Genetic screening of familial Mediterranean fever mutations in the Palestinian population

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ABSTRACT

Objectives: To investigate the spectrum of mutations and genotypes in the pyrin gene in familial Mediterranean fever (FMF) patients.

Methods: Blood samples of 511 suspected FMF patients, received from the Molecular Genetics Laboratory, Makassed Islamic Charitable Hospital, Mount Olives, Jerusalem during the period from June 1999 to August 2004, were investigated by genotyping 24 different MEFV mutations.

Results: Our work revealed the presence of 14 different mutations from the identified 24 mutations in the gene which are assembled in 6 homozygous, 9 heterozygous and 16 compound heterozygous genotypes. The homozygous genotypes represent the predominant format among our patients representing approximately 38% of the revealed genotypes. Interestingly, in 94 (31.4%) of the tested subjects, only one mutation in the pyrin gene could be identified while the other mutant allele remains unidentified. Moreover, the genotype of 3 (1%) patients revealed the presence of triplet mutations in the pyrin gene.

Conclusion: The results of our study clearly suggest that the origin of FMF among the Palestinian population is mostly homozygous. The identification of a significant number of patients with one known mutation indicates potentially the presence of new mutations in the gene which will be investigated in the future.

Saudi Med J 2005; Vol. 26 (5): 732-737

Familial Mediterranean fever (FMF) is an inherited inflammatory disease that is principally recognized in Jewish, Armenian, Turkish and Arab populations.[1] The characteristic intermittent clinical episodes of fever, peritonitis, pleurisy, rashes and arthritis are variable in their pattern, frequency, intensity and age of onset, as is the proportion of FMF patients in different ethnic groups who develop amyloidosis A (AA).[2] Since the release of chemotactic factors is one of the early events in an inflammatory response, therefore a defective inhibitor of these factors represent a highly susceptible target that can explain the observed clinical signs of the disease. Accordingly, a serine protease that inactivate complement component number 5a (C5a) and interleukin-8 was considered an immediate target,[3-6] which was eventually shown to display reduced activity in serosal fluids from FMF patients.[6,7-10] This finding suggests that the C5a/IL-8 inhibitor acts to prevent inappropriate inflammation in serosal tissues and therefore its reduced activity in FMF patients that could account for the typical attacks of sterile inflammation, which is one of the characteristics of the disease. Furthermore, colchicine an anti-inflammatory agent, was shown to be very effective in preventing the attacks of FMF as well as in the development of amyloidosis.[11] Although some occasional reports suggested that FMF can be inherited dominantly; however, these reports tended to be discounted on the basis that asymptomatic FMF carriers are very common in certain

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Received 20th October 2004. Accepted for publication in final form 13th February 2005.

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populations and thus, FMF can be better explained as pseudo-dominant inheritance in those patients.[12] The recent identification and cloning of the FMF gene, MEFV,[13,14] which is predominantly expressed only in neutrophils and encodes a protein named pyrin or marenostrin, enabled a precise molecular basis of the disease that is already used as a diagnostic marker.[12] In addition, diagnosis of FMF in patients also requires a high index of acute, reversible serosal attacks and family history when possible. Currently, 29 mutations are identified in the MEFV gene that is strongly and directly associated with the clinical expressions in FMF patients.[13]

In the present study, a comprehensive investigation was conducted to identify the spectrum of mutations and the associated genotypes in the pyrin gene that characterize FMF patients in the Palestinian population. The results showed that 14 of known mutations in the pyrin gene could be detected in our group of patients with a relatively high percentage of homozygous genotypes.

Methods. In our study, 511 suspected FMF patients were recruited from various hospitals, laboratories and private clinics. The subjects were requested to complete a questionnaire describing their residence, clinical manifestations of the disease including abdominal pain, fever, arthralgia, chest pain and back pain, number of attacks, family history and the familial relationship between their parents. All subjects were either individuals with clinical symptoms typical for FMF, those with the atypical form of FMF, or asymptomatic relatives of FMF patients. The study was conducted in the Molecular Genetics Laboratory, Makassed Islamic Charitable Hospital, Mount Olives, Jerusalem between June 1999 and August 2004.

Blood sampling and DNA extraction. Ethylenediaminetetraacetic acid blood samples (3-4 ml) were collected from all subjects, centrifuged, and DNA was extracted from the Buffy coat using Master pure Genomic DNA purification kit (Epicentre Technologies Co). The purified DNA was resuspended in Tris-ethylenediaminetetraacetic acid buffer (TE) and stored at -20^{0}C until use.

Polymerase chain reaction (PCR) amplification and sequencing. Polymerase chain reaction amplification and DNA sequencing were used to screen 24 known FMF mutations as follows: 18 in exon 10 (M694V, V726A, M680I, 2040G>C, M680Ib 2040G>A, M694I, T681I, M694DEL, I692DEL, K695R, A744S, R761H, S675N, M680L, Y688X, R653H, E656A, G678E, V704I), 3 in exon 2 (E148Q, E167D, T267I), 2 in exon 3 (P369S, R408Q), and one in exon 5 (F479L). Samples were initially analyzed utilizing Amplified Refractory Mutation System (ARMS) to screen for the most common mutations (M694V, V726A, M680I-a, M694I, E148Q). Homozygous individuals were excluded and the rest of the samples were then subjected to DNA sequencing of exon 10 utilizing an automated sequencer (ABI PRISM 377 Automated DNA sequencer). Other rare mutations outside exon 10 (P369S, R408Q, T267I, F479L, E167D) were identified by ARMS and confirmed by sequencing the exon in which the mutation is located.

Primers for 9 mutations (E148Q, T267I, P369S, R408Q, F479L, R653H, I692 del., M694del and M694I) were designed in our laboratory as described in **Table 1**. The primers for E167D, M680I-b, M694V, K695R, V726A, A744S and R761H were used as described before.[14,15] All PCR reactions were run in a final volume of 25ul, containing 200 or 400 ng of genomic DNA, 0.625U of Taq. Deoxyribonucleic acid polymerase (Takara) in 1X Buffer (50 mM potassium chloride, 1.5 mM magnesium chloride, 10 mM Tris-hydrochloride) with 0.2 mM deoxynucleoside 5'-triphosphate and 150ng of each primer. The PCR conditions included preheating for 10 minutes at 94°C, followed by 30 cycles of 94°C for 15 seconds annealing (mutation dependent) for 15 seconds 72°C for 30 seconds and a final extension step of 10 minutes at 72°C as described in **Table 2**.

The amplification of individual MEFV exons and flanking intronic sequences were performed in 100µl reaction containing 400-800 ng of Genomic DNA, 0.8 µg of each primer, 0.2 mM dNTPs mix, 1X PCR Buffer and 2.5U of Taq polymerase. The PCR conditions included an initial denaturation step of 10 minutes at 95°C, followed by 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 45 seconds, with a final extension step of 10 minutes at 72°C as outlined in **Table 2**. The PCR products were purified utilizing a commercially available kit (GFX™ PCR DNA and Gel Band Purification kit, Amersham, Pharmacia Biotech). The purified DNA was then subjected for sequencing using an ABI PRISM 377 automatic sequencer. The sequences were compared to the wild-type MEFV gene sequence retrieved from The National Center for Biotechnology Information Gene Bank.

Results. Among the 511 subjects included in the present study, 299 (58.5%) tested positive for one, 2 or 3 of the known FMF mutations in the pyrin gene. The various clinical characteristics of the disease were quite variable among these patients and distributed as follows; abdominal pain 281 (94%), fever 227 (76%), arthralgia 172 (57.5%), chest pain 125 (41.8%) and back pain 118 (39.5%). The onset of diagnosis ranged between 2-59 years and the male to female ratio was 1.3:1. Furthermore, 140 (46.8%) of the patients had a

Table 1 - Primers' sequences and lengths designed for Amplified Refractory Mutation System (ARMS) and sequencing products.

Mutation	Sequence [5`-3`]	Primer length	Primer type
ARMS primers			
E148Q	AGCCCCTGCAGCCTCCCCGCGGA	23	Common
	CAGCCTGCGGTGCAGCCAGCCCG	23	Normal
	CAGCCTGCGGTGCAGCCAGCCCC	23	Mutant
T267I	CTGCGCAGAAACGCCAGCTCCGC	23	Common
	TTCTGTTGCCGAGTCCAGATTCGCAGCTG	29	Normal
	TTCTGTTGCCGAGTCCAGATTCGCAGCTA	29	Mutant
R408Q	CCTGTAGCTCCCCTCTTTGCTGGACTGGTTTATATTGTG	39	Common
	AGTCTGAGTCAGGAGCACCAAGGCCACCG	29	Normal
	AGTCTGAGTCAGGAGCACCAAGGCCACCA	29	Mutant
P369S	CCTGTAGCTCCCCTCTTTGCTGGACTGGTTTATATTGTG	39	Common
	CATGAAAGGAAGAGCCCGGGAAGCCTAAGCC	31	Normal
	CATGAAAGGAAGAGCCCGGGAAGCCTAAGCT	31	Mutant
F479L	TTCCAGCACGGCTGATGGCAGAGCTG	26	Common
	TGTACTACTTCCTGGAGCAGCAAGAGCATTTC	32	Normal
	TGTACTACTTCCTGGAGCAGCAAGAGCATTTG	32	Mutant
R653H	TGGGATCTGGCTGTCACATTGTAAAAGGAGATGCTTCCTA	40	Common
	CCGAGTTTCCTCTCTGGCCGCCG	23	Normal
	CCGAGTTTCCTCTCTGGCCGCCA	23	Mutant
M680I-b	TTAGACTTGGAAACAAGTGGGAGAGGCTGC	30	Common
	ATTATCACCACCCAGTAGCCATTCTCTGGCGACAGAGCC	39	Normal
	ATTATCACCACCCAGTAGCCATTCTCTGGCGACAGAGCT	39	Mutant
I692del	GAAGATAGGTTGAAGGGGCCCAGAGAAAGAGCAGCTG	38	Common
	TCTGTCGCCAGAGAATGGCTACTGGGTGGTGATA	36	Normal
	TCTGTCGCCAGAGAATGGCTACTGGGTGGTGATG	36	Mutant
M694del	GAAGATAGGTTGAAGGGGCCCAGAGAAAGAGCAGCTG	38	Common
	CTGTCGCCAGAGAATGGCTACTGGGTGGTGATAATGA	37	Normal
	GCCAGAGAATGGCTACTGGGTGGTGATAATGAAGG	35	Mutant
M694I	CTGTATCATTGTTCTGGGCTCTCCG	25	Common
	GGACGCCTGGTACTCATTTTCCTTC	25	Normal
	GGACGCCTGGTACTCATTTTCCTTT	25	Mutant
Sequencing primers			
Ex-10	CCCGCAAAGATTTGACAGCTGTATC	25	Forward
	GGACAGATAGTCAGAGGAGCTGTGTTCTT	29	Reverse
Ex-2	CCCTAAACGTGGGACAGCTTCATC	28	Forward
	CCATTCTTTCTCTGCAGCCGATATAAAG	24	Reverse
Ex-3,4	AGGGGGTTTTTCCACTGCATGTCC	24	Forward
	GGCCATCAGCCACCTCTGACCTTA	24	Reverse
Ex-5,6	AGACCCCCATACCTCCCTGT	23	Forward
	GTACACCACAGGACCTCGCTGTA	20	Reverse

Table 2 - Polymerase chain reaction conditions for Amplified Refractory Mutation System (ARMS) and sequencing products.

Mutation	DNA (ng)	Additives		Tm[1]	Product size (bp)[2]
		DMSO %	Formamide %		
E148Q	400	-	5	62	227
E167D	200	-	5	60	259
T267I	200	2.5	5	60	221
P369S	200	-	5	67	352
R408Q	400	-	5	66	232
F479L	200	-	-	60	272
R653H	200	-	5	63	280
M680-a	400	-	5	63	219
M680-b	400	-	5	60	219
I692del	400	-	5	66	225
M694del	400	-	-	65	224
M694V	400	-	-	63	211
M694I	400	-	2.5	63	192
K695R	400	-	5	65	260
V726A	400	-	-	63	246
A744S	400	-	-	63	298
R761H	200	-	5	60	200
Exon10	100	-	-	55	435
Exon 2	200	3	-	55	731
Exon 3,4	200	3	-	55	899
Exon 5,6	200	3	-	55	781

DMSO - dimethyl sulfoxide, Tm[1] - annealing temperature, bp[2] - base pair

Table 3 - Spectrum of familial mediterranean fever mutations.

N	Mutations	Independent alleles	
		n	(%)
1	M694V	247	(49)
2	V726A	84	(16.7)
3	M694I	60	(11.9)
4	E148Q	43	(8.5)
5	M680I	20	(4)
6	P369S	13	(2.6)
7	R408Q	13	(2.6)
8	A744S	8	(1.6)
9	M680Ib	4	(0.8)
10	R761H	4	(0.8)
11	R653H	4	(0.8)
12	F479L	2	(0.4)
13	E167D	1	(0.2)
14	K695R	1	(0.2)
Total		**504**	**(100)**

positive family history of FMF while the parents of 133 (44.5%) proved to be relatives. Genetic analysis allowed the identification of 14 different mutations in 504 (84.3%) of the 598 independent tested alleles as shown in **Table 3**. The remaining 94 alleles (~16%) tested normal for all the 24 known mutations. The 5 most dominant mutations among this group included M694V (49%), V726A (16.7%), M694I (11.9%) and E148Q (8.5%), M680I (4.4%) that account for 90% of all detected mutations. The remaining 9 mutations (P369S, R408Q, A744S, M680Ib, R653H, K695R, E167D, F479L, and R761H) were much less represented and had frequencies ≤2.6% each. The various mutated genotypes of the pyrin gene, which is the characteristic of these patients are described in **Table 4**. It is very clear that the majority of the patients (38%) were homozygous of 6 mutations including the 4 predominant mutations with the 2 additional rare mutations (E148Q and R761H mutations). The genotypes of the remaining patients were divided into 2 groups; 91 patients (30.4%) were compound heterozygous for either 2 (29.4%) or 3 mutations (1%) while in the remaining patients (31.4%) only one of the 24 known mutations could be detected after screening the expected sites in the gene. Evidently, the M694V mutation was also the most dominant among all compound heterozygous genotypes. Interestingly, approximately one third of the patients (31%) revealed the presence of only one of the known mutations in the pyrin gene. These patients possess other unidentified mutations in the gene, express pseudo dominancy in the related mutations or are misdiagnosed with other FMF-like diseases.

Discussion. Evidently, identification of the spectrum of mutations in a gene that is directly linked to a common inherited disease represents a part of the genetic identity of inflicted populations. The cloning of the pyrin gene provided the necessary tool to investigate the genetic variation among FMF patients in the various populations. In the present study, the frequency of mutations and genotypes in the MEFV gene is described within FMF patients among Palestinian Arabs who are particularly susceptible to the development of the disease.[1] In our work, we screened for the expression of 24 known mutations in the MEFV gene among 511 suspected individuals which led to positive identification of one, 2, or 3 mutations in 299 (58.5%) subjects to confirm FMF among this group. These results revealed the existence of 14 different mutations in the pyrin gene. The most common founder of MEFV gene mutations includes M694V, V726A, M694I, M680I, and E148Q, accounting for 90% of the total mutant alleles and the remaining 10% mutations included M680Ib,

A744S, F479L, E167D, R761H, P369S, R408Q, K695R and R653H (**Table 3**). In addition, 94 of the total 598 alleles proved to be normal with respect to the identified mutations.

The mutational profile among the Palestinian patients is in concordance with other Arab populations, especially Lebanese patients where the 5 most frequent mutations are exactly the same but occur with different frequencies.[16] Notably, the M694V constitute the most common frequent mutation among our study comprising nearly half of the total mutated alleles (49%), while it represents approximately one third of the FMF alleles among Armenians, Turks and Arabs patients.[4,5] This mutation is over represented in North African Jews reaching an approximately 90% of the tested alleles;[18,19] however, it is detected only in 20% of Jordanian and 27% of Lebanese FMF alleles.[16,17] Presumably, the remarkably high frequency of the M694V mutation among our study group is attributed either to the relatively high rate of consanguinity especially that the homozygous M694V/M694V genotype accounts for 28.4% of the patients. Evidently, consanguineous marriages are an ancient custom that is widely spread among Palestinians and reaches approximately 44.5% among our tested group which is compatible with the national frequency that reaches almost 49.7% according to the official values of the Palestinian Central Bureau of Statistics, which indicates that the marriages among members of the same clan reaches about 49.3% (Personal contacts). In comparison to Jordanians, the M680I mutation comprises the third common mutation, while it is the fifth common mutation among our study group.[17]

Alternatively, the V726A mutation that was reported to be the most common mutation among both Ashkenazi Jews and Israeli Arab patients,[20,21] representing the second most common mutation among our study group and accounting for 16.7% of the MEFV alleles similar to the reported values among Jordanian FMF patients.[17] On the other hand, the M694I mutation which was found to be rare but confined to Arab patients and more specifically to Meghrebins,[16,21,22] was detected in 11.9% of the alleles, higher than that for Jordanians and close to the Lebanese FMF mutation profiles. Furthermore, the E148Q mutation, which was encountered among patients from several distinct origins,[20,23,24] was encountered in 8.5% of the mutant alleles among the patients in the present study. Mutations other than the 5 common mutations that usually encounter ≤1% of the FMF alleles each,[13] account for 10% of the total mutant alleles in our present work. Except for the P369S (2.6%), R408Q (2.6%) and A744S (1.6%), the rest of the rare mutations are found with similar frequencies as reported before.[13] The A744S mutation which was claimed to be over represented among Arabs[13,25] was found to be carried by 8 patients, representing 1.6% of the total described alleles.

Concerning the current revealed genotypes, the most common ones, namely M694V/M694V, M694V/V726A and M694V/-, are in accordance with the Lebanese genotypes with the exception that the V726A/- genotype being the third common among these patients.[16] One of the striking finding in the present study is the detection of 3 patients with the complex allele (E148Q/P369S/R408Q) that was described once in an Armenian patient.[13,23] Similarly, the P369S/R408Q genotype that was reported in one Armenian patient[13] was detected among 10 patients in our study. The remaining 212 suspected patients where no known mutations could be detected, this can be explained by one of the following possibilities. First, a proportion of these patients may carry other rare mutations that lie outside exon 10 such as R42W (exon 1), E230K, E148V, L110P (exon 2), and I591T (exon 9). Second, the presence of unidentified mutations in

Table 4 - Genotype of patients (N=511).

N	Genotype	Patients n	(%)
Homozygous			
1	M694V/ M694V	85	(28.4)
2	M694I/ M694I	13	(4.3)
3	M680I/M680I	7	(2.3)
4	V726A/V726A	6	(2)
5	E148Q/E148Q	2	(0.67)
6	R761H/ R761H	1	(0.33)
Total		**114**	**(38.10)**
Compound heterozygous			
7	M694V/V726A	31	(10.40)
8	M694V/E148Q	12	(4)
9	M694V/M694I	6	(20)
10	M694I/V726A	9	(3)
11	M694I/M680I	2	(0.67)
12	M694I/E148Q	5	(1.7)
13	V726A/M680I	2	(0.67)
14	V726A/M680Ib	4	(1.340
15	V726A/F479L	1	(0.33)
16	V726A/E167D	1	(0.33)
17	V726A/R761H	1	(0.33)
18	P369S/R408Q/E148Q	3	(1)
19	P369S/R408Q	10	(3.34)
20	M694I/ R761H	1	(0.33)
21	M694V/ M680I	2	(0.67)
22	M694V/ R653H	1	(0.33)
Total		**91**	**(30.4)**
Heterozygous			
23	M694V/-	25	(8.4)
24	E148Q/-	21	(7)
25	M694I/-	11	(3.7)
26	V726A/-	23	(7.7)
27	M680I/-	2	(0.67)
28	A744S/-	8	(2.7)
29	F479L/-	1	(0.33)
30	R653H/-	2	(0.67)
31	K695R/-	1	(0.33)
Total		**94**	**(31.4)**

other exons, introns the promoter or the 3' untranslated regions. Third, the etiopathology of at least some of these patients could be attributed to other periodic syndromes other than FMF including Hyperimmunoglobulinemia-D or the tumor necrosis factor-receptor associated syndrome. Fourth, in a group of clinically diagnosed FMF families, the disease may be linked to a different gene that may be responsible for some cases of FMF other than the known chromosomal MEFV region (16p13.3) as was shown previously.[26]

We believe that the present study provides comprehensive information on the MEFV mutational profile among Palestinian FMF patients in comparison to other populations. Definitely, extending our knowledge on the various genetic backgrounds in association of a disease provides an essential element that may help understand the variable expression of a disease among patients within a population or between the various inflicted populations. Definitely, it became obvious that determining the various MEFV gene mutations and their frequencies among different inflicted ethnic groups represents an important step in understanding the pathophysiology of this disease.

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