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ORIGINAL ARTICLES

Clinical and Molecular Diagnosis of MEFV Gene Mutations in Egyptian patients with Familial Mediterranean Fever

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ABSTRACT

Background: Familial Mediterranean Fever (FMF) is the most frequent periodic febrile syndrome among the autoinflammatory syndromes (AS). Nowadays it is considered as innate immunity disorders, characterized by absence of autoantibodies and autoreactive T lymphocytes. Familial Mediterranean fever (FMF) is an autosomal recessive condition caused by mutations in *MEFV* gene that encodes pyrin protein that primarily affect the population of the Mediterranean basin. Objectives: This study aims at the identification of MEFV gene mutations in Egyptian patients with Familial Mediterranean Fever. Subjects and Methods: Subjects included 56 Egyptian patients (26 males and 30 females) who were diagnosed formerly as FMF according to Tel-Hashomer criteria. Their age ranging from 4 -18 years old; the age of onset, however, was before the age of 10 years in most of the cases. They had been referred from health centers and hospitals of different regions of Egypt to the Molecular Genetics and enzymology Department, National Research Centre; for confirmation of diagnosis through molecular analysis. In this study, we analyzed the most common five mutations in the *MEFV* gene which were; M680I, M694V, M694I, V726A and E148Q using amplification refractory mutation (ARMS-PCR) and restriction fragment length polymorphisms (RFLP) techniques. Results: Most of the studied cases (62.5%) had attacks ranging from 1-3 days duration. The rate of recurrence was variable but (37.5%) of them suffered attacks 3-5 days with frequency ranging from 1-2 times/month in (71.4%) cases and was more in (33.9%) cases. Positive parental consanguinity was present in (17.8%) patients and positive family history for Familial Mediterranean fever (FMF) were present in (7.1 %) patients. The most common symptoms were abdominal pain (89.2%), fever (76.6%), arthritis (69.6%) and myalgia (25%). The most frequent mutations detected in our patients were M680I and M694I, and other mutations according to frequency were, V726A, M694V and E148Q. Positive mutations were detected in (71.4%) of our patients; (51.7%) of these patients were heterozygous, while (19.6%) were homozygous. The genotype-phenotype relationship showed that the M680I and M694I homozygous were associated with the most severe phenotype of the disease (earlier onset and more frequent arthritis and amyloidosis). Conclusion: The present study demonstrates distinct expression patterns of M680I and M694I mutation in the severe form of the disease indicating genotype/phenotype correlation. The identification of these mutations has opened up new ways of diagnosing these diseases through the molecular screening of mutations in the Egyptian population for proper genetic counselling and prenatal diagnosis. Diagnosis of FMF among Egyptian children cases although based mainly on clinical suspicion requires to be confirmed through detection of the corresponding mutation which can be easily made using the simple PCR techniques.

Key words: Familial Mediterranean Fever, Autoinflammatory disease, Inflammasome, Mutations, MEFV gene, PCR.

Introduction

Familial Mediterranean fever (FMF, OMIM*249100) is an autosomal recessive inherited auto-inflammatory disorder, characterized by short, recurrent, apparently unprovoked attacks of fever and serositis, or erysipelas-like skin lesions (Unal *et al*, 2012). FMF peritonitis, the most common manifestation of this disease, may resemble acute abdomen pain, leading to laparotomy and appendectomy that reveal only an inflamed peritoneum and neutrophilic exudates. If a surgical procedure is avoided, the attack resolves spontaneously. In these cases, proper genetic consultation may suggest the early introduction of colchicine (Settin *et al* 2007). FMF attacks may be triggered by common factors such as cold exposure, emotional or physical stress, infections or menstruation (Katsenos *et al* 2008).

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More than 15% of female patients experience perimenstrual attacks. It is proposed that oestrogens normally inhibit IL-6 production and mimic colchicine's effect on tubuli and adhesion molecules. During menstruation the protective effect of estrogen disappears, leading to the acute attack (Ben-Chetrit 2002).

FMF is the most frequent Periodic Febrile Syndrome among Autoinflammatory Syndromes (Ozen 2006). The disease is prevalent amongst populations surrounding the Mediterranean Sea such as Turks, Armenians, non-Ashkenazi Jews and Arabs (Ben-Chetrit and Touitou, 2009).

Familial Mediterranean Fever was first described in 1945 but the responsible gene (MEFV) was independently cloned by American and French groups in 1997 (The International FMF Consortium 1997 and The French FMF Consortium, 1997).

The protein encoded by the MEFV gene has been named pyrin by an American group for its role in anti-pyrexia (Shinkai *et al* 2005). Pyrin is expressed in the myeloid cell lineage and participates in inflammasome formation, a macromolecular complex, which has been defined as the linebacker of innate immunity. It has been hypothesized that the wild-type pyrin normally regulates inflammation via apoptotic speck-like protein. Its dysfunction determines auto-inflammatory syndromes. Pyrin consists of four domains; the most important are the N-terminal pyrin domain and the C-terminal *B20.3 domain*. The first one is intimately connected to protein-protein homotypic interactions, inflammation, apoptosis and immunity against tumors. The second one participates in the processes of innate immunity and is codified by sequences in exon 10 (Samuel and Ozen, 2006).

In FMF, however, the pyrin derived from the mutated gene seems to lose the ability to regulate the normal inflammatory process, particularly that part of the process due to the production of IL-1β and nuclear factor-kB (NF-kB) (Yao and Furst, 2008). N-terminal pyrin domain interacts with the ASC adaptor protein, regulating caspase-1 activation and consequently, IL-1β production. Mutations interfere with the role of the pyrin domain, allowing an uninterrupted inflammatory cascade (Lidar and Livneh, 2007). The MEFV gene is located on the short arm of chromosome 16 and includes 10 exons and it encodes 781-amino-acid protein. To date, 142 mutations have been identified in the MEFV gene, most of which are substitutions. Of these mutations, five account for more than 70% of FMF cases which are; V726A, M694V, M694I, M680I and E148Q and have different frequencies in classically affected populations. Forty-eight of the MEFV mutations are found in exon 10. Mutation E148Q in exon 2 was found to be the second most common mutation occurring in patients of several ethnicities with different haplotypes (Telatar and Grody, 2000). Exons 2 and 10 are the most frequent mutations of the MEFV gene. Half of the FMF population carries two mutations, 30% carry a single mutation and 20% carry a non identifiable mutation (Katsenos *et al* 2008).

The purpose of this study was to study the MEFV gene in the Egyptian population and to search for genotype/phenotype correlation.

Subjects and methods:

This study comprised 56 childhood cases with a suspected diagnosis of FMF. They manifested with recurrent high fever and abdominal pain, joint affection, myalgia, chest pain. These cases were referred from various hospitals and health centers of Egypt to the Molecular Genetics and Enzymology Department, National Research Centre for confirmation of diagnosis through molecular analysis. They included 26 males and 30 females. Their age ranged from 4 -18 years old. The age of onset, however, was before the age of 10 years in most of the cases. Informed consent was obtained from all parents according to the guidelines of the Ethical Committee of the National Research Centre (NRC).

The clinical manifestations of these cases were analyzed according to Tel-Hashomer clinical criteria for confirmation of the diagnosis (Pras, 1998 and Samli *et al*, 2006). Our study developed a score system of 10 items analyzing the clinical manifestations comprising family history, consanguinity, attack duration and frequency, fever, abdominal pain, chest pain, joint affection, myalgia and history of operations. This score has a maximum value for the diagnosis of 27 and a minimum of 10.

DNA extraction and purification:

Venous blood samples (~3 ml) were collected from all patients on EDTA (ethylenediamine tetraacetate) containing tubes. DNA was extracted promptly using DNA extraction and purification kit (Roche, Germany) according to the manufacturer's instructions and then stored at -20°C till use.

Quantification of genomic DNA:

Spectrophotometric optical densities of 260 nm and 280nm were used to investigate the DNA quantity. DNA purity was measured using the appropriate ratio of OD260: OD280 (1.65-1.85). Concentrations (ng/µl) and A260/A280 readings were recorded for each sample. The extracted DNA concentration was measured and

adjusted by dilution to conc. 20-25 ng/µl prior to PCR using deionized bi-distilled, sterile water (Fluka, Germany).

Mutation detection:

The presence of the five most common MEFV mutations was determined using amplification refractory mutation system–polymerase chain reaction (ARMS) and PCR–restriction fragment length polymorphism methods (RFLP) as previously described (The French FMF Consortium, 1997; Eisenberg *et al*., 1998 and Akar *et al*, 2000 and 2003).

The PCR amplification was performed in a final volume of 25 µL containing 100 ng of purified genomic DNA, 0.04 U of Ampli Taq Gold (Perkin-Elmer, Branchburg, New Jersey) and its 1× PCR buffer (contains 15 mmol of MgCl2 per L), 0.2 mmol of deoxynucleoside 5'-triphosphate mix per L (Gibco BRL, Gaithersburg, Maryland), and 1 pmol of each primer. Amplification conditions were kept the same for all of the ARMS tests, and the procedure was carried out as follows. The reaction was heated to 94 °C for 9 minutes for denaturation, followed by 35 cycles with denaturation at 94 °C for 10 seconds, annealing at 60 °C for 10 seconds, and extension at 72 °C for 30 seconds. A final extension was done for 10 minutes at 72 °C. PCR products and restriction enzyme–digested fragments were electrophoresed in a 2% agarose gel and observed by ethidium bromide staining.

Results:

This study included 56 Egyptian patients 26 (46.4%) were males and 30 (53.5%) were females. Patients were diagnosed formerly as FMF according to Tel-Hashomer criteria. Their age ranging from 4 -18 years old. The age of onset, however, was before the age of 10 years in most of the cases. Positive parental consanguinity was present in 10 (17.8 %) patients and positive family history of Familial Mediterranean fever (FMF) was present in 4 cases (7.1 %).

Attack duration was 1-3 days in most patients and it occurred in 35 cases (62.5%). The rate of recurrence was variable but 21 patients (37.5%) suffered from longer attacks (3-5 days). The frequency of attacks ranged from 1-2 times/month in 37 cases (71.4%), while 19 cases (33.9%) had a frequency of attacks more than 2-3times/month.

The main clinical characteristics of the patients were: abdominal pain found in 50 cases (89.2%), fever in 43 cases (76.7%), joints were affected during the attacks in 39 (69.9%) and myalgia in 14 cases (25%). Table 1; summarizes the phenotypic pictures of the study population.

Table 1: Demographic data and phenotypic expression of 56 patients with FMF.

Current age	4-18 years
Age of onset	< 10years
Positive Family history	7.1 (%)
Consanguineous marriage	17.8 (%)
Abdominal pain	89.2 (%)
Fever	76.7 (%)
Joint affection	69.9 (%)
Myalgia	25 (%)

MEFV mutations were studied using amplification refractory mutation system–polymerase chain reaction (ARMS) and PCR–restriction fragment length polymorphism methods (RFLP) as previously described. One and /or compound mutations were detected in 40 cases (71.4%) while 16 cases (28.6%) had no detected mutations. Homozygozity was represented in 11 patients (19.6%) and heterozygosity was found in 29 cases (51.7%).

The five common mutations of FMF detected in our study group revealed M680I and M694I were the most frequent mutations found in 13 cases (23.2%), followed by M726A present in 10 cases (17.9%) , M694V found in 9 cases (16%) and E148Q in 5 cases (8.9%) (Table 2 and 3) (Fig. 1).

The patients presenting with the homozygous form of M680I and M694I were associated with the most severe clinical form of the disease i.e. earlier age of onset, longer duration and higher frequency of the attacks. This confirms genotype/phenotype correlation. The same phenotype was also noticed in patients carrying the combination of M680I/M694I or M680I/M694V.

While milder manifestations were recorded in cases carrying the V726A homozygous genotype and E148Q homozygous or heterozygous genotype.

J. Appl. Sci. Res., 9(4): 3037-3044, 2012

Table II: Molecular genotyping of the five common mutations among the studied FMF Egyptian patients.

Genotype	Patients n=56	Frequency (%)
M694I/M680I	4	(7.1)
V726A/V726A	4	(7.1)
M680I/undef	4	(7.1)
M694V/undef	4	(7.1)
M694V/M680I	3	(5.3)
M680I/680I	3	(5.3)
M694I/M694I	3	(5.3)
M694I/undef	3	(5.3)
M694I/V726A	3	(5.3)
M680I/E148Q	2	(3.6)
E148Q/undef	2	(3.6)
V726A/undef	2	(3.6)
E148Q/E148Q	1	(1.7)
M694V/E148Q	1	(1.7)
M694V/V726A	1	(1.7)
Undef/undef	16	(28.6)

Table III: Alleles frequency in the studied patients.

Alleles	Frequency/alleles n=80	Frequency/cases n=56
M68I	16 (20%)	13(23.2%)
M694I	16 (20%)	13(23.2%)
M726A	14(17.5%)	10 (25%)
M694V	9(11.2%)	9(16%)
E148Q	5(6.2%)	5(8.9%)

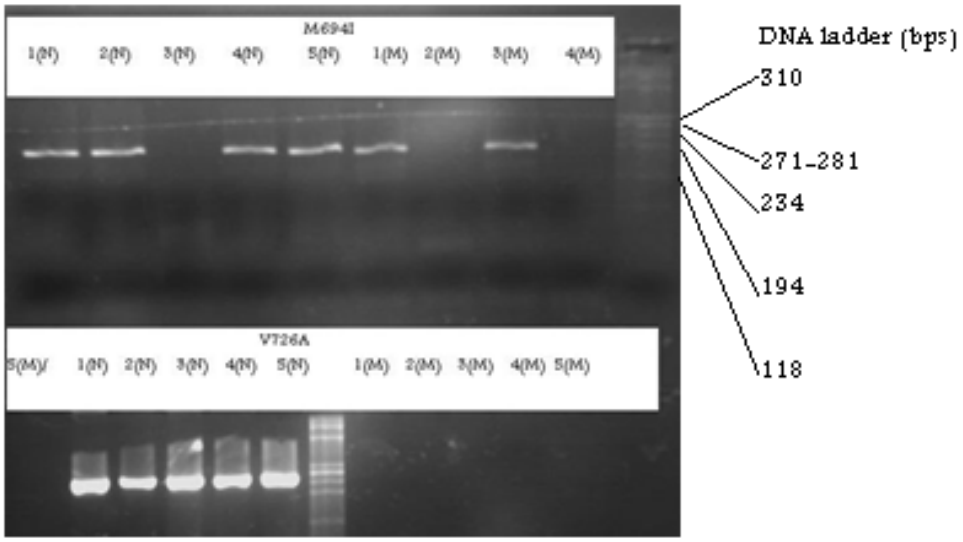

Fig. 1: DNA electrophoresis of amplified segments of FMF gene using normal and mutant primers corresponding to mutations M694I and V726A showing that cases no 1 and 3 are heterozygous and homozygous for M694I mutation respectively. All cases are normal for 726A. The used DNA ladder marker was phi X – 174 Hae III marker (M).

Discussion:

Familial Mediterranean fever (FMF) is an autosomal recessively-transmitted disease characterized by attacks of fever and serositis.

Traditionally, the diagnosis of FMF has been based on clinical manifestations. Following the cloning of MEFV, genetic analysis of its mutations has become a useful adjunct for establishing or confirming the diagnosis of FMF (Ben Chetrit *et al* 2002).

Consanguineous marriage could play an important role in the occurrence of FMF in Egypt at a relatively high rate (no exact estimate is available). The parental consanguinity and similarly affected family members of FMF in this study were detected in 17.8% (10/56) and 7. 1% (4/56) respectively. Dundar *et al*, 2012 described that there was consanguineous marriage in 16% of their studied patients. However, Inal and colleagues (2009) reported a higher rate of consanguinity (40%). Parental consanguinity and positive family history were also

reported by some other researchers in different ethnic groups , varying between 20 and 60% (Ozdemir *et al*, 2011).

In our study, the clinical spectrum of the FMF disease presented in most of the cases with an FMF attack duration of 1-3 days in 35cases (62.5%); longer attack duration (3-5 days) was found in 21cases (37.5%) with a frequency of 1-2 times/month in 37cases (66.07%), and more than 2 times/month in 19 cases (33.9 %). Shorter duration of attacks (2-3 days) was reported among Jewish cases (Gedalia *et al*, 1992). Italian cases had attack frequency of 12 attacks/year (La Regina *et al*, 2003). Manna *et al*, 2009 stated that in FMF, periodic attacks show inter- and intra-individual variability in terms of frequency and severity. Usually, they are triggered by apparently innocuous stimuli and may be preceded by a prodromal period (Manna *et al* 2009).

In this study, abdominal pain was the most common feature (89.2%) followed by fever (76.7%), joint affection by arthritis or arthralgia (69.9%) and myalgia (25%). In comparison with our study; Settin *et al*; found that abdominal pain was the most common symptom (87.9%) followed by fever (82%), arthritis or arthralgia (56.1%), chest pain (45%) and myalgia (6%). Laparotomy had been done during attacks for exploration or appendectomy in 27% of cases (Settin *et al*., 2007). In another study of Arab children Rawashdeh and Majeed, 1996 reported that 82% had recurrent abdominal pain, 43% had pleurisy, 37% had arthritis, 15% had cutaneous manifestations, 12% had splenomegaly and 4% had hepatomegaly. On the other hand, another Egyptian study was done by Zekri, *et al*, 2004 reported that the clinical features in his patients were fever (100%), abdominal pain (95%), arthritis (55%), pleurisy (40%) with no skin rash or pericarditis. Also they have reported that 25% of the cases had a past history of appendectomy or laparotomy. In another group of Arab patients, the most common manifestations were peritonitis (93.7%), arthritis (33.7%) and pleurisy (32%). The authors reported lack of manifestations of amyloidosis, skin lesions, organomegaly and lymphadenopathy (Barakat *et al*, 1986). Tunca *et al*, 2005 studied a large series of Turkish cases and noted that their clinical spectrum included peritonitis (93.7%), fever (92.5%), arthritis (47.4%), pleuritis (31.2%), myalgia (39.6%) and erysipelas- like erythema (20.9%).

The cloning of MEFV considerably improved our understanding of FMF population genetics. Over 200 sequence variants (mutations and polymorphisms) in the MEFV gene have been collected in the INFEVERS database (http://fmf.igh.crns.fr/ISSAID/invevers/) (Tchernitchko *et al*., 2005 and Milhavet *et al*., 2008). Four conservative missense mutations (M694V, M680I, M694I, and V726A) are located in exon 10, which, together with E148 Q mutation located in exon 2, represent the main five founder mutations and account for the vast majority of FMF chromosomes in typical FMF patients. In various recent studies, it has been reported that 70% to 80% of the total FMF cases consisted of these five mutations (Touitou, 2001; Majeed *et al*, 2002 and Tunca *et al*, 2005). These various mutations explain the different phenotypes in FMF patients (Shohat *et al*, 1999; Gershoni-Baruch *et al*, 2001; Kastner *et al*, 2005 and El-Garf *et al*, 2010). Analyzing the five common mutations of FMF in our study group revealed that, the most common mutations were M680I and M694I, followed by M726A, M694V and E148Q with a frequency of (23.2%), (17.9%), (16%) and (8.9%) respectively.

In another study in Egypt, Zekri *et al*, 2004 reported that the M694V mutation was detected in 100% and V726A mutation in 85% of their cases. Al-Alami *et al*, 2003 studied a mixed Arabic population sample from Egypt, Syria, Iraq and Saudi Arabia and found mutations in 53.4% of their cases with M694V and V726A mutations being the most common.

Moreover, in a Syrian study, 89% of their cases had one, two or three mutations. The allelic frequency of M694V, V726A and M680I mutations was 45.8%, 26% and 4.8% respectively (Mattit *et al*, 2006).

Furthermore, among Maghreb cases, the most frequent mutations were M694V and M694I. These account for different proportions of the MEFV mutations; (5% and 80%) in Algeria, (49% and 37%) in Moroccon, and (50% and 25%) in Tunisian patients. They hypothized that M694I mutation was specific to the Arab population from Maghreb (Belmahi *et al*, 2006).

In a mixed population of Jewish and Arab children with FMF, the M694V mutation was found in 92% of Jewish patients and in only 30% of the Arab patients. All other mutations were identified among 94% of the Arab patients, but with no particular prevalence for any one of them (Brik *et al*, 1999).

The M694V mutation was elevated among cases of Sephardi Jews (10.9%), and Oriental Jews (9.2%) while it was very low among Ashkenazis (0.8%). The most common mutation in an Italian study was M694V (16 %) (La Regina *et al* 2003).

Similarly, in Turkish cases, the mutation type M694V was the most frequent found in 51.4% of cases, especially in Non Ashkenazi Jewish followed by M680I mutation (14.4%) detected among the Armenian ancestry persons followed by mutation V726A (8.6%) found in Armenians, Ashkenazi Jews and Iraqi Jews (Tunca *et al*, 2005). In another study of Turkish cases, the frequency of the mutations was: M694V in 51.55%, M680I in 9.22% and V726A in 2.88% (Yilmaz *et al*, 2001). The difference in percentage between the results of this study and the previous may refer to the different number of cases in both studies.

Our findings showed that the allele mutation E148Q was less frequent among our patients since it was detected in (8.9%). This coincides with the study of Al-Alami and his colleagues (2003) who indicated that

E148Q has reduced penetrance in the Arab population and thus a proportion of the genetically affected individuals remain asymptomatic. Moreover, El Shanti *et al*., 2006 reported that E148Q mutation was the least penetrant and might be a polymorphism. It has been identified in Arab patients only and it is generally seen in healthy carriers (El Shanti *et al*, 2006). On the other hand in cases from Tunis 18% were positive for E148Q mutation (Touitou, 2003 and Chaabouni *et al*, 2007). On the contrary, among Lebanese patients Sabbagh *et al*., (2008) reported that the most important feature in their study was the relatively high frequency of E148Q that allowed them to question it as mutation rather than polymorphism. It was the second most common mutation in their study group (22.2%), while M694V mutation was found to be the most common (26.1%).

Lidar and Livneh (2007) reported that the role of the exon 2, E148Q mutation, as a disease-causing mutation is controversial. This non-founder mutation is found in populations in which FMF is distinctly rare, such as the Japanese, Chinese and Punjabi Indians. Additionally, E148Q homozygotes are rarely found in the FMF population.

FMF is a recessively inherited disease, finding two mutations does not necessarily confirm the disease. Phasing by analysis of the parent's DNAs is mandatory, especially if one of the two mutations is E148Q, a low-penetrance mutation up to 4% frequency in the general population. Not only may the phenotyping expression of the disease be attributable to a particular gene mutation, but the mutation also contributes to the disease severity (El Sayed and Al Jarallah, 2012).

Livneh (2011) found that the carriage of M680I, M 694V and M694I usually predicts a severe clinical course. Our results were consistent with this finding as we realized that the homozygous M680I mutation was associated with the most severe clinical spectrum of the disease. The same phenotype was noticed in M694I homozygous genotype and in patients carrying the combination of M680I/M694I or M680I/M694V. Similar reports suggested that the phenotype expression of M694V mutation was more severe than others (Koné Paut *et al*, 2000 and Shinar *et al*, 2000).

In recent studies, delineated that the severity of the disease is affected by environmental factors, and *MEFV* mutations are not the only cause of the disease. This suggestion was supported by comparing the disease activity of Turkish children living in Turkey with that of Turkish children living in Germany which showed a significantly more severe disease course in patients residing in their native country (Ozen *et al*, 2009). Thus, the genotype-phenotype correlation is very complex , with ethnic and environmental factors playing a role in the clinical outcome (Ureten *et al*, 2009 and Chae *et al*, 2009).

We therefore have reached the conclusion that the diagnosis of FMF among Egyptian cases, although based mainly on clinical suspicion, needs to be confirmed by the detection of the corresponding mutation. The identification of these mutations has opened up new ways of diagnosing these diseases for proper genetic counselling and prenatal diagnosis.

Conflict of interest

There was no conflict of interest to declare.

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