Molecular Diagnosis of Familial Mediterranean fever Among Subjects from Al-Qassim Region

Mahmoud El Sayed[1] and Badr Al Jarallah[2]

Departments of Biochemistry[1] & Internal Medicine[2], College of Medicine, Al Qassim University,
Sdr.mahmoud@yahoo.com

Abstract: This project was conducted to study Familial Mediterranean Fever (FMF) which is an autosomal recessive condition that primarily affect population of the Mediterranean basin , If undiagnosed effectively and treated with colchicine for life it may lead to serious consequences in terms of renal amyloidosis and renal failure . We aim to check for the presence of FMF mutations among clinically suspected cases among Saudi subjects in Al-Qassim region by PCR technique, also as an important step for family counseling and case management. The study is a pilot study to check for the presence of FMF mutations among suspected cases (24 cases) from Saudi subjects in Al Qassim region, The control subjects (7) were selected from healthy volunteers. We examined FMF mutations by PCR technique for MEFV gene analysis in order to establish a diagnosis of FMF by examining two common mutations, M694V and E148Q.. We found 8.3 % of cohort are positive for M694V mutation , and all cohort are negative for E148Q mutation . Moreover we found no correlation between clinical severity of the disease phenotype and the nature of the mutation. So genetic counseling by PCR technique provides a rapid, reliable, cost-effective, noninvasive, and sensitive test for establishing a diagnosis of FMF in symptomatic patients & also provides a rational basis for medical and genetic counseling and of FMF patients and their families.
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1. Introduction

Familial Mediterranean fever (FMF; MIM249100) is an autosomal recessive inherited auto-inflammatory disorder, which is frequent in populations originating from the Mediterranean basin **(Manukyan *et al*., 2008) .** It is a febrile disease characterized by acute, spontaneously resolving episodes of fever and pain caused by serosal inflammation and associated with mutations in the FMF gene, MEFV (**Tweezer-Zaks *et al*., 2007**). The MEFV gene was independently cloned by American and French groups in 1997: The protein encoded by the MEFV gene has been named pyrin by an American group for its role in anti-pyrexia. It is also called Marenostrin by the French group for the Latin word Marenostrum which stand for Mediterranean Sea. It has been hypothesized that the wild-type pyrin normally regulates inflammation via apoptotic speck-like protein. 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).** Pyrin belongs to a class of proteins involved in the regulation of apoptosis and inflammation. Its 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 located on short arm of chromosome 16 p13.3 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 (78 of them are missense, one – nonsense, 39 – silent mutations, 17 are located in introns, two – in UTS), one is duplication, two are insertions and two are deletions. Of these mutations, five account for more than 70% of FMF cases – V726A, M694V, M694I, M680I and E148Q7,26,27 and have different frequencies in classically affected populations. Forty-eight of the MEFV mutations so far identified are found in exon 10 (Yepiskoposyan and Harutyunyan, 2007). 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 mutation regions of the MEFV gene. Half of the FMF population carries two mutations, while 30% and 20% carry a single mutation and no identifiable mutation, respectively (**Katsenos *et al* ., 2008).**

The FMF disorder is characterized by recurrent episodes (exacerbations and remissions) of unprovoked inflammation involving the joints, the pleural and peritoneal cavities, And less frequently, the skin. FMF peritonitis, the most common manifestation of this disease, may resemble acute abdomen, leading to laparotomy and appendectomy that reveal only an inflamed peritoneum and

neutrophilic exudates. If a surgical procedure is avoided, the attack resolves spontaneously (**Settin *et al.*, 2007).**

In these cases proper genetic consultation may suggest early introduction of colchicine & enables early molecular confirmation of suspected cases, thus preventing undesirable abdominal surgery and unnecessary colchicine therapy. In addition, it enables rapid typing of FMF patients and their relatives, thereby allowing proper genetic and therapeutic consultation **(Eisenberg *et al*., 1998).**

And as many Arab populations have been investigated for MEFV gene mutations. However, data on Saudi FMF patients from Qassim region are still lacking. So the purpose of this study is to use PCR technique for MEFV gene analysis in order to establish a diagnosis of FMF in symptomatic patients to determine whether the clinical severity of the disease phenotype correlates with the nature of the mutation, and to perform a preliminary population genetic study in Qassim region.

2. Material and Methods

Material

The study is a pilot study to check for the presence of FMF mutations among suspected subjects (24 cases) from cohort of Saudi patients in Qassim region presenting to the Internal Medicine and Pediatric Departments of Qassim Hospitals & outpatient clinics of Qassim University during the years 2008-2009. The control subjects were selected from healthy volunteers.

The study and the control groups were matched in regard of age and sex. Informed consent had been obtained from patients and controls.

All patients were subjected to full analytic history and clinical examination, including: Age, sex, consanguinity, family history of FMF, attacks of abdominal pain, arthralgia or arthritis, chest pain, bone aches, renal affection, duration of attacks, effect of colchicines on frequency and duration of attacks, organ involvement, amyloidosis.

Clinical Scoring was evaluated according to Tel-Hashomer Criteria for diagnosis of FMF (**Samli *et al.*, 2006):**

Major Criteria:

(1) Recurrent febrile episodes accompanied by peritonitis, pleuritis, or synovitis; (2) Amyloidosis of A type without predisposing disease; (3) Favorable response to continuous colchicine treatment.

Minor Criteria:

(1) Recurrent febrile episodes; (2) Erysipelas - like erythema; (2) Positive history of FMF in first degree relative.

Definite Diagnosis = 2 major, Or 1 major + 2 minor**, Probable diagnosis** 1 major + 1 minor.

In this study we use standard PCR technique for MEFV gene analysis in order to establish a diagnosis of FMF by examining two mutations, M694V and E148Q and to determine whether the clinical severity of the disease phenotype correlates with the nature of the mutation among Saudi citizen.

1- DNA extraction and purification

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

2- Quantification of genomic DNA

Spectrophotometric optical densities of 260 nm and 280 nm 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).

3- Oligonucleotide primers

All primers used in this study were synthesized by (Tib Molbiol, Berlin, Germany) and obtained in a lyophilized state **(Zaks *et al*., 2003) .**

All primers were solved before use to obtain a final concentration of 20 pmol/µl of each. These primers make amplification for mutations that were coding regions of the E148Q and M694V mutation in the MEFV gene with the following sequences;

M694V:

Forward primer:

5'-ACTCTGTCGCCAGAGAATGGCTACTGGG TGGAGATAAATG-3'

Reverse primer::

5'-GTCAGGCCCCTGACCACCCACTGGACAG-3'

E148Q:

Forward primer:

5'-GCCTGAAGACTCCAGACCACCCCG-3'

Reverse primer:

5'-AGGCCCTCC-GAGGCCTTCTCTCTG-3'

4- DNA amplification & Mutation analysis

PCR was carried out on PCR system 2400 (Biometra, Whaman, UK).

For each series, a master mix was prepared. Master mixes were used to check for integrity of individual of materials. Confirmation of negative reactions by a second set of experiments was done as well.

Each DNA sample was tested for the two mutations (M694V &E148Q). All amplicons were stored at 4ºC until separation by gel electrophoresis.

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 (Roche, Germany) and its 1x PCR buffer (contains 15 mmol of MgCl2/L), 0.2 mmol of deoxynucleoside 5'-triphosphate mix/L (Roche, Germany), and 1 pmol of each primer.

The procedure was carried out as follows: The reaction was heated to 94°C for 10 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. Final extension was done for 10 minutes at 72°C.

Then the PCR products were separated by electrophoresis on a 2% agarose gel (Fluka, Germany). Ethidium bromide staining of the agarose gel was used to detect the amplified fragments.

5- Agarose gel electrophoresis examination for identification of PCR products

Together with the different amplicons were separated on 2% w/v agarose gel (LE, Roche)/TBE buffer stained with 0.01% ethidium bromide solution (0.5 mg/L). 10 µl of all amplicons and DNA marker were added stained before gel electrophoresis to 2 µl xylenecyanol dye solution (1 mg xylenecyanol, 400 mg sucrose and completed to 1 ml with water), and then subjected to electrophoresis for 45 min. The amplicons were made visible by ethidium bromide staining and documented using (Biostop, Germany).

The results from gel electrophoresis were visualized on a UV transillumination (254 nm) with a Phoretix workstation (Biostep, Germany).

Agarose gel preparation as well as electrophoresis were carried out using Tris-base/borate (TBE) buffer solution (pH 8.0), containing 45 mmol/L Tris-base / boric acid and 1 mmol/L EDTA adjusted with hydrochloric acid.

To determine the size of the DNA fragments, DNA of a known size (100 bp DNA marker, Roche, Germany) was used.

3. Results

Analysis of presenting clinical manifestations of studied patients, showed that non of them had a positive family history for FMF. Parental consanguinity was positive in 16.66% of these patients.

Recurrent febrile episodes and abdominal pain were reported in 95.8% of patients. Peritonitis affects 87.5% of all patients. Joints affection during attacks were: arthralgia (54.16%), arthritis (20.8%) and bone aches (83.3%). Chest pain was a symptom in 41.6% of patients. Pleuritis in 20.8% of patients. 20.8% from all patients underwent surgical operations 4.16% of them underwent laparotomy and appendectomy in 8.3% of them and tonsillectomy in 8.3% of patients. No Skin erythema was reported in all patients. Proteinuria suggestive for renal amyloidosis was found in 8.3% of patients. The results of examined FMF M694V and E148Q mutations showed that 8.3% (2 cases) had positive mutations for M694V mutation, while all our patients were negative for E148Q mutation. The normal controls were negative for previous two mutations.

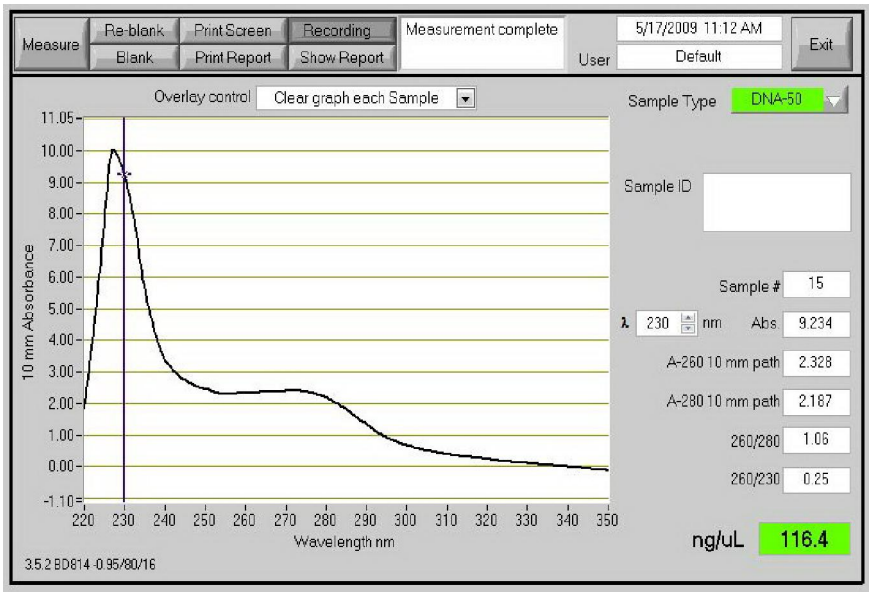

Figure (1): Showed example from the results obtained from DNA Nano Drop during DNA measured (determined) indicated the ration of 260/280 nano meter.

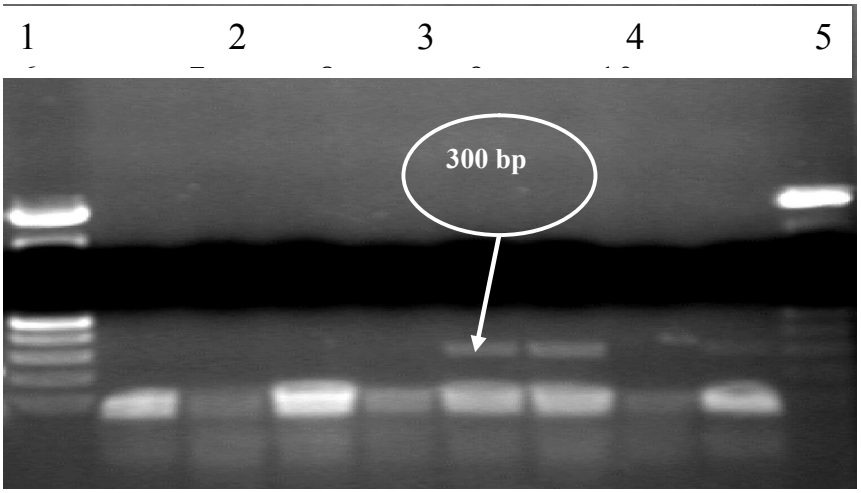

Figure (2): Detection of the MEFV gene in different blood samples. The size and location of the expected amplification product is indicated. DNA was extracted from different samples and examined by PCR-analysis using primer pair M694V for PCR-analysis. Line 1and10: DNA ladder100 bp, line 2: PCR control, Line 3-9 PCR products from patients samples. Lanes 6 and 7showed positive bands at 300 bp for M694V mutation. The positive samples were identified by using of PCR, the primer pair (Nurit Zaks *et al*., 2003) and able to amplifie 300 bp fragments from MEFV gene in blood samples DNA. Other patients samples were negative.

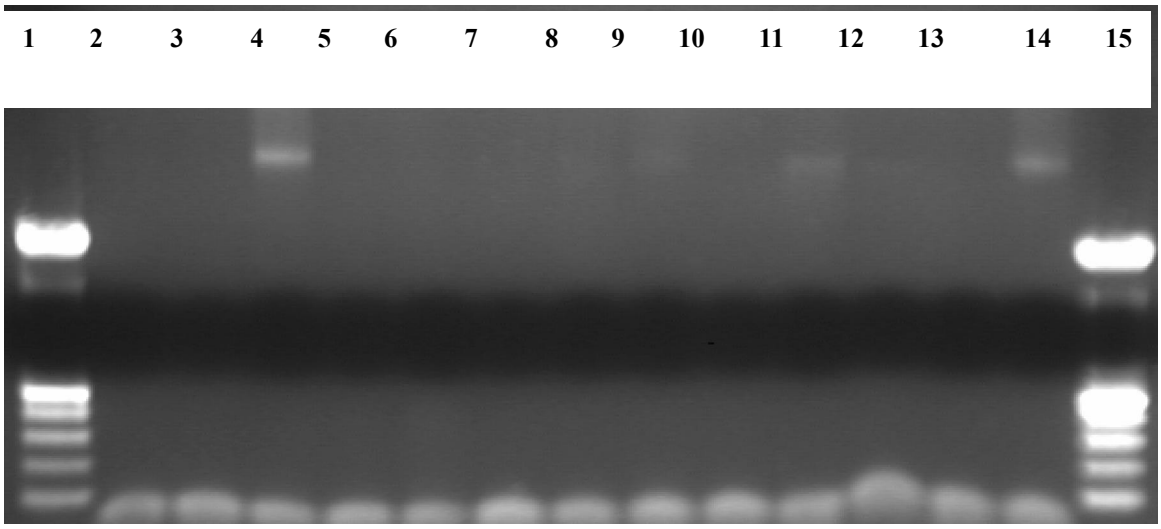

Figure (3): Detection of the MEFV gene in different blood samples. The size and location of the expected amplification product is indicated. DNA was extracted from different samples and examined by PCR-analysis using primer pair M694V for PCR-analysis. Line 1and 15: DNA ladder100 bp, Line 2: PCR control, Line 3-14 PCR products from patients samples. All samples were identified negative by using of PCR by the primer pair (Nurit Zaks *et al*., 2003).

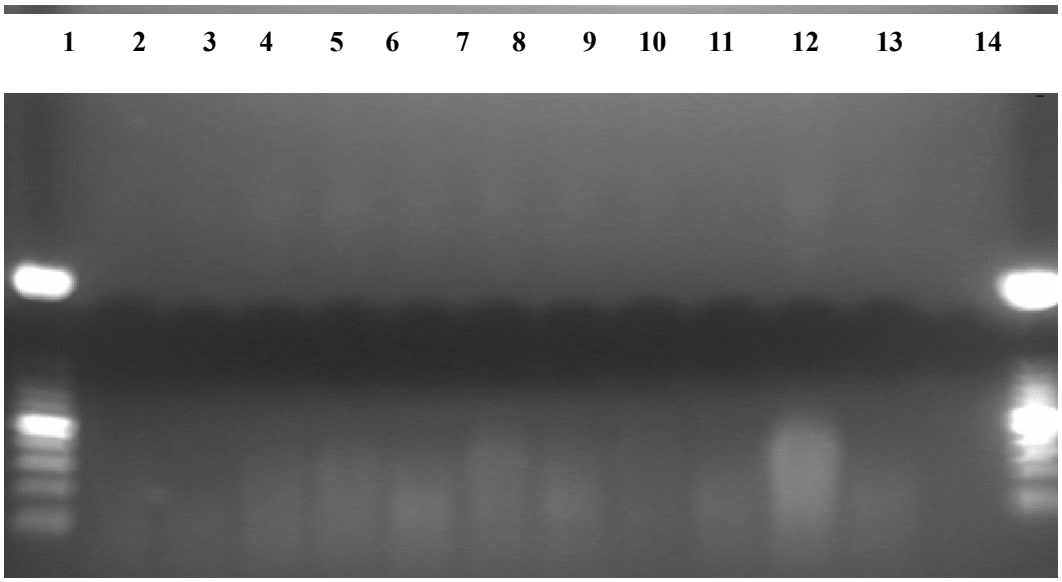

Figure (4): Detection of the MEFV gene in different blood samples. The size and location of the expected amplification product is indicated. DNA was extracted from different samples and examined by PCR-analysis using primer pair E148Q was used for PCR-analysis. Line 1and 14: DNA ladder100 bp, Line 2: PCR control, Line 3-12 PCR products from patients samples. All samples were identified negative by using of PCR by the primer pair (Nurit Zaks *et al*., 2003).

4. Discussion

Familial Mediterranean fever (FMF) is an autosomal recessively-transmitted disease characterized by attacks of fever and serositis (**Borman *et al*., 2009),** MEFV gene mutations are responsible for the disease. **(Jarjour, 2009)** This gene was discovered firstly in 1997 (Mediterranean FeVer – MEFV) and this has created possibilities to study the distribution of various mutations in geographically and ethnically different populations. **(Yepiskoposyan and Harutyunyan, 2007).**

Traditionally, the diagnosis of FMF has been based on clinical manifestations and the physician's experience. 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).**

In this study, recurrent febrile episodes and abdominal pain were the most common feature (95.8 %) followed by peritonitis (87.5 %), joint affection by bone aches (83.3 %) or arthralgia (54.16 %) or arthritis (20.8 %) and chest pain (41.6 %) or pleuritis (20.8 %). Interestingly, 20.8 % of cases had undergone surgery either appendicectomy (8.3 %) or tonsillectomy (8.3 %) or Spleenectomy (4.16 %), Proteinuria suggestive for amyloidosis AA (8.3 %) and skin erythema (4.16 %).

In comparison with this study, the only study in King Saudi Arabia was done by **Barakat *et al*. (1986)** conducted a retrospective study to assess the clinical profile, course and complications of familial mediterranean fever seen in 88 children over a period of 11 years. The group included 48 children (55%) who had onset before the age of 5 years (mean 4.9 years). Peritonitis occurred in 85% of children, arthritis in 50%, pleuritis in 33% and erysipelas-like lesions in 16%. Two children developed renal amyloidosis, and one third of the children were subjected to unnecessary operative surgery, reflecting the diagnostic difficulties. The arthritis was mono-articular in 80% and polyarticular in 20% of children with arthritis (Centre for Arab Genomic Studies).

In a 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*.). On the other hand, in another study on Arabs, 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.

In study among Egyptians, the clinical features 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 (**Zekri *et al*., 2004).**

Tunca *et al*. (2005) studied a large series of Turkish cases and noted that their clinical features included peritonitis (93.7%), fever (92.5%), arthritis

(47.4%), pleuritis (31.2%), myalgia (39.6%) and erysipelas-like erythema (20.9%).

We found positive M694V mutation in 8.3 % of all studied patients, which consistent with **El Shanti *et al*. (2006)** who found that M694V mutation is the most common MEFV mutation between Arabs, though it is less common in Arabs than in other ethnic groups.

Al-Alami *et al*. (2003) studied Arabic population from (Egypt, Syria, Iraq and Saudi Arabia) and found that only 53.4% are mutation positive. With mutational types M694V and V726A are the most common.

Another study from Jordan found that 59% had 1 or 2 mutations , of the studied mutations M694V, V726A, M680I, accounted for 38%, 26%, 10% respectively (**Majeed *et al*., 2005**).

Among Egyptians **Settin *et al*. (2007)** found that M694V is most common allelic mutation found followed by V726A then M680I (18.8 %, 17.42% 12.1% 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.

In Syria, 89% were positive either for one, two or three mutations. The allelic frequency of M694V, V726A, M680I mutations was in the form of 45.8%, 26%, 4.8% respectively **(Mattit *et al*., 2006).**

Among north Africa population, the most frequent mutations were M694V and M694I. These mutations account for different proportions of the MEFV mutations in Algeria (5%, 80%), Morocco (49%, 37%), and Tunisia (50%, 25%) patients. They pointed out that M694I mutation is specific to the Arab population from Maghreb **(Belmahi *et al*., 2006).**

We found that all studied patients are negative for E148Q Mutation which agree with study by **Al-Alami et al.,(2003)** who indicates that E148Q has reduced penetrance in the Arab population and thus a proportion of the genetically affected individuals remain asymptomatic.

El Shanti *et al*. (2006) reported E148Q mutation is the least penetrant and might be a polymorphism. It has been identified in Arab patients alone and in a complex allele with other exon 10 mutations, but is generally seen in healthy carriers. This may be noteworthy due to the use of the restriction endonuclease digestion test for the detection of the E148Q mutation that can lead to misdiagnosis in the presence of the E148V mutation, which could artificially increase the number of individuals identified as carriers of the E148Q mutation.

In **(2007) Lidar and Livneh** reported that around 80% of FMF patients have an identifiable MEFV mutation, 57% have two mutations, 26% have a single mutation, while 16% have no identifiable mutation and the majority of cases are caused by four mutations clustered on a single exon: M694V, V726A, M680I and M694I. The prevalence of which varies according to the population studied.

They also added 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 **(Lidar and Livneh, 2007).**

Al-Alami *et al*. (2003) found that the E148Q mutation was the most common among the healthy adult cohort, but was not present in affected individuals.

FMF is a recessively inherited disease, finding two mutations does not necessarily confirm the disease genetically. Phasing by analysis of the parent's DNAs is mandatory, especially if one of the two mutations is E148Q, a low-penetrance mutation frequent (up to 4%) in the general population (**Touitou, 2003).**

Moreover we found no correlation between clinical severity of the disease phenotype with the nature of the mutation, which is the same results obtained by **Telatar and Grody (2000)** whom found no consistent correlations between particular mutations and the type or the severity of FMF symptoms. The sole exception seems to be a significant association between amyloidosis and the M694V mutation. Aside from that association, however, specific MEFV mutations are not the sole determinants of phenotypic features such as age of onset, frequency or duration of attacks, expression of particular symptoms, or response to colchicine.

Yepiskoposyan and Harutyunyan (2007) reported FMF inflammatory attacks can be triggered by stress and extreme physical exercise. In general, the effect of environment on the inflammatory attacks in FMF is not surprising and is also seen in other cyclic conditions such as sickle cell anemia. However, in contrast to the latter, where only one mutation exists, in FMF, predisposition to the influence of environment is dependent on which mutations are present. This is confirmed by stronger predisposition in the M694V homozygotes.

This study is a preliminary genetic study in Qassim region which must be followed by further studies to help to know exactly the size of the prevalence of disease in Qassim region so prevent many unnecessary, unrevealing, and sometimes risky diagnostic and exploratory procedures .

In conclusion FMF is mainly a clinically diagnosed disease with validated diagnostic criteria

and it necessitating a high index of suspicion in patients from high-risk ethnic groups. If a surgical procedure is avoided, the attack resolves spontaneously. In these cases, proper genetic consultation may suggest early introduction of colchicines. Genetic counseling by PCR technique provides a rapid, reliable, cost-effective, noninvasive, and sensitive molecular genetic test for established a diagnosis of FMF in symptomatic patients and provides a rational basis for medical and genetic counseling and clinical treatment of FMF patients and their families.

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