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Molecular investigation of MEFV gene polymorphisms among patients with familial mediterranean fever-like symptoms

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Abstract

The diagnosis of familial Mediterranean fever (FMF) is primarily based on clinical standards. The purpose of this study was to investigate the relevance of Mediterranean fever (MEFV) genetic testing in the diagnosis of FMF as well as to identify the most frequent variant alleles and their relationship to clinical symptoms in Egyptian patients. Egyptian patients with a clinical suspicion of having FMF were studied in order to determine MEFV genotypes. Each patient was meticulously evaluated through an extensive collection of their medical history, a thorough clinical examination, and a series of laboratory tests, encompassing CBC, ESR, and CRP measurements. The MEFV variant screening procedure included the use of reverse dot blot hybridization. The average age of our patients when they were given a diagnosis was 22.8 ± 1.404 years old. The predominant clinical manifestations identified were abdominal pain, fever, and arthralgia. Molecular interrogation of the MEFV gene unveiled that a significant proportion of the cohort, constituting 72 individuals (60%), displayed heterozygosity, whereas a smaller fraction, comprising 12 subjects (10%), demonstrated homozygosity and an equivalent number (10%) exhibited compound heterozygosity. Pertaining to the distribution of allele variants, E148Q emerged as the most prevalent, succeeded by M694I, accounting for 12.5% of the cases, and M680I (G/A), representing 10.41%. This notable prevalence of heterozygous genotypes among the Egyptian demographic, preliminarily identified as potential FMF cases, underscores the imperative for molecular diagnostics to enhance the precision of FMF identification.

Keywords: Familial mediterranean fever, Reverse dot blot hybridization, Variant, MEFV gene.

1. Introduction

Familial Mediterranean fever (FMF), an autosomal recessive illness, is characterized by recurrent fever and pain brought on by peritoneal, synovial, or pleural inflammation [1]. A significant complication stemming from FMF includes the emergence of systemic amyloidosis, subsequently leading to impairment of renal function [2]. The disease has predominantly been documented among individuals hailing from the Mediterranean basin, especially among populations including Sephardic Jews, Arabs, Armenians and Turks [3, 4]. The diagnosis of FMF has always been based on the clinical characteristics and exclusion of alternative causes of periodic fever, which highlights the need for genetic diagnostics to identify the patient with atypical symptom pattern [5].

The MEFV gene is responsible for encoding a protein comprised of 781 amino acids, referred to as Marenostrin or Pyrin. Optimal expression of the pyrin gene is observed in a range of cells, including dendritic cells, eosinophils, neutrophils, monocytes, and synovial fibroblasts [6, 7].

Pyrin has a key role in apoptosis and inflammatory pathways. This entity serves as the catalyst for the assembly of the pyrin inflammasome complex, which plays a pivotal role in the activation and release of the proinflammatory cytokines IL-1β and IL-18, ultimately leading to an inflammatory variant of cell death, designated as pyroptosis [8] Mutated pyrin causes an exaggerated inflammatory response by uncontrolled secretion of interleukin-1 (IL-1), a proinflammatory cytokine interleukin that is central in the pathogenesis of FMF [4].

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To date, more than 300 MEFV gene sequence variants have so far been documented. The most common mutations are in exon 2 (E148Q and E148V) and exon 10 (M694V, M680I, V726A, and M694I). The frequency of MEFV gene mutations may vary depending on race [9, 10].

While most reports of FMF describe autosomal recessive inheritance, some have reported specific heterozygous mutations with dominant inheritance. According to these results, a single variant can impact protein expression levels in a way that is comparable to patients who have mutations impacting both alleles [11].

A wide array of clinical benchmarks, encompassing Tel-Hashomer, simplified Livneh, Yalcinkaya and Ozen, Turkish pediatric criteria, Eurofever and the collaborative Eurofever/Paediatric Rheumatology International Trials Organization (PRINTO) guidelines, have been established to facilitate the diagnosis of FMF and its differentiation from other autoinflammatory conditions. Moreover, the 2015 recommendations from the Single Hub and Access Point for Pediatric Rheumatology in Europe (SHARE) advocate for the utilization of genetic testing as a supplementary tool in confirming an FMF diagnosis predicated on clinical manifestations, albeit it does not possess the capacity to conclusively negate such a diagnosis [12]. The aim of this study was to investigate the role of MEFV genetic testing in diagnosis of FMF and to characterize the most frequent variant alleles and their association with clinical symptoms among Egyptian patients sample.

2. Materials and methods

From April 2023 to June 2023, 120 Egyptian patients of varying ages, were recruited from outpatient clinics of General Medicine and Pediatric Rheumatology, Benha University hospitals after signing informed consent and approval of the study by the Research Ethics Committee, Faculty of Medicine, Benha University (Rc.7.3.2023).

The determination of the sample size was conducted through the application of Epi-info software, version 3.1.9.2, drawing upon the findings from a preceding investigation by Fentoğlu et al. (2017), which elucidated a mutation prevalence rate of 92.7% in the MEFV gene among patients afflicted with Familial Mediterranean Fever [13]. The minimum number of patients needed was 105, and the sample size was increased to 120 to compensate for possible laboratory failures. Confidence level and margin of error were adjusted at 95% and 5%, respectively. Every participant, exhibiting symptoms akin to FMF and clinically presumed to be afflicted with FMF based on the updated FMF diagnostic criteria, was incorporated into the study [14]. They were included in the research and underwent MEFV genotyping at the molecular biology unit, faculty of medicine, Benha University. Patients with other autoimmune or autoinflammatory diseases, hematological or solid malignancy, chronic liver, kidney or pulmonary diseases, or any other comorbid disorders are excluded from the study. Potential participants were advised about the nature of the research, its significance, and its potential advantages. All patients were subjected to:

I- Thorough history taking; demographics (sex and age at diagnosis), family history (consanguinity of parents, family history of FMF), the presence of fever, recurrent typical attacks of FMF (including peritonitis, pleuritis and arthritis).

II- Full clinical examination.

III- The criteria delineated by Yalcinkaya and Ozen [14] for the establishment of FMF diagnosis necessitate the confirmation of a minimum of two among five specified criteria. These encompass:

- Fever (axillary temperature >38°C, 6-72 h of duration, ≥three attacks)
- Abdominal pain (6-72h duration, ≥three attacks)
- Chest pain (6-72h duration, ≥three attacks)
- Oligo-arthritis (6-72h duration, ≥three attacks)
- Family history of FMF (Yalcinkaya et al., 2009)

Laboratory assessments, including ESR, CBC, and CRP, were also conducted and documented. For molecular analysis, a small sample of peripheral blood (3-5 ml in an EDTA vacutainer) was taken from each individual. The Vienna FMF Strip Assay® kit (Austria) was used to perform reverse dot blot Hybridization analysis on all samples to determine the MEFV genotyping.

2.1. Reverse dot blot Hybridization

The procedure for Reverse dot blot Hybridization analysis entailed the following stages: (1) Amplification via PCR employing biotinylated primers; (2) The binding of the PCR amplified fragments to a testing strip that has distinct oligonucleotide probes anchored in a configuration of parallel lines; and (3) The identification of the biotinylated sequences through the application of streptavidin-alkaline phosphatase (Conjugate) alongside color substrates. 15 ul of PCR amplification mix, 5 ul of diluted Taq DNA polymerase (1U), and 5 ul of DNA template were used. Temperature cycling: Pre-PCR conditions were 94˚C for 2 minutes, thermocycling (35 cycles): 94˚C for 15 sec, 58 ˚C for 30 seconds and 72 ˚C for 30 seconds, then 72 ˚C for 3 minutes for final extension. After that, PCR products were incubated (45 ˚C) in a shaking water bath with test strips that included immobilized allele-specific probes for 30 minutes to induce hybridization. After incubation, two rounds of washing were performed, then the conjugate solution was added, and the mixture was incubated once more for 15 minutes at room temperature. After 15 minutes in the dark on a rocker or orbital shaker, the color developer was applied, and the incubation process was repeated. A purple staining appeared with positive reactions. To identify the genotype, we compared the results from our test strip to those from the reference strip provided by the manufacturer (Figure 1). The eight most frequent polymorphism regions of the MEFV gene were tested.

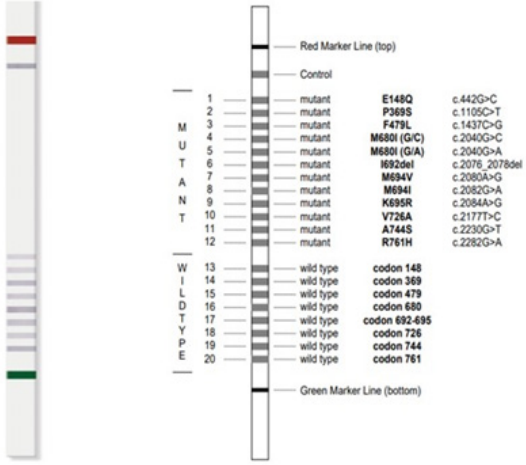

Fig. 1. FMF strip assay.

2.2. Statistical analysis

Data were revised, coded, and analyzed using SPSS package version number 20. Data were tested for normality with the Shapiro-Wilk test. Quantitative data are depicted as mean values accompanied by the standard deviation (SD). Qualitative data were expressed as frequencies (n) and percentages (%). Fisher's exact test was used to test the association between qualitative variables. P-value ≤ 0.05 was considered significant.

3. Results

The present study was conducted on 120 unrelated patients with FMF-like symptoms, 64 (53.3%) male patients and 56 (46.7%) female patients with a mean age of 22.8 ±1 4.04 years ranging from 2 to 66 years. The clinical features and laboratory findings of patients with FMF are described in Table 1.

Family history was positive in 70 % of our cases. Fever was the most prevalent manifestation (83.3%) followed by abdominal pain (80%) then arthritis (16.7%). Mean values of Hg, WBCs, Platelets, ESR & CRP were 10.9 ± 0.94, 10.7 ± 2.69, 269 ± 55.7, 30.9 ± 13.4 & 20.4 ± 20.2 respectively.

No mutation was found in 20% (24/120) of FMF patients, 10% (12/120) were homozygotes, 10% (12/120) compound heterozygotes and 60% (72/120) heterozygotes (Table 2).

In our study group, fever was associated mainly with homozygous, compound heterozygous mutants (100%) and then heterozygous mutant (88.9%) (P value 0.005). Abdominal pain was associated mainly with heterozygous mutant (88.9%) followed by wild variant (83.3%) and then compound heterozygous (66.7%) (P value 0.01). Arthritis was associated mainly with homozygous mutant (66.7%) and compound heterozygous (33.3%) (P value 0.002). Positive family history was associated with compound heterozygous (100%) followed by heterozygous mutant (83.3%) and then homozygous mutant (66.7%) (P-value 0.001) (Table 3).

Considering allele frequency, the frequencies of the most common alleles were as follows: E148Q (18.75%), M694I (12.5%) and M680I (G/A) (10.41 %). Allele frequencies for V726A, M694V, and P369S were 8.33%, 6.25%, and 6.25% respectively (Table 4 and Figure 2).

Among the most common alleles reported in our study, E148Q, M694v and P369S were associated with early disease onset, being E148Q the most frequent one. While M694I and v726a alleles were associated with late disease onset (Table 5).

Table 1. Demographic data, clinical characteristics and laboratory findings of the studied group.

Parameter	FMF patients (n =120)
Sex	
Male	64 (53.3%)
Female	56 (46.7%)
Age (years)	22.8 (2-66 years)
Family History	84 (70%)
Clinical manifestations:	
Fever	100 (83.3%)
Abdominal pain	96 (80%)
Arthritis	20 (16.7%)
Laboratory findings	
Hemoglobin (g/dl)	10.9 ± 0.94
WBCs (x10^3/ml)	10.7 ± 2.69
Platelets (x10^3/ml)	269 ± 55.7
ESR (mm/h)	30.9 ± 13.4
CRP (mg/dl)	20.4 ± 20.2

Data are expressed as the mean ± SD for Hemoglobin, WBCs, Platelets, ESR, and CRP and as number (%) for all other parameters. ESR, erythrocyte sedimentation rate; CRP, C-reactive protein.

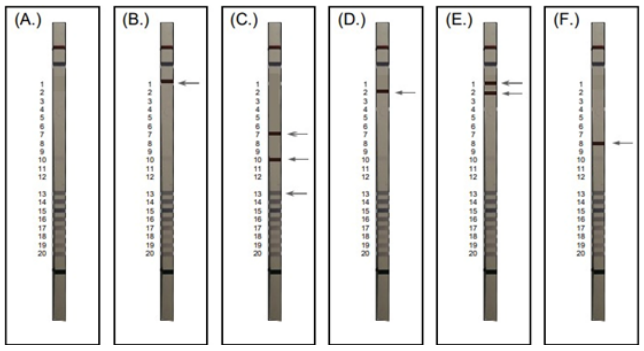

Fig. 2. MEFV variants. A: Wild type. B: Heterozygous E148Q. C: Compound Heterozygous M694V&V726A. D: Heterozygous P369S. E: Compound Heterozygous E148Q&P369S. F: Heterozygous M694I.

Table 2. Prevalence of predominant MEFV variants in patients with FMF.

Variant	Genotype	Number	% (within mutation)	% (within total mutant cases)
Heterozygous N=72 (75 %)	E 148Q	24	33.3	25
	M680I (G/A)	20	27.8	20.8
	M694I	12	16.7	12.5
	P369S	8	11.1	8.3
	V726	8	11.1	8.3
Compound Heterozygous complex N=12 (12.5%)	E148Q, P369S	4	33.3	4.2
	M694I, V726A	4	33.3	4.2
	M694V, V726A	4	33.3	4.2
Homozygous mutant N= 12 (12.5%)	E 148Q	4	33.3	4.2
	M694I	4	33.3	4.2
	M694V	4	33.3	4.2
Total		96		(%)100

Table 3. Association of genetic mutations with clinical manifestations and family history.

Parameter	Total (120)	Wild (24)		Homozygous mutant (12)		Heterozygous mutant (72)		Compound heterozygous mutant (12)		Test value	p-value
		No	%	No	%	No	%	No	%		
Fever											
Yes	100	12	50	12	100	64	88.9	12	100	9.31	0.005*
No	20	12	50	00	0	8	11.1	0	0	*(FET)*	
Abdominal pain											
Yes	96	20	83.3	4	33.3	64	88.9	8	66.7	9.18	0.01*
No	24	4	16.7	8	66.7	8	11.1	4	33.3	*(FET)*	
Arthritis											
Yes	20	4	16.7	8	66.7	4	5.6	4	33.3	12.8	0.002*
No	100	20	83.3	4	33.3	68	94.4	8	66.7	(FET)	
Family history											
Yes	84	4	16.7	8	66.7	60	83.3	12	100	19.7	0.001**
No	36	20	83.3	4	33.3	12	16.7	0	0	*(FET)*	

**Significant p≤ 0.05, **highly significant p≤ 0.001, FET=Fisher Exact Test.*

Table 4. Distribution of MEFV genotypes and allele frequencies among cases with mutation.

Genotypes	Allele frequency (n)	Allele Frequency (%)
E148Q	36	18.75 %
M680I (G/A)	20	10.41
M694I	24	12.5 %
P369S	12	6.25%
V726	16	8.33 %
M694V	12	6.25%
Wild allele	72	37.5%
Total	192	100 %

Table 5. Distribution of Diagnosis Age and Prevalence of Predominant MEFV Genotypes Among Patients with FMF.

Genotype	Age at diagnosis in years							
	1-15 N=24		16-30 N=44		31-45 N=20		>45 N=8	
	N	%	N	%	N	%	N	%
E148Q	12	50	12	27.3	4	20	0	0
E148Q, P369S	0	0	4	9.1	0	0	0	0
M680I(G/A)	4	16.7	8	18.2	8	40.	0	0
M694I	0	0	12	27.3	0	0	4	50
M694I, v726a	0	0	0	0	0	0	4	50
M694v	4	16.7	0	0	0	0	0	0
M694V, v726a	0	0	4	9.1	0	0	0	0
P369S	4	16.7	0	0	4	20	0	0
V726A	0	0	4	9.1	4	20	0	0

4. Discussion

Since MEFV was shown to be the mutant gene for FMF, genetic investigation has proven to be useful for verifying the occasional diagnosis given by clinical findings [10, 15]. Subjects harboring both homozygous and compound heterozygous mutations, in addition to those presenting with singular mutations, have been discerned [16, 17].

Egyptian patients with FMF-like symptoms were included in our study, and they were all examined for the MEFV gene variants M694I, V726A, M680I, E148Q, and M694V. This study was conducted on 120 patients: 64 males (53.3%) and 56 females (46.7%) with mean age ± SD is 22.8 years ± 14.04. Male to female ratio of 1.14,

which is consistent with prior research showing that males are more likely to be diagnosed with FMF than females by a factor of around 3/2 (1.5) due to the disease's lower penetrance in females [18, 19, 20]. However, a higher frequency of FMF female sex prevalence was observed in other studies performed on Italians and Arabs [21, 22].

In this study, family history was positive in 70 % which is consistent with Gursoy et al., (2023) results as they reported the presence of family history of the disease in 68.1% of their study group [23]. Although family history is an important element in FMF diagnosis criteria, El Gezery et al. (2010) and Yilmaz et al. (2009) reported presence of FMF family history in only 25.3% and 4% respectively [20, 24].

Within our patient population, fever emerged the most commonly observed symptom, manifesting in 83.3% of the patients, while abdominal pain ranked as the second most frequent symptom, affecting 80% of the patients, followed by arthritis in 16.7% of the cases. This distribution aligns with findings by Almalky et al. (2021) and Ates et al. (2022), who reported fever (96.3%, 100%), abdominal pain (90.9%, 93.3%), and arthritis (75.8%) as the most frequent symptoms during episodes [25, 26]. However, this mismatches with data from Farag et al. (2020) [27], Lotfy et al. (2016) [28] and Tunca et al. (2005) [29] who found that abdominal pain was the most common followed by fever, chest pain, and arthritis.

Within the scope of the current investigation, elevated levels of CRP and ESR were observed among FMF patients amidst an acute attack, a finding that aligns with the research outcomes presented by Yorulmaz et al. (2019) [30], Dinçer et al. (2022) [31], and Ates et al. (2022) [26]. These studies collectively underscored that CRP and ESR levels were markedly increased in individuals experiencing an FMF acute episode in comparison to periods devoid of attacks.

In this study, ninety-Six individuals (80%) were found to carry MEFV mutations that cause FMF, this is in line with Arpacı et al., 2021 results as they detected MEFV mutations in 78.7% of their study group [9]. Results of this study demonstrated that 72 (75%) were found to be heterozygous (carrying just one mutation), 12.5% were homozygous, and 12.5% were compound heterozygous (carrying many variants; variants on both alleles).

The findings of this study are congruent with the observations made by El Roz et al. (2020) [19], which delineated that a majority, precisely 63.7%, exhibited heterozygosity, while a lesser proportion of 9.20% were found to be homozygous, and a significant 27.01% harbored multiple MEFV mutations, thereby being categorized as either compound heterozygotes (mutations present on both alleles) or possessing a complex allelic configuration (multiple mutations situated on the same chromosome). In stark contrast, research conducted by El Hawary et al. (2015) revealed a markedly lower incidence, with only 23% of the subjects in their cohort being identified with a solitary heterozygous mutation [32].

In the patient sample under research, the most common alleles were E148Q (18.75%), followed by M694I (12.5%) and M680I (G/A) (10.41%). The allele frequencies of M694V, P369S, and V726A were 6.25%, 8.33%, and 6.25%, respectively, indicating lower frequency of these alleles. These findings came in line with those reported by El Roz et al. (2020) [19] as they found that the frequency of E148Q and M694I were 17.9 % and 11.8% respectively. Corresponding with these findings, Kırnaz et al., 2022 reported that the frequency of E148Q was 17.49% **[33]** and Arpacı et al. (2021) reported that the frequency of M694V was 6.51% in their study groups [9]. Another study conducted by Migita et al. (2014) [34] found that the frequency of P369S allele was 6.1% among FMF patients which was very close to our results. Of the 12 screened MEFV genetic variants in this study, E148Q was associated with early disease onset (12/24, 50%) which is inconsistent with what was previously reported by El Roz et al. (2020) [19] as they found that the frequency of E184Q was associated with delayed onset age. The relatively small number of patients included in the study and the requirement for multiple centers' collaboration in data collecting were the study's limitations.

5. Conclusion

In the patient sample under research, 80% had MEFV genetic mutations, of which 75% were found to be heterozygous, 12% were homozygous, and 12% were compound heterozygous. In this study, E148Q (18.75%) was the most prevalent allele, followed by M694I (12.5%) and M680I (G/A) (10.41%). The frequencies of M694V, P369S, and V726A alleles were found to be lower, with respective values of 6.25%, 8.33%, and 6.25%. In addition, the study's findings indicate that while MEFV genetic testing is an important diagnostic tool, it is still not able to confirm the diagnosis in all patients because 20% of the patient sample lacked mutations. This underscores the necessity of screening for the presence of new mutations to further strengthen the usefulness of MEFV genetic testing in the diagnosis of FMF. Future research could further examine the presence of other variants that could not be detected by the Reverse dot blot Hybridization, using the more advanced next-generation sequencing technique.

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Declaration of competing interest
The authors declare no conflict of interest.

Data Availability Statement
On reasonable request, the supporting data of this study's findings can be provided by the corresponding author.

Declaration of Generative AI and AI-assisted technologies
During the preparation of this work, the authors did not use any AI-assisted technology

Authors' contributions
Study Design: (Nashwa Ahmed); **Data Collection:** (Nashwa Ahmed, Arwa Amer); **Statistical Analysis:** (Nashwa Ahmed, Mai Elmahdy); **Data Interpretation:** (Nashwa Ahmed, Walaa El Gazzar, Arwa Amer, Medhat Elamawy, Hiam Eleleimy, Ola El-Shimi, Mai Elmahdy, Marwa El-sayed, Shaymaa Abdelrahman); **Manuscript Preparation:** (Nashwa Ahmed, Walaa El Gazzar, Arwa Amer, Medhat Elamawy, Hiam Eleleimy, Ola El-Shimi, Mai Elmahdy, Marwa El-sayed, Shaymaa Abdelrahman); **Literature Search:** (Nashwa Ahmed, Walaa El Gazzar, Arwa Amer, Medhat Elamawy, Hiam Eleleimy, Ola El-

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