.1)	
rs28940579 (V726A)					1.000
TT (wild-type)	12 (92.3)	28 (90.3)	20 (95.2)	60 (92.3)	
TC	1 (7.7)	2 (6.5)	1 (4.8)	4 (6.2)	
CC	0	1 (3.2)	0	1 (1.5)	
rs3743930 (E148Q)					0.790
GG (wild-type)	11 (84.6)	28 (90.3)	18 (85.7)	57 (87.7)	
GC	2 (15.4)	3 (9.7)	3 (14.3)	8 (12.3)	
rs61732874 (A744S)					0.583
GG (wild-type)	13 (100.0)	30 (96.8)	19 (90.5)	62 (95.4)	
GT	0	1 (3.2)	2 (9.5)	3 (4.6)	
rs104895097 (R761H)					1.000
GG (wild-type)	13 (100.0)	30 (96.8)	21 (100.0)	64 (98.5)	
GA	0	1 (3.2)	0	1 (1.5)	
A744S/V726A					0.853
GG/CC	0	1 (3.2)	0	1 (1.5)	
GG/TC	1 (7.7)	2 (6.5)	0	3 (4.6)	
GG/TT	12 (92.3)	27 (87.1)	19 (90.5)	58 (89.2)	
GT/TC	0	0	1 (4.8)	1 (1.5)	
GT/TT	0	1 (3.2)	1 (4.8)	2 (3.1)	
R761H/V726A					0.952
GA/TC	0	1 (3.2)	0	1 (1.5)	
GG/CC	0	1 (3.2)	0	1 (1.5)	
GG/TC	1 (7.7)	1 (3.2)	1 (4.8)	3 (4.6)	
GG/TT	12 (92.3)	28 (90.3)	20 (95.2)	60 (92.3)	
M680I/E148Q					0.775
AA/GG	0	0	1 (4.8)	1 (1.5)	
GA/GC	0	1 (3.2)	0	1 (1.5)	
GA/GG	1 (7.7)	6 (19.4)	2 (9.5)	9 (13.8)	
GG/GC	2 (15.4)	2 (6.5)	3 (14.3)	7 (10.8)	
GG/GG	10 (76.9)	22 (71.0)	15 (71.4)	47 (72.3)	
A744S/E148Q					0.880
GG/GC	2 (15.4)	3 (9.7)	2 (9.5)	7 (10.8)	
GG/GG	11 (84.6)	27 (87.1)	17 (81.0)	55 (84.6)	
GT/GC	0	0	1 (4.8)	1 (1.5)	
GT/GG	0	1 (3.2)	1 (4.8)	2 (3.1)	
Mutation status (overall)					0.009*
Mutation (s) detected	5 (38.5)	25 (80.6)	18 (85.7)	48 (73.8)	
Mutation (s) not detected	8 (61.5)	6 (19.4)	3 (14.3)	17 (26.2)	

*(*P*<0.05)

markers in the studied patients with FMF such as ESR, CRP, and SAA were increased during the FMF attack period by 84.6, 76.9, and 21.5%, respectively. On the contrary, the findings observed by Barut *et al.* (2018) displayed an increase in ESR and CRP (by 13.9 and 11%, respectively). This variation in the results might be attributed to the difference in the time of the analysis, as we took the samples during the FMF attack, whereas that of Barut *et al.* (2018) was during remission.

Although the clinical symptoms and the course of the illness are still the main tools of FMF diagnosis, the

Table 3 Comparison between disease severity grades and allele frequencies in the studied patients

	Severity [*n* (%)]			Total (*n*=130)	*P**
	Mild (*n*=26)	Moderate (*n*=62)	Severe (*n*=42)		
rs28940580 (M680I)					0.668
A	1 (3.8)	7 (11.3)	4 (9.5)	12 (9.2)	
G	25 (96.2)	55 (88.7)	38 (90.5)	118 (90.8)	
rs28940578 (M694I)					0.028*
A	1 (3.8)	11 (17.7)	12 (28.6)	24 (18.5)	
G	25 (96.2)	51 (82.3)	30 (71.4)	106 (81.5)	
rs61752717 (M694V)					0.689
A	26 (100.0)	60 (96.8)	42 (100.0)	128 (98.5)	
G	0 (3.8)	2 (3.2)	0	2 (1.5)	
rs28940579 (V726A)					0.858
C	1 (3.8)	4 (6.5)	1 (2.4)	6 (4.6)	
T	25 (96.2)	58 (93.5)	41 (97.6)	124 (95.4)	
rs3743930 (E148Q)					0.712
C	2 (7.7)	3 (4.8)	3 (7.1)	8 (6.2)	
G	24 (92.3)	59 (95.2)	39 (92.9)	122 (93.8)	
rs104895097 (R761H)					1.000
A	0	1 (1.6)	0	1 (0.8)	
G	26 (100.0)	61 (98.4)	42 (100.0)	129 (99.2)	
rs61732874 (A744S)					0.589
G	26 (100.0)	61 (98.4)	40 (95.2)	127 (97.7)	
T	0	1 (1.6)	2 (4.8)	3 (2.3)	

*(*P*<0.05)

Table 4 Comparison of *MEFV* gene expression level in familial Mediterranean fever patients and controls

	Patients (*n*=65)	Controls (*n*=26)	*P*
MEFV expression			0.001
Median (Q1, Q3)	0.314 (0.22, 0.753)	1.676 (0.624, 5.112)	

Table 5 Comparison of *MEFV* gene expression level in patients with familial Mediterranean fever with identified mutation and patients without mutation

	With mutation (s) (*n*=48)	No mutations (*n*=17)	*P*
MEFV expression			0.165
Median (Q1, Q3)	0.311 (0.216, 0.566)	0.458 (0.224, 3.158)	

demonstration of the *MEFV* gene mutations is essential to diagnose suspected cases (Onen, 2006). M680I, M694V, M694I, V726A, and E148Q mutations are the most common detected mutations accounting for 65–95% of the cases. The rest of the mutations are rarely present in different populations. Significant variation in the carrier frequency of FMF is found in ethnic and racial groups (Ben-Chetrit and Touitou, 2009; Kallinich *et al.*, 2017; Sönmez *et al.*, 2017). Our results showed that the M694I mutant allele was the most encountered in the studied patients with a frequency of 30.8%, which agrees with previous studies in Arabian countries, including Egypt, Algeria, Morocco, and Tunisia (Belmahi *et al.*, 2006; Ibrahim *et al.*, 2010). These results were contradictory to other studies that described other mutations as being the most frequently detected mutations in patients with FMF (Gheita and Eesa, 2019). Moreover, a previous Egyptian study found that the most common mutation was E148Q allele and was associated with a severe phenotype and high SAA (Mansour *et al.*, 2019). However, our study revealed a significant correlation between the M694I mutant allele and different severity grades; the mutant allele was more common in patients who had moderate or severe symptoms. Nevertheless, no association was found among the different mutant alleles and high SAA. This diversity in the distribution of *MEFV* mutations may be owing to the variation in the sample size among the different studies.

The evaluation of the expression profile of *MEFV* gene is important to address the involvement of this gene in the pathogenesis of FMF and additionally to assess its correlation with the mutation pattern and clinical phenotype. In this study, the expression level of *MEFV* gene in the patients with FMF was significantly lower compared with the healthy controls, which is consistent with the results of many previous studies (Notarnicola *et al.*, 2002; Kirectepe *et al.*, 2011; Tozkir *et al.*, 2014; Dogan *et al.*, 2019). However, this association was absent in a study performed by Booty *et al.* (2009). The difference in gene expression patterns across different ethnic groups may be attributed to variation in the prevalence of putative regulatory variants among these groups. Additionally, different experimental conditions (the cell type used for RNA extractions, sample size, instrument, primer sets used for qPCR, and the data analysis methods) could play a notable role in displaying some variations in the results of different groups (Grandemange *et al.*, 2011).

In our study, the patients with FMF were divided into two groups. One group included patients with FMF carrying the mutant allele, and the other group included patients with FMF with undetected mutations. The *MEFV* mRNA expression level was lower in the group of patients carrying the mutant allele than in those with undetected mutation, although the difference was not statistically significant. This finding agrees with the studies of Üstek *et al.* (2007) and Kirectepe *et al.* (2011). Nevertheless, Tozkir *et al.* (2014) found a borderline significant difference in *MEFV* gene expression between patients with FMF with and those without *MEFV* mutations. There may be regulatory variants in the different ethnic studies that may account for the changes in the gene expression among these studies.

Our results revealed no association between changes in gene expression and the elevated SAA levels. This is probably owing to the small number of patients with elevated SAA levels in our study group. A significant association was reported between the disease severity and low *MEFV* mRNA levels, especially in patients with homozygous M694V and heterozygote M694V (Notarnicola *et al.*, 2002). However, in our study, when the *MEFV* mRNA levels were plotted versus the clinical severity score, no correlation was detected in the entire group of patients with FMF. These differences may be owing to the limited sample size in our study. Furthermore, environmental factors may contribute to as much as 12% of the phenotypic variation and may trigger epigenetic changes that are proven to be of a crucial clinical significance in patients with FMF (Kirectepe *et al.*, 2011; Ben-Zvi *et al.*, 2012).

Conclusion

This study delineates the reduction in the level of *MEFV* gene expression in Egyptian patients with FMF. Our findings suggest that *MEFV* gene expression regulation may be one of the mechanisms involved in FMF pathogenesis, which may be clinically valuable in developing new therapeutic strategies.

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Conflicts of interest

There are no conflicts of interest.

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