Am. J. Hum. Genet. 65:88–97, 1999

MEFV-Gene Analysis in Armenian Patients with Familial Mediterranean Fever: Diagnostic Value and Unfavorable Renal Prognosis of the M694V Homozygous Genotype—Genetic and Therapeutic Implications

Cécile Cazeneuve,[1] Tamara Sarkisian,[3] Christophe Pêcheux,[5] Michel Dervichian,[7] Brigitte Nédelec,[5] Philippe Reinert,[2] Alexandre Ayvazyan,[4] Jean-Claude Kouyoumdjian,[1] Hasmik Ajrapetyan,[3] Marc Delpech,[5] Michel Goossens,[1] Catherine Dodé,[5] Gilles Grateau,[6] and Serge Amselem[1]

[1]Service de Biochimie et de Génétique Moléculaire and the Institut National de la Santé et de la Recherche Médicale (Unité 468), Hôpital Henri-Mondor, and [2]Service de Pédiatrie, Centre Hospitalier Intercommunal de Créteil, Créteil, France; [3]Department of Genetics, Yerevan State University, and [4]Erebuni Medical Center, Yerevan, Armenia; [5]Service de Biochimie Génétique, Hôpital Cochin, and [6]Service de Médecine Interne, Hôpital de l'Hôtel-Dieu, Paris; and [7]Service d'Hépato-Gastro-Entérologie, Centre Hospitalier de Villeneuve-Saint-Georges, France

Summary

Familial Mediterranean fever (FMF) is a recessively inherited disorder that is common in patients of Armenian ancestry. To date, its diagnosis, which can be made only retrospectively, is one of exclusion, based entirely on nonspecific clinical signs that result from serosal inflammation and that may lead to unnecessary surgery. Renal amyloidosis, prevented by colchicine, is the most severe complication of FMF, a disorder associated with mutations in the MEFV gene. To evaluate the diagnostic and prognostic value of MEFV-gene analysis, we investigated 90 Armenian FMF patients from 77 unrelated families that were not selected through genetic-linkage analysis. Eight mutations, one of which (R408Q) is new, were found to account for 93% of the 163 independent FMF alleles, with both FMF alleles identified in 89% of the patients. In several instances, family studies provided molecular evidence for pseudodominant transmission and incomplete penetrance of the disease phenotype. The M694V homozygous genotype was found to be associated with a higher prevalence of renal amyloidosis and arthritis, compared with other genotypes (P = .0002 and P = .006, respectively). The demonstration of both the diagnostic and prognostic value of MEFV analysis and particular modes of inheritance should lead to new ways for management of FMF—including genetic counseling and therapeutic decisions in affected families.

Received February 2, 1999; accepted for publication April 29, 1999; electronically published May 26, 1999.

Address for correspondence and reprints: Dr. Serge Amselem, Service de Biochimie et de Génétique Moléculaire, Hôpital Henri-Mondor, 51 Avenue du Maréchal de-Lattre-de-Tassigny, 94010 Créteil, France. E-mail: amselem@im3.inserm.fr

0002-9297/99/6501-0013$02.00

Introduction

Familial Mediterranean fever (FMF) is an autosomal recessive condition (MIM 249100) that primarily affects populations surrounding the Mediterranean basin, the disease being restricted essentially to Armenian, Sephardic Jewish, Turkish, and Arab populations (Sohar et al. 1967). The frequency of heterozygotes, as deduced from the prevalence of the disease, is extremely high in those populations, reaching 1:7 among Armenians (Rogers et al. 1989). This disease is characterized by recurrent episodes of fever and serosal inflammation manifested by sterile peritonitis, arthritis, and/or pleurisy, sometimes associated with erysipelas-like erythema. The major complication of FMF is amyloidosis, mainly renal, which develops over years and progresses to terminal renal failure (Sohar et al. 1967).

Given the absence of pathognomonic clinical symptoms and of any specific biochemical abnormality, the diagnosis of FMF is, at present, one of exclusion; it can be made only retrospectively, and is based entirely on clinical criteria (Livneh et al. 1997). It is, however, of prime importance to ascertain this diagnosis, for the following reasons. First, the symptomatology of FMF may mimic that of other affections—such as acute peritonitis, appendicitis, cholecystitis, or arthritis—thereby leading to unnecessary exploratory surgery (Sohar et al. 1967). Second, an effective therapy is available: daily and lifelong administration of colchicine not only reduces the frequency and severity of attacks (Dinarello et al. 1974; Zemer et al. 1974) but also prevents amyloidosis and transplantation for renal failure (Zemer et al. 1986). Furthermore, the rare observation of renal amyloidosis as the first and sometimes only manifestation of the disease (Blum et al. 1962; Sohar et al. 1967; Saatci et al. 1997) emphasizes these diagnostic difficulties and raises

questions with regard to the value of developing presymptomatic diagnostic tests for use in FMF families.

Recently, a candidate gene for FMF (designated "MEFV"), located on the short arm of chromosome 16 (Pras et al. 1992; French FMF Consortium 1996; Levy et al. 1996), has been identified independently by two consortia following a positional cloning approach (French FMF Consortium 1997; International FMF Consortium 1997). This gene, which spans ~15 kb of the 16p13.3 region, is composed of 10 coding exons. The 3.7-kb MEFV transcript expressed in polynuclear leukocytes predicts a protein that has been named both "marenostrin" (French FMF Consortium 1997) and "pyrin" (International FMF Consortium 1997) and that consists of 781 residues. Although the function of the marenostrin/pyrin protein is unknown, several lines of evidence suggest a possible role in the regulation of inflammation (French FMF Consortium 1997; International FMF Consortium 1997). In these first studies (French FMF Consortium 1997; International FMF Consortium 1997), four conservative missense mutations (i.e., M680I, M694V, M694I, and V726A), which are clustered in exon 10 and are associated with ancestral haplotypes, were recovered exclusively on carrier chromosomes, thereby suggesting that the isolated gene was involved in FMF, a hypothesis further supported by the recent description of nonfounder MEFV mutations (Bernot et al. 1998). However, no unambiguous mutations—such as nonsense, frameshift, or splice mutations—have been described thus far; in addition, to date, there are no available in vitro assays or animal models by which to investigate the mutations recently identified in humans.

The identification of the MEFV gene prompted us to investigate a large cohort of independent Armenian patients, with the following aims: (i) to evaluate the interest of MEFV gene analysis to establish a diagnosis of FMF, (ii) to determine whether the clinical severity of the disease phenotype correlates with the nature of the mutation, and (iii) to determine whether such molecular investigations both lead to new ways of managing this disease and further confirm the involvement of the MEFV gene in FMF.

Patients and Methods

Patients

We investigated 90 patients (age 4–68 years [mean 28.8 years]; male:female ratio 1.2:1.0) from 77 unrelated FMF families originating from Armenia, representing a total of 163 independent alleles. Of these 77 families, 16 were living in France; the remaining 61 families (70 patients) were living in Armenia. The diagnosis of FMF was made according to established clinical criteria (Livneh et al. 1997). None of the families were selected through genetic-linkage analyses using MEFV-gene markers. A parent-to-offspring transmission of the disease phenotype was documented in three families.

Clinical features prior to onset of colchicine therapy (renal amyloidosis, arthritis, fever, peritonitis, pleurisy, erysipelas-like rash, and diarrhea) were recorded on a standardized form. Since colchicine was not easily available in Armenia, all but one of the patients living in that country did not have access to daily administration of colchicine. Informed consent was obtained from all patients or their parents.

Mutation Analysis

Genomic DNA was isolated from peripheral leukocytes, by standard procedures. Different methods were used to screen for MEFV mutations, according to the strategy described in figure 1*a*. The M694V and V726A mutations (French FMF Consortium 1997; International FMF Consortium 1997) were screened for by *Hph*I and *Alu*I digestions, respectively, performed on PCR products generated by primers pM694V-f (5′-AGAATGGCTACTGGGTGGAGAT-3′) and p10A-r (5′-AGAGAAAGAGCAGCTGGCGAATGTAT-3′). The E148Q mutation (Bernot et al. 1998) was screened for by *Bsr*I digestion of PCR products generated by primers p2-f (5′-CCGCAGCGTCCAGCTCCCTG-3′) and pE148Q-r (5′-GCTTCCTCGACAGCCCCCTCCCGGACT-3′). The pM694V-f and pE148Q-r primers are mismatched primers: the pM694V-f primer abolishes the *Hph*I restriction site that is located 9 bp upstream from the M694V mutation site that creates a *Hph*I restriction site, whereas the pE148Q-r primer introduces a *Bsr*I restriction site in the presence of the E148Q substitution.

Denaturing gradient gel electrophoreses (DGGE) of PCR-amplified products of exons 5 and 10 were performed within the framework of an exhaustive scanning approach designed to screen all the MEFV coding exons and their flanking intronic sequences (Cazeneuve et al. 1998). These experiments were performed to analyze both the major part of exon 10 (fragment 10A, consisting of 212 nucleotides surrounding the four clustered mutations [i.e., M680I, M694V, M694I, and V726A], and fragment 10B, consisting of 185 nucleotides allowing screening for mutations in the 3′ end of exon 10) and the entire exon 5 together with flanking intronic sequences. The DGGE conditions were determined by means of the MELT87 and SQHTX programs, kindly provided by L. Lerman. The primer sets used to amplify fragment 10A, fragment 10B, and exon 5 were p10A-f (5′-$[GC]_{40}$GCATGGATCCTGGGAGCCTG-3′) and p10A-r, p10B-f (5′-$[GC]_{50}$CGTGGACTACAGAGTTGGAAGC-3′) and p10B-r (5′-ATACAAGGCCAGAAGCAGG-3′), and p5-f (5′-$[GC]_{50}$CTGGGGGTTCC-

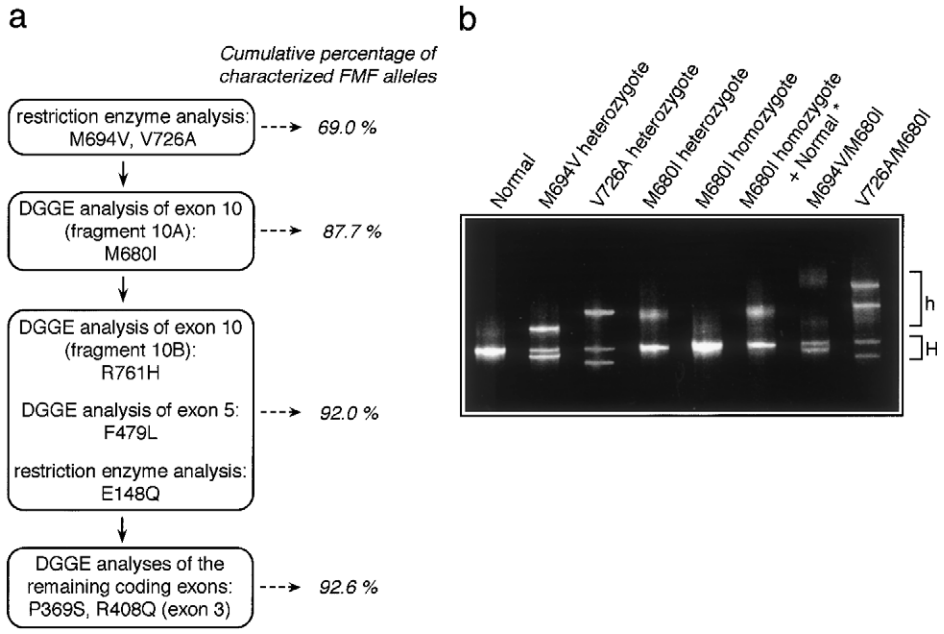

Figure 1 Strategy used to screen for MEFV-gene mutations with one representative DGGE experiment. *a*, Screening strategy. The strategy includes restriction-enzyme analysis of PCR-amplified fragments, which was first performed to screen for the M694V and V726A mutations (see Patients and Methods section). In the absence of these mutations, a DGGE-based screening system was performed to analyze 212 nucleotides of exon 10 (fragment 10A) surrounding the four initially described mutations (i.e., M694V, V726A, M680I, and M694I); to further analyze the MEFV gene, two DGGE experiments were performed to screen for sequence variations in the 3′ end of exon 10 (fragment 10B) and the entire exon 5 together with flanking intronic sequences; the E148Q mutation was screened for by restriction-enzyme analysis; all the remaining coding sequences and intronic boundaries were subsequently scanned for mutations by means of DGGE analysis. *b*, Representative DGGE migration patterns of 10A PCR-amplified fragment of exon 10 from patients presenting with various genotypes. H = homoduplexes; h = heteroduplexes. The asterisk (*) refers to the particular situation in which the PCR products did not display a shift in mobility; they thus were systematically mixed with a PCR product from a normal control (v/v) and then were subjected to a cycle of melting and reassociating to generate heteroduplexes that, in this case, point to the presence of the M680I mutation.

TGGACATCC-3′) and p5-r (5′-GAGCTGGGAGCC-TGAGGCAT-3′), respectively. Fifteen microliters of the PCR products were subjected to electrophoresis at 230 V during 5 h in a 6.5% polyacrylamide gel containing a linearly increasing denaturing gradient (range 20%–70% [for fragments 10A and 10B] or 20%–80% [for exon 5]). The FMF alleles that remained uncharacterized were subsequently screened for mutations in all other MEFV coding exons and flanking splice junctions (i.e., exons 1–4 and 6–9 and the 5′ part of exon 10), by means of DGGE, with experimental conditions that are available on request. The PCR products that did not display a shift in mobility were systematically mixed with a PCR product from a normal control (v/v) and were subjected to a cycle of melting and reassociating, to generate heteroduplexes, in order to improve the sensitivity of the DGGE assay. All samples displaying a shift in mobility were subsequently directly sequenced.

MEFV-Genotype Determination

The MEFV genotype of each affected individual carrying at least two different mutations was accurately identified by different means, depending on both the availability of parental DNA samples and the location and nature of the two MEFV mutations. When DNA samples from relatives were not available for study, we analyzed the PCR products spanning the two identified mutations, using the following procedures: determination of the genotype of patients who carried the M694V and V726A mutations was performed by means of DGGE analysis of fragment 10A, as described above (the two mutated homoduplexes migrate at positions different from that of the wild-type sequence); since the M680I mutation resulted in homoduplexes that could not be discriminated from wild-type homoduplexes (i.e., isostable mutation), the genotype of patients who carried both the M680I and M694V mutations was accurately identified by means of a double digestion of the PCR products generated, by primers p10A-f and pM694V-r (5′-GCTGGACGCCTGGTACTCATTTTCGTTAA-3′), with restriction enzymes *Hpa*I and *Hin*fI; a similar approach was designed to accurately identify the genotype of patients carrying the V726A and M680I mutations, by use of primers pM680I-f (5′-GACATCCATA-

Table 1

MEFV Gene–Sequence Variations in Armenian Patients

A. Spectrum of MEFV-Gene Mutations

Mutation	No (%) of Independent Alleles
M694V	72–74 (44.8)[a]
V726A	39 or 40 (24.2)[a]
M680I	30 or 31 (18.7)[a]
F479L	4 (2.5)
E148Q	2 (1.2)
R761H	1 (.6)
E148Q-P369S-R408Q[b]	1 (.6)
Unidentified allele	12 (7.4)
Total	163 (100)

B. MEFV Gene Polymorphisms

Nucleotide Variant[c]	Codon	Location
306, T→C	102	Exon 2
414, A→G	138	Exon 2
495, C→A	165	Exon 2
605, G→A	202	Exon 2
942, C→T	314	Exon 3
1356+44, A→G	...	Intron 4
1422, G→A[d]	474	Exon 5
1428, A→G[d]	476	Exon 5
1503, C→T	501	Exon 5
1518, C→T	506	Exon 5
1530, T→C[d]	510	Exon 5
1588−69, G→A	...	Intron 5
1759+8, C→T	...	Intron 8
1760−30, T→A	...	Intron 8
1764, G→A	588	Exon 9

[a] In two independent patients, it was impossible to determine which FMF allele (M694V or V726A in one patient and M694V or M680I in the other patient) was inherited from the affected parent.
[b] Identified in *cis*.
[c] Newly identified variations are underlined.
[d] Always associated in *cis*.

AGCAGGAAAGGGAAGAT-3′) and p10A-r, with restriction enzymes *Alu*I and *Dpn*II; primers p10C-f (5′-GAGGTGGAGGTTGGAGACAA-3′) and p10B-r were used to determine whether the M680I and R761H mutations were located on the same allele or on two different MEFV alleles; in the latter case, the PCR products were analyzed by enzymatic digestion by restriction enzyme *Nla*III. pM694V-r and pM680I-f are mismatched primers that allow enzymatic identification of the M694V and M680I mutations, respectively. To precisely determine the genotype of the patient who carried three different MEFV mutations, we cloned the PCR fragment generated by primers p2-f and p4-r (5′-CGGGGAC-CCCTGCTCACT-3′), which encompasses the three mutated sites.

Statistical Analysis

The statistical significance of differences between groups was calculated by either the χ^2 test or Fisher's exact test. All statistical tests were two-sided.

Results

Characterization of Sequence Variations in the MEFV Gene: Diagnostic Value of MEFV-Gene Analysis

By means of the strategy described in figure 1*a*, 23 different sequence variations were identified in our population sample. Seven of them (listed in table 1A) consist of seven mutations—M694V, V726A, M680I, R761H, F479L, E148Q, and P369S—that have been described elsewhere (French FMF Consortium 1997; International FMF Consortium 1997; Bernot et al. 1998; Cazeneuve et al. 1998; Samuels et al. 1998; Aksentijevich et al. 1999), whereas one, R408Q (G→A transition at nucleotide 1223), is new and was found to be associated with the E148Q and P369S mutations in the same patient. The 15 remaining sequence variations, 5 of which are new, are polymorphisms (i.e., silent substitutions or intronic sequence variations) (table 1B). A representative DGGE experiment illustrating the characterization of different FMF alleles is shown in figure 1*b*.

The MEFV genotype of each individual carrying at least two different mutations was accurately identified by different means, depending on the availability of parental DNA samples and the location and nature of MEFV mutations (see Patients and Methods section). This analysis showed that, in our population of patients, the eight identified missense mutations accounted for 93% of the 163 independent FMF alleles, with the M694V substitution representing nearly half of the characterized alleles (table 1A). Fourteen different genotypes were characterized among 85 of the 90 patients (table 2); the 85 patients included 77 independent patients and 8 patients who shared one allele with an affected relative,

Table 2

Genotypes at the MEFV Locus in Armenian Patients

Genotype	No (%)
M694V/M694V	18 (21.2)
M694V/V726A	22 (25.9)
M694V/M680I	13 (15.3)
V726A/M680I	9 (10.6)
M680I/M680I	4 (4.7)
V726A/V726A	3 (3.5)
V726A/F479L	3 (3.5)
M694V/E148Q	2 (2.4)
M680I/R761H	1 (1.2)
M680I/F479L	1 (1.2)
M680I/unidentified allele	1 (1.2)
M694V/unidentified allele	2 (2.4)
V726A/unidentified allele	2 (2.4)
E148Q-P369S-R408Q[a]/unidentified allele	1 (1.2)
Unidentified allele/unidentified allele	3 (3.5)
Total	85[b](100)

[a] E148Q, P369S, and R408Q were associated in *cis*.
[b] Includes 77 independent patients and 8 patients who shared one allele with an affected relative.

Table 3

Clinical Features of FMF Patients with One or Two Uncharacterized FMF Alleles

Patient	Genotype	Clinical Features[a]						
		Renal Amyloidosis	Arthritis	Fever	Peritonitis	Pleurisy	Erysipelas-like Rash	Diarrhea
1	Unidentified allele/unidentified allele	−	−	+	+	−	−	−
2	Unidentified allele/unidentified allele	−	−	+	+	−	+	−
3	Unidentified allele/unidentified allele	−	+	+	+	−	−	−
4	M694V/unidentified allele	−	−	+	+	−	−	+
5	M694V/unidentified allele	−	+	+	+	+	−	−
6	M680I/unidentified allele	−	+	+	−	−	−	−
7	M680I/unidentified allele	−	−	+	+	−	−	Not available
8	V726A/unidentified allele	−	+	−	+	−	+	−
9	Complex allele[b]/unidentified allele	−	−	+	+	−	−	−

[a] For each patient, recurrent attacks (i.e., at least three of the same type) associating at least two of the major established clinical criteria (Livneh et al. 1997) were documented; the presence or absence of each clinical sign is indicated by a plus sign (+) or a minus sign (−), respectively.

[b] E148Q, P369S, and R408Q mutations in *cis*.

whereas the 5 remaining patients belonged to families with affected siblings carrying identical MEFV genotypes. The determination of the genotype in individuals with at least two different MEFV mutations revealed that all patients with two mutations were indeed compound heterozygotes, whereas the patient with three mutations (E148Q, P369S, and R408Q) actually carried all three mutations in *cis* on the same allele (tables 1A and 2). The M694V homozygous and compound heterozygous (M694V/other mutation) genotypes were displayed by almost two-thirds of the patients. In this mutation-screening procedure, both FMF alleles were characterized in 76 (89%) of the 85 patients. After a complete screening for mutations of all MEFV coding exons and intronic boundaries by means of DGGE, the two FMF alleles remained unidentified in three patients, whereas one FMF allele was uncharacterized in six patients (table 2); the disease features of these nine patients are presented in table 3. In addition, in three families, pseudodominant transmission was clearly demonstrated, with the patient's affected parent carrying two mutated MEFV alleles (fig. 2*a–c*).

Surprisingly, three healthy relatives of affected children from three different families, who were investigated within the framework of a systematic analysis, were found to carry two mutated MEFV alleles: two of these healthy relatives—one mother (individual CI_2, age 51 years) and one father (individual DI_1, age 46 years) of affected children—displayed the V726A/M680I compound-heterozygous genotype, whereas the remaining healthy female relative (individual EII_1, age 9 years) displayed the M694V/M680I compound-heterozygous genotype (fig. 2*c–e*).

Phenotype-Genotype Correlations: Prognostic Value of MEFV-Gene Analysis

To determine whether the clinical severity of the disease correlated with the MEFV genotype, we compared the clinical features among subgroups of patients with the three major genotypes (i.e., M694V homozygous, M694V/V726A compound heterozygous, and M694V/M680I compound heterozygous), as well as in M694V homozygous patients versus patients with other genotypes. Results of phenotype-genotype correlations are

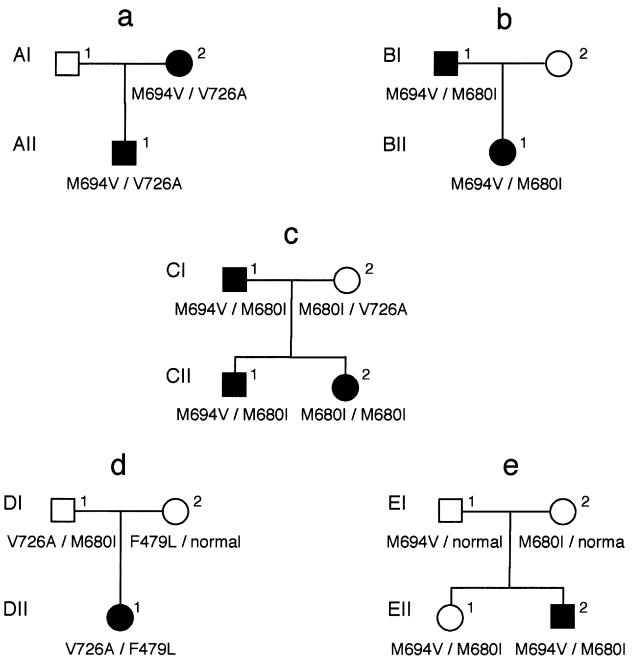

Figure 2 Pedigrees illustrating atypical modes of inheritance in FMF. Pseudodominant transmission of the FMF phenotype is shown in pedigrees a–c. Incomplete penetrance of the disease phenotype is shown in pedigrees c–e (individuals CI_2, DI_1, and EII_1, each of whom carried two mutated MEFV alleles, are clinically asymptomatic). Note the presence of both pseudodominant transmission and incomplete penetrance in family c. The DNA samples from individuals AI_1 and BI_2 were not available for study.

Table 4

Clinical Features Associated with MEFV Mutations in Armenian Patients with FMF

Genotype	Renal Amyloidosis[a] (48.5%[b])		Arthritis (60%[b])		Fever (91%[b])		Peritonitis (90%[b])		Pleurisy (57%[b])		Erysipelas-Like Rash (11.5%[b])		Diarrhea (19%[b])	
	Frequency	*P*	Frequency	*P*	Frequency	*P*	Frequency	*P*	Frequency	*P*	Frequency	*P*	Frequency	*P*
M694V/M694V	10/10		18/21		20/21		19/21		9/20		4/20		4/19	
M694V/V726A	1/8		12/24		20/24		22/24		18/23		1/24		2/24	
M694V/M690I	2/4		7/13		13/13		13/13		8/13		1/13		2/11	
Overall[c]		.0009[d]		.009[d]		>.05		>.05		>.05		>.05		>.05
M694V/M694V	10/10		18/21		20/21		19/21		9/20		4/10		4/19	
Other	6/23		36/69		62/69		62/69		41/68		6/68		11/60	
Overall[e]		.0002		.006		>.05		>.05		>.05		>.05		>.05

[a] Both all the patients with renal amyloidosis and all the patients without amyloidosis who had not undergone colchicine therapy at age <40 years have been taken into account.

[b] Overall frequency.

[c] *P* values apply to all three genotypes, unless otherwise indicated.

[d] Patients with the M694V/V726A and the M694V/M680I genotypes were grouped for this calculation.

[e] *P* values apply to the two genotypes.

summarized in table 4. In addition, to identify the clinical signs associated with each FMF mutation, phenotype-genotype correlations between the groups of patients with one or two M694V, V726A, or M680I alleles and the group of patients who did not carry the particular mutation were also studied.

The prevalence of renal amyloidosis was assessed by taking into account both (*a*) all the patients with renal amyloidosis and (*b*) those without renal amyloidosis who had not undergone colchicine therapy at age <40 years, since amyloidosis usually appears at age <40 years (Sohar et al. 1967) (table 5). MEFV-gene analysis revealed that renal amyloidosis was not observed in the three patients who had one or two uncharacterized alleles (table 5). We found a higher point prevalence of renal amyloidosis in M694V homozygous patients than in patients with other genotypes (10/10 vs. 6/23 [$P = .0002$]); the difference was also significant when M694V homozygous patients were compared with M694V/V726A compound-heterozygous patients (10/10 vs. 1/8 [$P = .0005$]). However, there was no significant variation, in terms of the point prevalence of renal amyloidosis, when patients bearing one or two M694V alleles were compared with patients who did not carry the M694V allele; the same conclusion was obtained both (*a*) when patients with one or two V726A alleles were compared with patients who did not carry the V726A mutation and (*b*) when patients with one or two M680I alleles were compared with patients who did not carry the M680I mutation (data not shown).

The M694V homozygous patients also presented more frequently with arthritis, compared with patients who had other genotypes ($P = .006$); the difference was also significant when M694V homozygous patients were compared with patients carrying the two other major genotypes (i.e., M694V/V726A and M694V/M680I) ($P = .009$) (table 4). With regard to the prevalence of either fever, peritonitis, pleurisy, erysipelas-like rash, or diarrhea, there was no significant difference either between the M694V homozygous, the M694V/V726A compound-heterozygous, and the M694V/M680I compound-heterozygous subgroups of patients (table 4) or in patients with one or two M694V, V726A, or M680I alleles versus patients who did not have the particular mutation (data not shown).

Discussion

We tested the diagnostic value of MEFV-gene analysis in FMF patients belonging to a population that displayed several important characteristics: the studied population consisted of a large number of independent families;

Table 5

Point Prevalence of Renal Amyloidosis, According to Genotypes at the MEFV Locus

Genotype	Prevalence[a]
M694V/M694V	10/10
M694V/V726A	1/ 8
M694V/M680I	2/ 4
V726A/M680I	2/ 3
V726A/V726A	1/ 3
M680I/R761H	0/ 1
M694V/unidentified allele	0/ 2
M680I/unidentified allele	0/ 1
E148Q-P369S-R408Q[b]/unidentified allele	0/ 1
Total	16/33

[a] All the patients presenting with renal amyloidosis have been taken into account, as have those without amyloidosis who had not undergone colchicine therapy at age <40 years (see text).

[b] The E148Q, P369S, and R408Q mutations were associated in *cis*.

none of these families were selected through linkage analyses using MEFV-gene markers; rather, all the patients were included in this study solely because they fulfilled the established clinical criteria for the diagnosis of FMF. Delineation of the spectrum of MEFV mutations in our cohort of Armenian patients led to the identification of (*a*) three mutations (i.e., M694V, V726A, and M680I) of the four located in exon 10 that were initially reported (French FMF Consortium 1997; International FMF Consortium 1997); (*b*) the more recently reported mutations—E148Q, F479L, and R761H, located in exons 2, 5 and 10, respectively (Bernot et al. 1998); and (*c*) the P369S mutation, located in exon 3 (Aksentijevich et al. 1999). The R408Q mutation, which was identified in the present study, was present on only one FMF allele. Although the functional significance of this mutation remains to be demonstrated, it is noteworthy that the substitution replaces a charged residue by a neutral amino acid; in addition, this nonconservative mutation lies in a region that comprises the C-terminal residues of the evolutionarily conserved B-box zinc-finger domain potentially involved in protein-protein interactions (Reddy et al. 1992); furthermore, when compared with residues occupying the same position in other members of the B-box family, Arg408 follows His407, which belongs to the conserved His/Cys residues potentially involved in metal binding. Nevertheless, residue 408 is not evolutionarily conserved: several members of the B-box family, including MID1, a putative transcription factor mutated in Opitz G/BBB syndrome (Quaderi et al. 1997), Staf50, an interferon-induced protein (Tissot and Mechti 1995), and rpt-1, a protein involved in the down-regulation of the interleukin-2 receptor (Partaca et al. 1988), contain a glutamine residue at this position; however, this striking observation is not sufficient to exclude any pathological consequences of the R408Q mutation, since a similar situation exists for the M680I, V726A, and R761H mutations located in another evolutionarily conserved domain of the protein, the B30.2 domain.

Molecular tools, which were developed to screen all MEFV coding exons and flanking intronic boundaries for mutations, represent the first laboratory tests of diagnostic value for the disease, with the eight identified mutations accounting for 93% of the 163 independent FMF alleles and with both FMF alleles being characterized in 89% of our patients. Such a diagnostic value of MEFV-gene analysis was not documented in a recent study performed in a non-Ashkenazi Jewish population of patients (Eisenberg et al. 1998); however, the difference between the latter study—which was designed to search for only three MEFV mutations in a smaller population of independent patients—and our study may result from MEFV allelic differences between two populations that do not share the same genetic background. In light of our results, in the Armenian population, it is, in return, now conceivable to design prospective studies to accurately evaluate the different sets of clinical criteria for the diagnosis of FMF (Livneh et al. 1997), by use of the result of MEFV-gene analysis as the first objective diagnostic criterion. By use of this molecularly based diagnostic procedure, one FMF allele remained uncharacterized in only six patients, and no mutation was detected in three patients, even after the screening of all MEFV coding regions and intronic boundaries. Several hypotheses may account for these observations: (i) in these patients, sequence variations may exist in the unexplored regions of the MEFV gene (i.e., intronic sequences, promoter and untranslated regions); (ii) although it is clearly established that DGGE is a highly sensitive technique for mutation detection (Cotton 1997), we cannot rule out the possibility that, in rare instances, a few mutations remained undetected by this experimental procedure; (iii) given the absence of any objective criterion for the diagnosis of FMF, the FMF-like symptomatology presented by these patients may result from a different disease, although, in these nine patients, the clinical diagnosis was still clear in retrospect. However, FMF shares several clinical features with other periodic-fever syndromes, such as hyperimmunoglobulinemia D with periodic-fever syndrome (MIM 260920) and dominantly inherited familial Hibernian fever (MIM 142680). Although, so far, these periodic-fever syndromes have not been reported in patients of Armenian ancestry, we can anticipate that, in this population, MEFV mutation analysis would be helpful for differential diagnosis of these clinically related syndromes.

One striking feature of FMF is the recent description of complex alleles with two mutations associated in *cis*—that is, alleles E148Q-I692del and E167D-F479L (Bernot et al. 1998), E148Q-V726A (Bernot et al. 1998; Aksentijevich et al. 1999), and E148Q-P369S (Aksentijevich et al. 1999). In this study, we identified the first FMF allele containing three different mutations in *cis:* E148Q-P369S-R408Q, the R408Q mutation having not been previously identified on other P369S or E148Q alleles, an observation that further documents the molecular diversity of the complex alleles found in FMF. In theory, such alleles could arise either sequentially, by the occurrence of independent mutational events, or by intragenic recombinations between simple alleles; in FMF, MEFV haplotype analyses rather favor the latter hypothesis (Bernot et al. 1998; Aksentijevich et al. 1999). The description of complex alleles may also have important clinical consequences: this unusual feature raises the question as to the extend to which the MEFV-gene analysis should be completed when two different mutations have been identified (i.e., are the two mutations located on the same allele? do other mutations exist in the same gene?). In addition, in the absence of functional

studies, one cannot exclude the possibility that some of the mutations identified in complex alleles either produce a mild effect or are simple polymorphisms; this could especially be the case for the E148Q and/or P369S mutations, which, in several instances, have been found in association with other mutations (Bernot et al. 1998; Aksentijevich et al. 1999; present study); this may also account for the high carrier frequency with reduced penetrance just recently reported for E148Q and P369S in the Ashkenazi Jewish population (Aksentijevich et al. 1999).

In the course of our study, in three unrelated families three healthy relatives (one male and two females) of affected children were found to carry two mutated MEFV alleles. These observations, which were documented with likely disease-causing mutations (i.e., V726A and M680I) in two adults, provide molecular evidence for incomplete penetrance of the disease phenotype and point out the likely existence of modifier factors; in the healthy 9-year-old girl with the M694V/M680I genotype, a delayed onset of the disease cannot be ruled out, although her brother, who carries the same genotype, has exhibited typical FMF disease since age 3 years. One part of the modifier factors may be related to sex; this is indeed suggested by the unbalanced male: female ratio (1.20:1.00) in our population of patients, an observation that already has been reported by others (Saatci et al. 1997) and that, in theory, may result from the incomplete penetrance of the disease phenotype in females and/or an increased embryonic death of female zygotes with two mutated MEFV alleles.

In three families, MEFV-gene analysis showed that the parent-to-offspring transmission of the disease phenotype was explained by the presence of two mutated MEFV alleles in the affected parent. These molecular data, which further clarify the atypical mode of inheritance reported by others (Rogers et al. 1989; Yuval et al. 1995), demonstrate a pseudodominant transmission of the disease. Pseudodominant transmission and incomplete penetrance of the disease phenotype should, therefore, be taken into account when FMF families are investigated within the framework of molecular diagnosis; in addition, as shown in this study (fig. 2*c*), the mode of inheritance of the disease phenotype can be further complicated by the association of both pseudodominant transmission and incomplete penetrance within the same nuclear family.

To interpret the correlation data that we observed between the genotype and the clinical symptomatology, it is important to emphasize that all investigated patients were of Armenian ancestry; they were therefore expected to share a common genetic background, thereby allowing correlation studies in a relatively homogeneous population; in addition, the occurrence of renal amyloidosis, which is a long-term complication of the disease, could not have been prevented in the patients who did not have access to daily administration of colchicine. When both all the patients with amyloidosis and those without amyloidosis who had not undergone colchicine therapy at age <40 years were considered, the prevalence of renal amyloidosis was 16/33 (48.5%); the high rate of this renal complication, which was observed only in patients who had not undergone regular colchicine therapy, further underscores the critical importance of the treatment (Zemer et al. 1986).

One of the most striking results of this study is the strongly unfavorable prognostic value that the M694V homozygous genotype has with respect to renal function. The prevalence of renal amyloidosis is indeed higher in M694V homozygous patients than in patients with other MEFV genotypes ($P = .0002$). This statistically significant association of the M694V homozygous genotype with renal amyloidosis was not shown in the recent studies that investigated populations of patients who either had been undergoing colchicine therapy for many years (Dewalle et al. 1998; Samuels et al. 1998) or were genetically heterogeneous with a limited number of patients carrying the M694V homozygous genotype (Samuels et al. 1998). In other respects, no significant difference in the frequency of amyloidosis was observed in our patients carrying one or two M694V alleles versus patients who did not carry the M694V mutation; similar results were obtained with the V726A or M680I mutation. The presence of two M694V alleles was, therefore, associated with a higher severity of the disease, as judged by the higher prevalence of this major complication, as well as by the higher frequency of arthritis ($P = .006$). It is also striking to note that renal amyloidosis was absent in the three patients who had not undergone colchicine therapy at age <40 years and who had one or two uncharacterized FMF alleles; the study of a larger cohort of patients would be decisive in demonstrating whether the presence of, at most, one of the MEFV mutations recovered in the present study is a favorable prognostic factor. Taken together, such a diagnostic and prognostic value of MEFV-gene analysis also further confirms the involvement of the MEFV gene in FMF.

The availability of a molecularly based FMF diagnosis, the clear-cut demonstration of both the incomplete penetrance and the pseudodominance of the disease phenotype, and the prognostic value of MEFV-gene analysis have several major clinical implications: MEFV-gene analysis performed within the framework of genetic counseling may accurately identify affected individuals as early as the first attack, thereby not only providing the possibility to start colchicine therapy precociously but also preventing unnecessary investigations, including invasive procedures such as laparotomies. In addition, MEFV genotyping may also identify individuals in their

presymptomatic phase, raising the pertinence of starting a prophylactic colchicine therapy. However, a proportion of these individuals with two mutated MEFV alleles—a proportion that has to be determined in a large cohort of healthy relatives of FMF patients—will never present with FMF symptoms. In other respects, one should also keep in mind the report of renal amyloidosis in FMF patients without attacks of serositis (Blum et al. 1962; Sohar et al. 1967; Saatci et al. 1997); at first glance, this observation might encourage colchicine therapy in asymptomatic individuals with two MEFV mutations, especially if they carry the M694V homozygous genotype, which is strongly associated with renal amyloidosis. Although it is clear that the molecular investigations performed in FMF families will therefore lead to new ways of managing the disease, large population-based screening studies will be necessary to define the best therapeutic attitude to adopt in each affected family.

Acknowledgments

This work was supported by Assistance Publique - Hôpitaux de Paris grant PHRC AOM 97201 and by grants from the Association Française contre les Myopathies and the Fondation Calauste Gulbenkian (Lisbon). We are grateful to the families for agreeing to participate in the study. We are indebted to Drs. J. Medaxian and H. Koushkian for their valuable assistance, and we thank Dr. E. Girodon for critical reading of the manuscript and Dr. P. Rémy for helpful discussions.

Electronic-Database Information

Accession numbers and URL for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for familial Hibernian fever [142680], FMF [249100], and hyperimmunoglobulinemia D syndrome with periodic fever [260920])

References

Aksentijevich I, Torosyan Y, Samuels J, Centola M, Pras E, Chae JJ, Oddoux C, et al (1999) Mutations and haplotype studies of familial Mediterranean fever reveal new ancestral relationships and evidence for a high carrier frequency with reduced penetrance in the Ashkenazi Jewish population. Am J Hum Genet 64:949–962

Bernot A, da Silva C, Petit J, Cruaud C, Caloustian C, Castet V, Ahmed-Arab M, et al (1998) Non-founder mutations in the MEFV gene establish this gene as the cause of familial Mediterranean fever. Hum Mol Genet 7:1317–1325

Blum A, Gafni J, Sohar E, Shibolet S, Heller H (1962) Amyloidosis as the sole manifestation of familial Mediterranean fever (FMF). Ann Intern Med 57:795–799

Cazeneuve C, Girodon E, Reinert P, Vivien P, Bitoun P, Gaudelus J, Delfraissy J-F, et al (1998) Familial Mediterranean fever: molecular epidemiology—identification of new marenostrin-pyrin mutations. Am J Hum Genet Suppl 63:A355

Cotton RG (1997) Slowly but surely toward better scanning for mutations. Trends Genet 13:43–46

Dewalle M, Domingo C, Rozenbaum M, Ben-Chetrit E, Cattan D, Bernot A, Dross C, et al (1998) Phenotype-genotype correlation in Jewish patients suffering from familial Mediterranean fever (FMF). Eur J Hum Genet 6:95–97

Dinarello CA, Wolff SM, Goldfinger SE, Dale DC, Alling DW (1974) Colchicine therapy for familial Mediterranean fever: a double-blind trial. N Engl J Med 291:934–937

Eisenberg S, Aksentijevitch I, Deng Z, Kastner D, Matzner Y (1998) Diagnosis of familial Mediterranean fever by a molecular genetics method. Ann Intern Med 129:539–542

French FMF Consortium, The (1996) Localization of the familial Mediterranean fever gene (FMF) to a 250-kb interval in non-Ashkenazi Jewish founder haplotypes. Am J Hum Genet 59:603–612

——— (1997) A candidate gene for familial Mediterranean fever. Nat Genet 17:25–31

International FMF Consortium, The (1997) Ancient missense mutations in a new member of the RoRet gene family are likely to cause familial Mediterranean fever. Cell 90: 797–807

Levy EN, Shen Y, Kupelian A, Kruglyak L, Aksentijevich I, Pras E, Balow JE Jr, et al (1996) Linkage disequilibrium mapping places the gene causing familial Mediterranean fever close to *D16S246*. Am J Hum Genet 58:523–534

Livneh A, Langevitz P, Zemer D, Zaks N, Kees S, Lidar T, Migdal A, et al (1997) Criteria for the diagnosis of familial Mediterranean fever. Arthritis Rheum 40:1879–1885

Partaca R, Freeman GJ, Schwartz J, Singh RP, Kong QT, Murphy E, Anderson Y, et al (1988) rpt-1, an intracellular protein from helper/inducer T cells that regulates gene expression of interleukin 2 receptor and human immunodeficiency virus type 1. Proc Natl Acad Sci USA 85:2733–2737

Pras E, Aksentijevich I, Gruberg L, Balow JJ, Prosen L, Dean M, Steinberg A, et al (1992) Mapping of a gene causing familial Mediterranean fever to the short arm of chromosome 16. N Engl J Med 326:1509–1513

Quaderi N, Schweiger S, Gaudenz K, Franco B, Rugarli E, Berger W, Feldman G, et al (1997) Opitz G/BBB syndrome, a defect of midline development, is due to mutations in a new RING finger gene on Xp22. Nat Genet 17:285–291

Reddy B, Etkin L, Freemont P (1992) A novel zinc finger coiled-coil domain in a family of nuclear proteins. Trends Biochem Sci 17:344–345

Rogers D, Shohat M, Petersen G, Bickal J, Congleton J, Schwabe A, Rotter J (1989) Familial Mediterranean fever in Armenians: autosomal recessive inheritance with high gene frequency. Am J Med Genet 34:168–172

Saatci U, Ozen S, Ozdemir S, Bakkaloglu A, Besbas N, Topaloglu R, Arslan S (1997) Familial Mediterranean fever in children: report of a large series and discussion of the risk and prognostic factors of amyloidosis. Eur J Pediatr 156: 619–623

Samuels J, Aksentijevich I, Torosyan Y, Centola M, Deng Z, Sood R, Kastner D (1998) Familial Mediterranean fever at the millenium. Medicine 77:268–297

Sohar E, Gafni J, Pras M, Heller H (1967) Familial Mediterranean fever: a survey of 470 cases and review of the literature. Am J Med 43:227–253

Tissot C, Mechti N (1995) Molecular cloning of a new interferon-induced factor that represses human immunodeficiency virus type 1 long terminal repeat expression. J Biol Chem 270:14891–14898

Yuval Y, Hemo-Zisser M, Zemer D, Sohar E, Pras M (1995) Dominant inheritance in two families with familial Mediterranean fever (FMF). Am J Med Genet 57:455–457

Zemer D, Pras M, Sohar E, Modan M, Cabili S, Gafni J (1986) Colchicine in the prevention and treatment of the amyloidosis of familial Mediterranean fever. N Engl J Med 314: 1001–1005

Zemer D, Revach M, Pras M, Modan B, Schor S, Sohar E, Gafni J (1974) A controlled trial of colchicine in preventing attacks of familial Mediterranean fever. N Engl J Med 291: 932–934