

# Phenotype-genotype updates from familial Mediterranean fever database registry of Mansoura University Children' Hospital, Mansoura, Egypt

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**BACKGROUND:** Familial Mediterranean fever (FMF) is autosomal recessive disease that affects people from Mediterranean region, Europe and Japan. Its gene (Mediterranean fever [MEFV]) has more than 100 mostly non-sense mutations.

**OBJECTIVES:** The objective of the following study is to provide some phenotype-genotype correlates in FMF by categorizing the Egyptian FMF cases from Delta governorates after analysis of the four most common mutations of MEFV gene (M680I, M694I, M694V, V726A).

**SUBJECTS AND METHODS:** Clinically, suspected FMF cases using Tel-Hashomer criteria were enrolled in the study. Cases were referred to Mansoura University Children's Hospital that serves most of the most middle Delta governorates, in the period from 2006 to 2011. Subjects included 282 males and 144 females, mean age of onset  $9.3 \pm 2.2$  years. All cases were analyzed for these mutations using amplification refractory mutation system based on the polymerase chain reaction technique. Five FMF patients agreed to undergo renal biopsy to check for development of amyloidosis. Analysis of data was carried out using SPSS (SPSS, Inc., Chicago, IL, USA).

**RESULTS:** Mutation was found in 521 out of 852 studies alleles, the most frequent is M694V (35.4%) followed by M694I, V726A and M680I. 11 cases were homozygous; 7 M694V, 3 M680I and only one M694I case. Severe abdominal pain occurred in 31 (7.28%) but severe arthritis in 103 cases (24.2%). Strong association was found between arthritis and homozygous mutant compared with single and double heterozygous (72.7% vs. 33.3% and 20.24%,  $P < 0.001$ ). Four amyloid cases were M694V positive.

**CONCLUSION:** M694V allele is the most common among Egyptian FMF especially those with amyloidosis. We recommend routine check for amyloidosis in FMF cases to statistically validate this link.

**Key words:** Amyloidosis, familial Mediterranean fever, Mediterranean fever gene, M694V

## Introduction

Familial Mediterranean fever (FMF, OMIM 249100) is the most common auto-inflammatory disease; it is inherited as an autosomal recessive disorder. It predominantly affects populations living in the Mediterranean region, especially North African Jews, Armenians, Turks and Arabs. It is not uncommon in Italians, Spanish, Portuguese, French, Greeks and also described in Northern Europeans and Japanese.<sup>[1]</sup>

FMF gene (Mediterranean fever [MEFV], OMIM 608107) is the only gene currently known to be associated with FMF, being mapped on the short arm of chromosome 16. It encodes a 781 amino acid protein known as pyrin.<sup>[2]</sup> More than 100 mutations in MEFV gene have been reported, the majority of are missense and located in exons 2 and 10.<sup>[3]</sup> Four common MEFV gene mutations (M680I, M694V, M694I and V726A) can be rapidly and accurately characterized using the amplification refractory mutation system based on the polymerase chain reaction (ARMS-PCR) technique.<sup>[4]</sup> The carrier frequency for MEFV mutations is quite high

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ranging 37-39% in Armenians and Iraqi Jews, to 20% in Turks, North African, Ashkenazi Jews and Arabs.<sup>[5-7]</sup>

FMF comprises two phenotypes; type 1 is characterized by recurrent short self-limiting episodes of inflammation and serositis causing inter- and intra-familial variable clinical severity, amyloidosis ending by renal failure is the most serious complication that can also be prevented by prophylactic colchicines administration.<sup>[8]</sup> FMF type 2 is characterized by amyloidosis as the first clinical manifestation of FMF in an otherwise asymptomatic individual.<sup>[9]</sup> Amyloidosis can be diagnosed by detecting the characteristic amyloid protein stained by Congo red in a biopsy specimen of involved tissue (mouth, rectum, fat, kidney, heart, or liver). A needle aspiration biopsy of fat just under the skin of anterior abdominal wall, originally developed at Boston University, offers a simple and less invasive method to diagnose systemic amyloidosis.<sup>[10]</sup>

Diagnosis of FMF was based on ethnicity, family history, clinical manifestations and response to colchicines, however, haplotyping of MEFV gene using the screening molecular methods (ARMS-PCR) provided a further strong diagnostic tool.<sup>[11-13]</sup> Due to the presence of many limitations for the application of Tel-Hashomer criteria in pediatrics, use of a new set of clinical data that comprised fever, abdominal pain, chest pain, arthritis and family history of FMF yielded accurate diagnosis of FMF with a sensitivity of 86.5% and specificity of 93.6% for two or more criteria among the carriers of two MEFV mutant alleles.<sup>[14]</sup>

The current study aims at molecular characterization of the clinically suspected FMF cases registered in Mansoura University Children's Hospital (MUCH) that serves most of middle Delta governorates of Egypt. Using the readily available molecular screening method (ARMS-PCR) previously mentioned,<sup>[13]</sup> the four most common mutations limited to exon 10 of MEFV gene (M680I, M694V, M694I and V726A) will be tested with the aim to provide some epidemiologic updates of our registry which could aid in understanding about phenotype-genotype correlations.

## Subjects and Methods

All the clinically suspected cases of FMF (543) based on the clinical scoring system of Tel-Hashomer or that

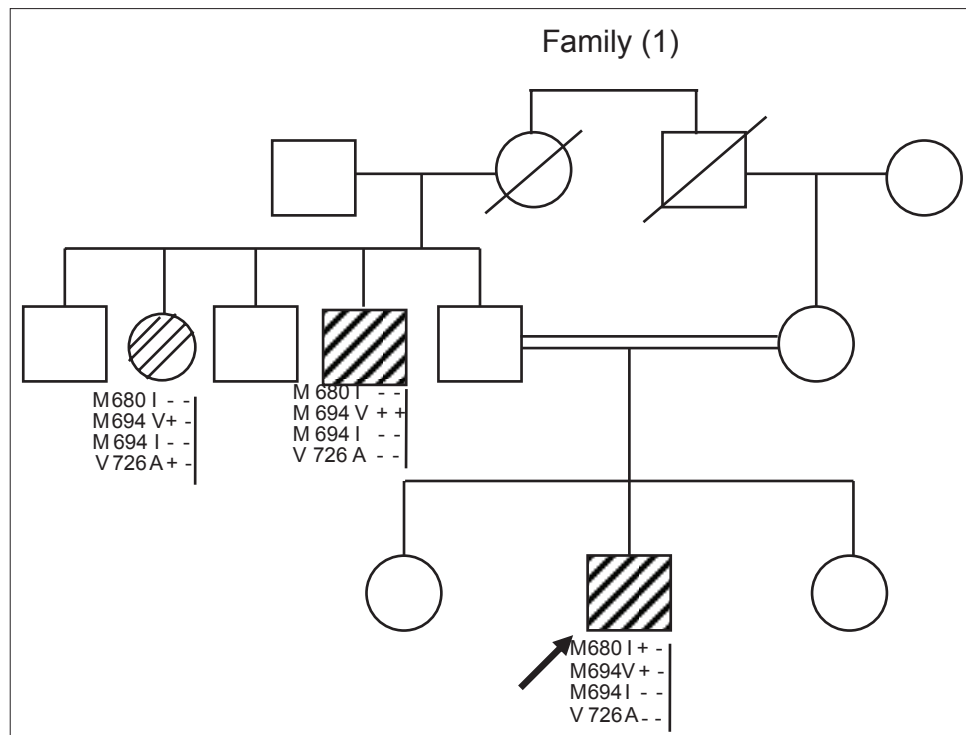
adopted by Settin *et al.*<sup>[13]</sup> were enrolled in the study. Cases clinically categorized as less probable or those currently genotyped negative by ARMS were excluded from the study, they included 117 individuals. As 80% of the enrolled cases with FMF were in the pediatrics age group, we used the diagnostic clinical set of criteria (fever, abdominal pain, chest pain, arthritis and family history of FMF) that had been proved to be more practical and specific (specificity equals 64.5% and 93.6% with  $\geq 1$  and  $\geq 2$  criteria, respectively) in Turkish children who had two mutant MEFV alleles, compared with Tel-Hashomer criteria which has many limitations in children lowering its specificity to only 54.6%,<sup>[14]</sup> however, Tel-Hashomer criteria still have a high sensitivity in pediatrics (98.8%). Cases were referred to Pediatric Genetics Unit, MUCH in the period from 2006 to 2011, with the aim to confirm the diagnosis and initiate specific therapy of FMF. MUCH serves most of the middle Delta governorates (Dakahlia, Gharbia, Sharkia and Damietta). Written informed consent had been taken from all cases before hand to be enrolled in the survey study.

## Statistical methods

Data were analyzed using SPSS for windows, version 16.0 (SPSS, Inc., Chicago, IL, USA). Exploration of quantitative parameters showed preserved normality (Kolmogorov-Smirnov test). Chi-square test was used for testing frequency differences between groups and as test of association between categorical variables. Our subjects included 282 males and 144 females (2:1). Their age mean  $\pm$  standard deviation (SD) was  $19.39 \pm 8.8$  years with the age of onset mean  $\pm$  SD was  $9.3 \pm 2.2$  years; mostly before age of 10. Some cases were clustered in single unrelated families to the extent that up to 3-5 cases had been diagnosed in some target families [Figures 1 and 2]. Moreover, most of the studied index cases (380 out of 426) showed positive consanguinity (89.2%). Family history, clinical examination as well as routine investigations of the enrolled cases (426) were reviewed and correlated with the molecular genotyping.

## Renal biopsy

Five FMF patients aged 45-60 years agreed to undergo renal biopsy as an initial assessment for their long standing illness (duration = 35-45 years) to rule out



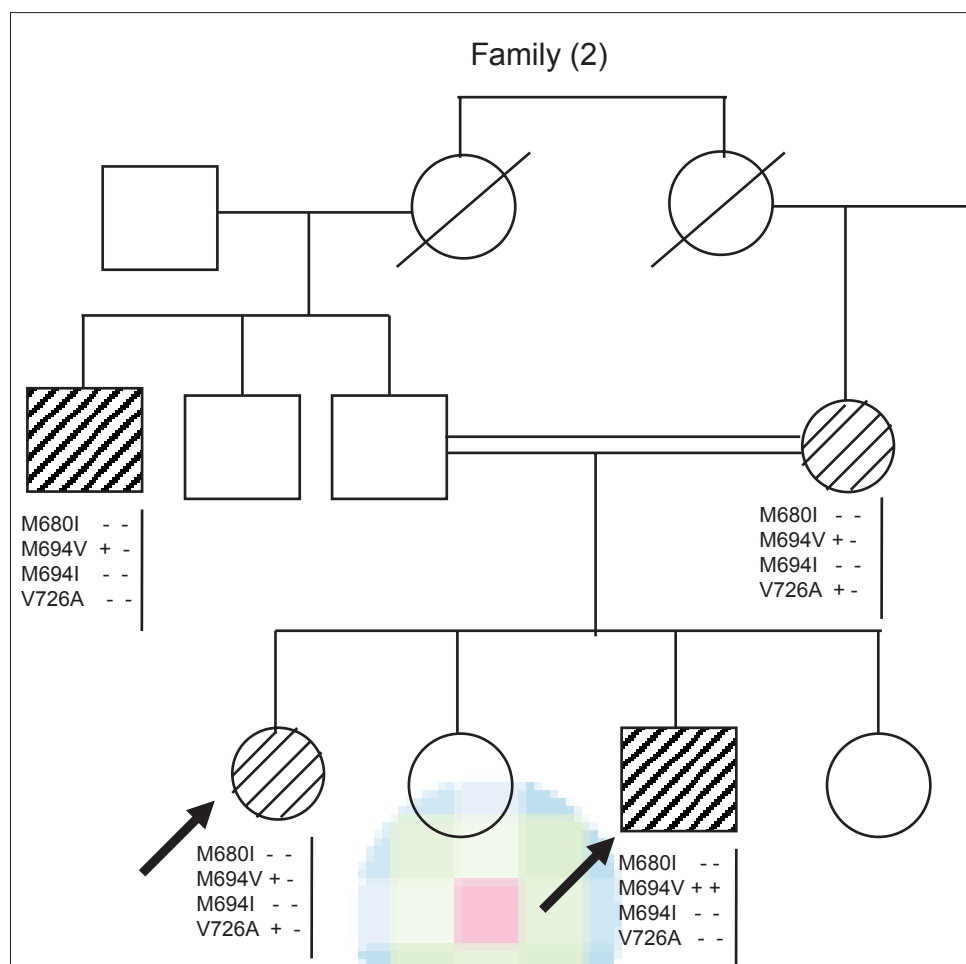
**Figure 1: Pedigree of a multiplex familial Mediterranean fever (FMF) family (three cases), proband is a male 12 years old genotyped M694V/M680I, started to complain at the age of 9.5 years. There is positive consanguinity. His uncle is genotyped homozygous M694V; his aunt is genotyped M694V/V726A. So, allele M694V is common in all affected members. Empty squares and circles are no-tested, filled squares and circles are symptomatic FMF cases**

the possibility of development of renal amyloidosis, all of them had affected offspring(s) or affected first degree relative(s) with FMF.

#### Mutation analysis

Using the readily available molecular screening method (ARMS-PCR), four missense mutations limited to exon 10 of MEFV gene were tested namely M680I, M694V, M694I and V726A. These mutations are clustered within 46 amino acids (codon 680 to codon 726) of a predicted 781-amino acid protein (pyrin), these mutations were responsible for a large percentage of mutations in the two initial studies of FMF families.<sup>[15,16]</sup> Deoxyribonucleic acid (DNA) was extracted from anticoagulated whole blood by using the DNA purification Capture Column Kit (Gentra kit). DNA was amplified using specific oligonucleotide primers mutation detection (M680I, M694V, M694I, V726A) by ARMS-PCR technique.<sup>[4]</sup> ARMS assay comprises two complementary reactions, each conducted with the same substrate DNA. One reaction includes an ARMS primer specific for the normal DNA sequence and cannot amplify mutant DNA at a given locus. The second reaction includes

a mutant-specific primer and cannot amplify normal DNA. The same common primer is used in both reactions. The lack of PCR products according to use of a specific mutation primer set in patients suspected of carrying the mutation for FMF suggests that the patient in question is not carrying the mutation being probed, however, an appropriate internal PCR control should be run to show that the DNA is amplified. Therefore, the complementary reaction with the normal primer set serves as an internal control for PCR amplification and discriminates between homozygous and heterozygous cases. Each DNA sample was tested for the four mutations. The PCR amplification was performed in a final volume of 25  $\mu$ l containing 4  $\mu$ l of purified genomic DNA, mixed with of 13  $\mu$ l of dream Taq Green PCR Master Mix (2x), (Fermentas, USA) mixed with 4  $\mu$ l of each primer (10 pmol). Amplification conditions were kept the same for all of ARMS tests and the procedure was carried out as follows; reaction was heated to 94°C for 10 min for denaturation, followed by 35 cycles with denaturation at 94°C for 10 s, annealing 60°C for 10 s and extension at 72°C for 30 s. Final extension was done for 10 min at 72°C. The amplified products were separated by



**Figure 2: Pedigree of a multiplex familial Mediterranean fever (FMF) family presented with positive consanguinity and two affected offsprings; 24-years woman (typed like her mother M694V/V726A) and 15-year-old boy (typed as homozygous M694V). Their uncle had moderate FMF symptoms (58 years), genotyped as M694V/Unidentified. Allele M694V is common in all affected members. Empty squares and circles are non-tested, filled squares and circles are symptomatic FMF cases**

electrophoresis on a 2.5% agarose gel, ethidium bromide staining, to detect the amplified fragments. List for the used primers in different alleles is shown in Table 1.

## Results

Analysis of the four most common mutations was found positive in 521 out of a total 852 tested alleles (64.67%) among the studies Egyptian cases of FMF. The remainder 331 alleles (35.33%) represent either unidentified less common alleles or novel unknown alleles, which could be the candidate for sequencing analysis of MEFV gene or linkage to map other responsible genes.

Among the 95 cases having two mutant MEFV alleles, 45% showed more than four criteria (fever, abdominal

pain, arthritis and family history), 64% showed more than three criteria, 82% showed only two criteria. Carriers of only one mutant allele (331) showed the following percentages relative to the number of clinical criteria (30%, 66% and 80% with more than 4, 3 and 2 criteria respectively), almost equals to those having two alleles; a finding that could be explained by the presence of another untested allele or the differential impact of mutant alleles in expression of clinical symptoms.

The most frequent allele is M694V (302/852; 35.4%) which is distributed among 295 cases, but only found in a homozygous state among seven cases. The next common alleles, in a descending order, are M694I, V726A and M680I in frequencies of 10.7, 7.9 and 7.2% respectively. The total number of homozygous is 11 cases; distributed as seven cases M694V/M694V, three cases M680I/M680I



and only one case M694I/M694I. No patient had been genotyped as V726A/V726A [Table 2 and Figure 3].

Males are twice more common than females with a ratio of 2:1 (284 males and 142 females). Their age of clinical presentation mostly fall in the range between 7 and 34 years (mean  $\pm$  SD,  $19.39 \pm 8.8$  years), however disease was reported in patients beyond age of 50. Age of onset may start as early as 6 years but not beyond 10 (mean  $\pm$  SD,  $9.3 \pm 2.2$  years). Multiplex involvement, familial clustering of many cases, was shown in some families [Figures 1 and 2].

Severe abdominal pain to the degree of trunk bending and bed restriction was found in only 31 cases (7.28%) however severe and clinically evident arthritis was found in 103 cases (24.2%). A strong association was shown between the severity of arthritis and homozygous mutant allele when compared with heterozygous [Table 3]. Moreover, this strong association was further confirmed when homozygous were compared with double heterozygous and single alleles; 72.7% versus 33.3% and 20.24% respectively [Table 4].

Out of the five cases who underwent renal biopsy, four were found positive for amyloid deposition of different grades, all of them were genotyped positive for M694V (3 were M694V/M694V and the fourth was

M694V/unidentified). The 5<sup>th</sup> case was heterozygous V726A and amyloid negative thus suggesting a possible link between amyloidosis and M694V allele or at least when this allele existed in a homozygous state.

## Discussion

FMF is a multi-systemic disease affecting many races in the Mediterranean coast as well as others. It is

**Table 1: The primers used**

Mutation type	Primer sequence
M680I	
Common	5'-TTAGACTTGGAAACAAGTGG GAGAGGCTGC-3'
Mutant	5'-ATTATCACCACCCAGTAGCCATTCTCTGG CGACAGAGCG-3'
Normal	5'-ATTATCACCACCCAGTAGCCATTCTCTGG CGACAGAGCC-3'
M694V	
Common	5'-TGACAGCTGTATCATTGTTCTGG GCTCTCCG-3'
Mutant	5'-TCGGGGGAACGCTGGACGCCTGGTACTCA TTTTCTTCCC-3'
Normal	5'-TCGGGGGAACGCTGGACGCCTGGTACTCA TTTTCTTCTC-3'
M694I	
Common	5'-TATCATTGTTCTGGGCTC-3
Mutant	5'-CTGGTACTCATTTTCCTTC-3
Normal	5'-CTGGTACTCATTTTCCTTT-3
V726A	
Common	5'-TGGAGGTTGGAGACAAGACACATGGATCC-3'
Mutant	5'-TGGGATCTGGCTGTACATTGTAAAAGGAG ATGCTTCCTG-3'
Normal	5'-TGGGATCTGCTGTACATTGTAAAAGGAG ATGCTTCCTA-3'

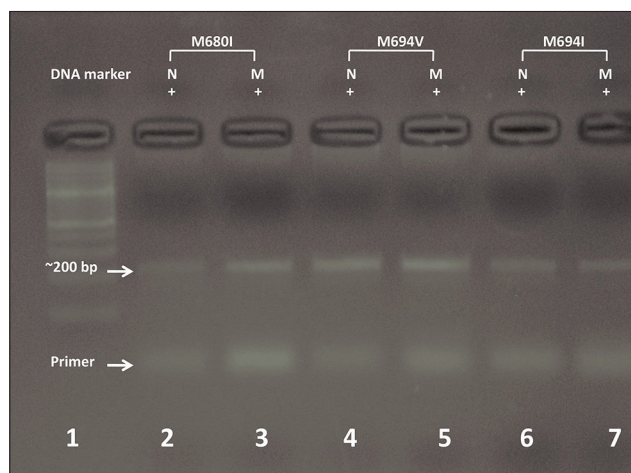
Sequence of common primers, mutant DNA primers, and normal DNA primers used in ARMS- PCR to detect M680I, M694V, M694I and V726A mutations

**Table 2: Molecular genotyping of the four common mutations of MEFV gene among Egyptian patients with FMF**

Genotype	Frequency/ cases	Allelic frequency			
		M680I	M694V	M694I	V726A
M680I/unidentified*	23	23	-	-	-
M680I/M680I	3	6	-	-	-
M680I/M694V	23	23	23	-	-
M680I/M694I	2	2	-	2	-
M680I/V726A	7	7	-	-	7
M694V/unidentified*	218	-	218	-	-
M694V/M694V	7	-	14	-	-
M694V/M694I	25	-	25	25	-
M694V/V726A	22	-	22	-	22
M694I/unidentified*	57	-	-	57	-
M694I/M694I	1	-	-	2	-
M694I/V726A	5	-	-	5	5
V726A/unidentified*	33	-	-	-	33
V726A/V726A	-	-	-	-	-
Total	426	61	302	91	67

Number of heterozygous 331, double heterozygous 84 and homozygous 11.

\*Unidentified alleles constitute 331 (out of the totally studied 852 alleles), they comprise less common un-tested alleles and unknown alleles. FMF: Familial Mediterranean fever, MEFV: Mediterranean fever



**Figure 3: Deoxyribonucleic acid (DNA) electrophoresis of amplified segments of familial Mediterranean fever gene (~200 bp, upper arrow) using normal and mutant primers corresponding to each of the four studied mutations, the distal fragment is that of primer (bottom arrow). Lane 1 is a marker DNA, lanes 2, 3 heterozygous mutants M680I, lanes 4, 5 heterozygous mutant M694V and lanes 6, 7 heterozygous mutants M694I**

**Table 3: Genotype frequency versus the common symptoms**

Genotype	Number of cases	N (%)	
		Severe abdominal pain	Severe arthritis
M680I/unidentified	23	1 (4.3)	6 (26.1)
M680I/M680I	3	0	3 (100)
M680I/M694V	23	3 (13.04)	11 (47.8)
M680I/M694I	2	0	0
M680I/V726A	7	1 (14.29)	1 (14.29)
M694V/unidentified	218	19 (8.7)	44 (20.18)
M694V/M694V	7	1 (14.29)	4 (57.14)
M694V/M694I	25	0	7 (28)
M694V/V726A	22	1 (4.5)	9 (40.9)
M694I/unidentified	57	5 (8.77)	11 (19.29)
M694I/M694I	1	0	1 (100)
M694I/V726A	5	0	0 (0)
V726A/unidentified	33	0	6 (18.18)
Total		31	103
Significance		$\chi^2=8.96$ ; $P=0.71$	$\chi^2=33.2$ ; $P=0.001^*$

\* $\chi^2$  test; significant if  $<0.05$

autosomal recessive as confirmed by the presence of consanguinity in most cases (380/426, 89.2%). About 83 families of the current report were multiplex (data are not shown in tables), familial clustering of FMF cases in some families is worth for extensive prospective study to analyze the differences between these families against those with only sporadic cases.

Up to the best of our knowledge, this is the largest cohort of Egyptian FMF cases but analyzing only the four most common MEFV mutant alleles.<sup>[17]</sup> Severe abdominal pain was found in only 31 cases (7.28%), however, clinically evident severe arthritis was found in 103 cases (24.2%). In a previous report of a small FMF sample (42 cases) published from our center, severe abdominal pain was reported in 66.7%, severe arthritis in only 15.2%, but up 40.9% of cases had variable articular complaints without actual arthritis.<sup>[13]</sup> This apparent difference could be due to the differences in sample size and selection criteria; however both studies showed M694V allele to be the most prevalent among Egyptian FMF cases.

Currently, a strong association was found between arthritis and homozygous mutant alleles M680I, M694V, M694I when compared to heterozygous or double heterozygous alleles; 72.7% versus 20.24% and 33.3%, respectively. Homozygous M680I was described in three cases all of them had arthritis, homozygous M694V was defined in seven cases (being the most common allele 35.3%) with only four cases had arthritis.

**Table 4: Number of alleles versus the common symptoms**

Number of alleles	Number of cases	N (%)	
		Severe abdominal pain	Severe arthritis
Only one allele heterozygous <sup>a</sup>	331	25 (7.5)	67 (20.2)
Two different alleles=double heterozygous	84	5 (5.95)	28 (33.3)
Two similar alleles=homozygous	11	1 (9.1)	8 (72.7)
Total	426	31 (7.28)	103 (24.2)
Significance		$\chi^2=0.31$ ; $P=0.86$	$\chi^2=20.8$ ; $P<0.001^*$

<sup>a</sup>Some of this group may involve undetermined alleles causing FMF, \* $\chi^2$  test; significant if  $<0.05$

Comparing results of our registry to that from other Egyptian centers were not consistent; M694V allele had been found in all FMF patients (20 cases) who originate from Cairo.<sup>[18]</sup> In a work from Alexandria, Egypt, analysis of 316 FMF cases for 12 mutants showed that the most common mutations to be M694V, V726A, M694I, M680I and E148Q.<sup>[17]</sup> They found mutations in 182 (57.6%) patients; 20 were homozygous, 80 were compound heterozygous and 82 had only one identifiable mutant allele. The most common allele was M694I (34%) followed by E148Q (22.7%), V726A (15.6%), M680I (12.1%) and M694V (7.8%). They linked the M694V allele to disease severity and occurrence of amyloidosis.<sup>[17]</sup> Another Egyptian work analyzed the 12 known MEFV mutations, in a cohort of 136 Egyptian FMF cases, they found 132 cases (97.1%) had one of the five common mutations V726A, M694V, M680I, E148Q and M694I in the following frequency 41.2, 32.4, 29.4, 25 and 20.6%, respectively, thus classifying M694V allele as the second common mutations.<sup>[19]</sup>

According to our results, the most common allele was M694V (found in 295/426, 69.3%) however its homozygous state is relatively rare (found in only seven cases out of 295, 2.4%). The four diagnosed cases of amyloidosis among our chronic FMF patients all were genotyped M694V, thus suggesting the hypothetical link between this allele and development of amyloidosis in FMF disease. However, this link should not be generalized due to the following reasons: (1) M694V allele is quiet frequent in of our cohort, (2) tissue diagnosis of amyloidosis was not done in all cases. Knowing the multi-factorial pathogenesis of amyloidosis as well as

its rarity along the course of Egyptian FMF cases,<sup>[17]</sup> one should speculate the link of this complication to homozygous M694V allele rather than its presence in any heterozygous state (as 3 out of 4, 75% of the amyloid cases were homozygous M694V). A long prospective study should be contemplated to compare the outcome of homozygous, heterozygous or even compound heterozygous FMF cases having M694V allele as regards the development of amyloidosis. We recommend the use of fine-needle aspiration biopsy of anterior abdominal wall fat to survey for amyloidosis,<sup>[10]</sup> in all FMF cases and hence improve the productivity of the statistical linkage between M694V allele and amyloidosis. Recently, this association is proven through a comprehensive review of 27 papers from 20 centers, including 3505 Turkish FMF subjects, 189 out of 400 patients with amyloidosis (47%) were homozygous M694V ( $P < 0.0001$ ). This strong association has been confirmed also in Armenia and Israel.<sup>[20]</sup> These results should raise the necessity to carefully consider the early treatment of asymptomatic or mildly symptomatic patients with homozygous M694V, especially in countries where amyloidosis is rare like Egypt, moreover treatment in such cases should not be given on an on demand basis.

Going beyond Egypt but still within the Mediterranean coast, in Jordan, the most common allele was M694V, V726A, M680I, accounted for 38%, 26%, 10% respectively.<sup>[21]</sup> In Israeli-Arab cases, the most prevalent mutation was V726A, followed by M680I and M694V.<sup>[22]</sup> However, in Syria, the allelic frequency of M694V, V726A, M680I mutations was 45.8%, 26%, 4.8% respectively.<sup>[23]</sup> M694V and M694I alleles were the most common alleles in Arabs of Maghreb.<sup>[24]</sup> The most common mutation in an Italian sample was M694V allele; 16%.<sup>[25]</sup> Recently, in a cohort of 883 FMF Turkish citizens of Aegean region, screening for the 12 most common MEFV gene mutations had been done; M694V was found the most common allele followed by E148Q, M680I and V726A in a frequency of 48.4, 16.5, 13.5 and 9.7%, respectively.<sup>[26]</sup>

Beyond Mediterranean coast, screening the 12 common MEFV mutations using FMF strip assay test in a cohort of 36 Iranian FMF patients; 10 were homozygous (6 were M680I/M680I), 20 were compound heterozygous and 5 were single heterozygous. The

most frequent allele was M680I, followed by M694V and V726A.<sup>[27]</sup> These results share some features with ours; the significant percentage of homozygous M680I/M680I cases and the relative high frequency of M694V allele.

To sum up our Egyptian FMF registry in MUCH showed the following features: (1) M694V allele is the most prevalent, (2) not all the homozygous cases are M680I/M680I as previously estimated, (3) M694V/M694V genotype could constitute a potential risk for amyloidosis, to be confirmed in a further study. As the diagnosis of FMF is mainly clinical and the frequency of clinical symptoms in relation to the number of alleles in our cohort is almost the same, we recommend the use of pediatric clinical diagnostic criteria of Yalçinkaya *et al.*<sup>[14]</sup> in conjunction with the four most common MEFV mutations in Egypt as a cost benefit practice.

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