ARTHRITIS & RHEUMATISM
Vol. 60, No. 6, June 2009, pp 1851–1861
DOI 10.1002/art.24569

Familial Mediterranean Fever With a Single *MEFV* Mutation

Where Is the Second Hit?

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Objective. **Familial Mediterranean fever (FMF) has traditionally been considered an autosomal-recessive disease; however, it has been observed that a substantial number of patients with clinical FMF possess only 1 demonstrable *MEFV* mutation. The purpose of this study was to perform an extensive search for a second *MEFV* mutation in 46 patients diagnosed clinically as having FMF and carrying only 1 high-penetrance FMF mutation.**

Methods. ***MEFV*** **and other candidate genes were sequenced by standard capillary electrophoresis. In 10 patients, the entire 15-kb *MEFV* genomic region was resequenced using hybridization-based chip technology. *MEFV* gene expression levels were determined by quantitative reverse transcription–polymerase chain reaction. Pyrin protein levels were examined by Western blotting.**

Results. **A second *MEFV* mutation was not identified in any of the patients who were screened. Haplotype analysis did not identify a common haplotype that might be associated with the transmission of a second FMF allele. Western blots did not demonstrate a significant difference in pyrin levels between patients with a single mutation and those with a double mutation; however, FMF patients of both types showed higher protein expression as compared with controls and with non-FMF patients with active inflammation. Screening of genes encoding pyrin-interacting proteins identified rare mutations in a small number of patients, suggesting the possibility of digenic inheritance.**

Conclusion. **Our data underscore the existence of a significant subset of FMF patients who are carriers of only 1 *MEFV* mutation and demonstrate that complete *MEFV* sequencing is not likely to yield a second mutation. Screening for the set of the most common mutations and detection of a single mutation appears to be sufficient in the presence of clinical symptoms for the diagnosis of FMF and the initiation of a trial of colchicine.**

Familial Mediterranean fever (FMF; OMIM no. #249100) is an autosomal-recessive autoinflammatory disease characterized by episodic, self-limiting attacks of fever, along with abdominal pain, pleurisy, arthritis, and a distinctive rash (1). Systemic amyloidosis is the most severe manifestation of the disease, commonly affecting the kidneys (11% of cases), and sometimes the adrenals, intestine, spleen, lung, and testis (2). Of the known hereditary periodic fevers, FMF is the most prevalent and best characterized. FMF is common in Middle Eastern populations, including Sephardic and Ashkenazi Jews, Turks, Armenians, and Arabs, and it is not uncommon in other Mediterranean populations, such as Italians, Spanish, Portuguese, French, and Greeks. FMF cases have also been described in many other populations, including Northern Europeans and Japanese (1).

The carrier frequency of *MEFV* mutations is

Supported by the Intramural Research program of the National Institute of Arthritis and Musculoskeletal and Skin Diseases.

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Drs. Kastner and Aksentijevich hold a patent on the gene for familial Mediterranean fever, for which they receive royalties.

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Submitted for publication June 16, 2008; accepted in revised form March 9, 2009.

quite high in the 4 classically affected populations, ranging from 37–39% in Armenians and Iraqi Jews to 20% in Turks, North African and Ashkenazi Jews, and Arabs. The high prevalence of carriers in multiple Middle Eastern and Mediterranean populations suggests that heterozygosity may confer a selective advantage. Despite high carrier frequencies in these populations, the prevalence of FMF is less than expected, indicating either that the disease is underdiagnosed or that disease-associated mutations have reduced penetrance.

The gene responsible for FMF, designated *MEFV*, encodes a 781–amino acid protein known as pyrin (alternatively, marenostrin) (3,4). Pyrin is primarily expressed in polymorphonuclear cells, cytokine-activated monocytes, dendritic cells, and synovial fibroblasts (5,6). Pyrin affects the inflammatory response by regulating the processing of mature interleukin-1β (IL-1β), a potent pyrogenic cytokine. Depending on the experimental system used, pyrin has been shown to act as both as an inhibitor and an activator of IL-1β processing (7–9).

To date, over 50 disease-associated mutations have been identified in *MEFV*, with the majority of mutations being missense changes and more than half clustering in exons 2 and 10 (10). A subset of *MEFV* mutations (usually, E148Q in exon 2 and M680I, M694I, M694V, and V726A in exon 10) may account for as many as 80% of FMF cases in classically affected populations (11); however, it has been observed that a substantial number of patients with clinical FMF (up to 30%, depending on the population) possess only 1 demonstrable mutation despite sequencing of the entire coding region (12–16). These patients with a single mutation often have a typical disease history and respond well to colchicine, the standard treatment for FMF.

One explanation for this phenomenon is a lack of sensitivity in screening techniques. The majority of FMF patients in classically affected populations are screened for a limited number of mutations, which account for a majority of carrier chromosomes in a given population. This approach typically targets only the most prevalent *MEFV* mutations in a specific population; thus, rare or novel mutations can be overlooked. Another possibility is that the second disease-associated mutation may reside in the noncoding (intronic) or regulatory regions of *MEFV*, possibly affecting messenger RNA (mRNA) expression or splicing. The entire genomic region encompassing the *MEFV* transcript is 15 kb in size; thus, it is not practical for diagnostic sequencing using standard techniques. Although most disease-associated mutations are missense nucleotide changes, the possibility of genomic rearrangements (e.g., deletions or copy number variations) cannot be excluded as another mechanism of disease. However, a recent study using multiplex ligation-dependent probe amplification (MLPA) failed to identify any *MEFV* copy number variations in a large cohort of 216 patients with FMF, suggesting that *MEFV* copy number variations do not contribute to the pathogenesis of FMF (17).

Two recent reports have raised the question of dominant inheritance in FMF. Booth et al (15) described a single mutation associated with the disease in 3 unrelated British patients (M694del) and the M694I–E148Q complex allele in 2 FMF families of Turkish and Indian ancestry. Complete *MEFV* sequencing failed to identify any coding region abnormality in the other allele in any of these cases. In addition, a 3-generation Spanish family with 5 affected members presenting with severe disease and amyloidosis was found to transmit a novel *MEFV* mutation, H478Y, in a clearly dominant manner (16). The existence of the second common disease-associated *MEFV* mutation was ruled out by intragenic haplotype analysis of the affected members. Taken together, these data strongly suggest that a single-gene recessive model of inheritance is incapable of fully describing the broad spectrum of *MEFV*-associated phenotypes.

There is also evidence suggesting genetic heterogeneity of FMF in Turkish and Armenian populations. A subset of Turkish FMF families appeared to be unlinked to chromosome 16p13.3, where *MEFV* resides (18), and a divergence from Hardy-Weinberg equilibrium, with a relative excess of patients without FMF mutations, has been described among Armenian patients from Karabakh (14).

In previous studies, only a few groups have attempted to search for FMF mutations in the entire coding region of *MEFV* (12–16). In this study, we performed an extensive search for the second *MEFV* mutation in 46 patients clinically diagnosed as having FMF and carrying only 1 known high-penetrance FMF-associated mutation.

PATIENTS AND METHODS

Patients. The 46 patients included in this study were diagnosed clinically as having FMF. About half of the patients (28 of 46) were seen at the National Institutes of Health (Bethesda, MD); thus, only a subset of patient records was available for the retrospective study. For participation in the study, all patients (or parents or legal guardians if the patient

was a minor) provided written informed consent, as approved by the Institutional Review Board of the National Institute of Arthritis and Musculoskeletal and Skin Diseases/National Institute of Diabetes and Digestive and Kidney Diseases.

Mutation analysis. Genomic DNA was isolated from peripheral blood leukocytes using a Gentra Puregene Blood kit (Qiagen, Valencia, CA). In 46 patients, fluorescence sequencing was performed on the coding regions and splice junctions of *MEFV* with BigDye Terminator version 3.1 chemistry on an ABI 3100 Genetic Analyzer (both from Applied Biosystems, Foster City, CA). The sequencing data were analyzed with Sequencher 4.6 (Gene Codes, Ann Arbor, MI). In 10 patients, a hybridization-based resequencing system (Hychip; Callida Genomics, Sunnyvale, CA) was used to analyze 15 kb of the genomic sequence encompassing all 10 exons, all 9 introns, and 1 kb of upstream sequence likely to include the promoter region of *MEFV*.

Control DNA genotyping. The allele frequencies of novel mutations were evaluated in a panel of 376 or 750 Caucasian control DNA samples or in a panel of 382 Jewish control DNA samples from the New York Cancer Project (NYCP) panel using mass spectrometry (homogeneous MassExtend assay; Sequenom, San Diego, CA).

Gene expression analysis. Total RNA was isolated from peripheral blood mononuclear cells (PBMCs) using TRIzol reagent (Invitrogen, Carlsbad, CA) and standard chloroform extraction. The PBMCs were processed within 1 hour of the blood draw. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA). For allele expression, 5 sets of primers were designed to produce overlapping amplicons covering the entire coding region of *MEFV*. These amplicons were then sequenced using the method described above. The relative expression of *MEFV* was measured by real-time RT-PCR using TaqMan Gene Expression Assays and a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA). Primers (assay ID Hs00925528-g1) targeting exon junction 9–10 were used, and the expression level was compared with the level of β_2-microglobulin (β_2m), which served as an internal control. The primer set was validated for its efficiency using a standard serial dilution of cDNA.

Protein study. Patients were all under treatment when sampling was performed. Patient granulocytes were purified from 10–20 cc of whole blood using a standard Dextran sedimentation protocol. Purified cells were treated with diisopropylfluorophosphate (Sigma) to prevent proteolysis. Total protein from the cell lysates was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis for Western blotting with an antipyrin polyclonal antibody. The intensity of the pyrin band was measured by densitometry (Bio-Rad GS800 Scanner with Quantity One software; Bio-Rad, Richmond, CA) and normalized to GAPDH, which was used as an endogenous control.

Structural modeling. A structure for the caspase activation and recruitment domain (CARD) domain of apoptosis-associated speck-like protein with a caspase 1 recruitment domain (ASC) was modeled on the crystal structures of 2 other CARD domains from the proteins RAIDD (19) and Apaf-1 (20). A structural alignment was first generated using the Fugue algorithm (21), and then energy minimization was performed using an implementation of GROMOS (Groningen Molecular Simulation) in the Swiss-Model program (22). Structural images were created using the program PyMOL (http://www.pymol.org).

RESULTS

Clinical features of patients with 1 mutation. Table 1 summarizes the clinical features of 28 patients for whom detailed clinical histories were available. The remaining patients were not evaluated at the National Institutes of Health, and the respective referring physicians made the clinical diagnosis of FMF. In most patients, genetic testing was done after the clinical diagnosis of FMF was already established.

Within this cohort of patients with a single mutation, a variability of phenotype and some inconsistency in responsiveness to colchicine treatment was observed. All of the patients met the clinical criteria for FMF, based on the experiences of physicians at the Sheba Medical Center of Tel Hashomer in Israel (23), although most of them presented with an incomplete abdominal attack (abdominal pain without frank peritonitis) as the major criterion of the disease. We adopted a conservative approach in defining the abdominal attacks as incomplete, since no patients were seen at the National Institutes of Health during an attack. Three patients did not report any abdominal pain during an attack but presented with pleurisy and/or arthralgia.

The age at onset of the disease in these 28 patients was variable, ranging from age 1 month to age 40 years. The duration of attacks was less than 1 week, with the majority lasting 2–3 days. Sixteen of the 28 patients reported arthralgia, and 11 patients developed skin rash. Response to colchicine therapy was either complete or partial in 84% of the patients (21 of 25), whereas 4 patients carrying the M694del mutation did not respond or were intolerant to treatment. Information on colchicine responsiveness was unavailable for 3 patients. Fourteen patients (56%) had a complete response, while 7 patients (28%) had periodic attacks of inflammation, although less frequently while receiving treatment (Table 1).

Most of the 46 study patients were carriers of the M694V or the V726A mutation. Included in this study were patients with apparently dominantly inherited disease and with uncommon ancestry. For example, patients 1803 and 1804 were a father and son of German-Welsh ancestry. Patients 407 and 408 were a mother and daughter of mixed European ancestry. All 4 of these patients were carriers of a single M694del mutation.

Table 1. Clinical symptoms of familial Mediterranean fever in patients with only 1 identified *MEFV* mutation*

Mutation	Patient ID	Ethnicity	Sex	Age at onset	Duration of attacks	FMF symptoms								Response to colchicine†
						Fever	Abdominal pain	Pleuritis	Pericarditis	Rash	EE	Myalgia	Arthralgia	
M694V/?	27‡	Armenian	F	30 years	3–7 days	N	N	Y	N	Y	Y	N	Y	Complete
M694V/?	108	Armenian	M	1 month	3 days	Y	N	N	N	N	NA	N	Y	Complete
M694V/?	116‡	Sephardic/Ashkenazi Jewish	F	Childhood	2–3 days	Y	Y	Y	Y	N	N	N	N	Partial
M694V/?	139	Sephardic Jewish	F	12 years	NA	Y	Y	Y	N	N	N	N	N	Partial
M694V/?	171	Italian	F	14 years	2–3 days	Y	Y	N	N	N	N	N	Y	Complete
M694V/?	234	Greek	F	Childhood	NA	Y	Y	N	N	Y	N	Y	Y	Complete
M694V/?	248‡	Sephardic Jewish	F	17 years	3–5 days	Y	Y	Y	N	Y	Y	N	Y	Partial
M694V/?	337	Mixed European/ Iraqi Jewish	M	6 years	1–2 days	Y	Y	Y	N	N	N	N	N	NA
M694V/?	381	Italian	F	16 years	4–7 days	Y	Y	N	N	Y	N	N	N	NA
M694V/?	395‡	Russian/Ashkenazi Jewish	M	19 years	1–2 days	Y	Y	Y	N	N	N	N	N	Complete
M694V/?	817	Italian	F	17 years	NA	N	Y	Y	N	N	Y	N	N	Complete
M694V/?	1198	Jordanian/English	M	5 years	2–3 days	Y	Y	Y	N	N	N	Y	Y	Partial
M694V/?	1312‡	Turkish	M	20 years	<24 hours	Y	Y	N	N	Y	N	N	N	Complete
M694V/?	1454	Italian/Irish/German	M	12 years	3–4 days	Y	Y	Y	N	N	N	N	N	NA
M694V/?	1630‡	Armenian/European	F	13 years	2–3 days	Y	Y	N	N	Y	N	N	Y	Complete
M694del/?	407‡	Mixed European	F	1 year	3–5 days	Y	Y	Y	N	N	N	Y	N	None
M694del/?	408	Mixed European	F	10 years	3–4 days	Y	Y	Y	N	N	N	Y	Y	None
M694del/?	1803	German/Welsh	M	30 years	1–2 days	Y	Y	N	N	N	N	N	N	None
M694del/?	1804	German/Welsh/ Hispanic	M	1 month	3–4 days	Y	Y	N	N	N	N	N	N	Intolerant
V726A/?	1313	Jewish	M	Childhood	3–4 days	Y	Y	N	N	Y	N	N	N	Complete
V726A/?	1044	Jewish/Dutch	M	4 years	3–5 days	Y	N	Y	N	Y	N	N	Y	Partial
V726A/?	785	Ashkenazi Jewish	M	8 years	3–5 days	Y	Y	N	N	Y	N	N	Y	Complete
E148Q, V726A/?	977‡	Ashkenazi Jewish	F	13 years	5–7 days	Y	Y	N	N	N	N	N	Y	Complete
E148Q, V726A/?	1532	Sephardic Jewish	M	Childhood	2–3 days	Y	Y	Y	N	N	N	N	Y	Complete
E148Q, V726A/?	1533	Ashkenazi Jewish	M	Childhood	2–3 days	Y	Y	Y	N	Y	Y	N	Y	Complete
E148Q, V726A/?	1587	Ashkenazi Jewish	M	40 years	2–3 days	N	Y	N	N	N	N	N	Y	Complete
R653H/?	569‡	Lebanese/Irish	F	1 year	5 days	Y	Y	N	N	Y	N	N	Y	Partial
R761H/?	1048	Mixed European/ Jewish	M	1 year	2–3 days	Y	Y	N	N	N	N	N	Y	Partial

* FMF = familial Mediterranean fever; EE = erysipelas erythema; NA = not available.
† Response to colchicine therapy was defined as complete in patients who were taking colchicine regularly but may have had very infrequent attacks and partial in patients who had only less frequent attacks. Intolerant was defined as unable to tolerate colchicine therapy because of side effects.
‡ One of the 10 patients in whom the entire 15-kb genomic region encompassing *MEFV* was sequenced using hybridization-based chip technology (clinical record not available in 1 patient).

Table 2. Intragenic *MEFV* haplotype analysis of familial Mediterranean fever patients with affected first-degree relatives

Mutation/haplotype, patient	D102D	G138G	A165A	R202Q	R314R	E474E	Q476Q	D510D	Intron 6 c.1610+96C>T	Intron 8 c.1760−30A>T	Exon 9 P588P
Med/A haplotype	C	G	A	A	C	G	A	T	C	A	A
Patient 116											
M694V	C	G	A	A	C	G	A	T	C	A	A
Second haplotype	**T**	**A**	**C**	**G**	**C**	**G**	**A**	**T**	**C**	**T**	**A**
Patient 139											
M694V	C	G	A	A	C	G	A	T	C	A	A
Second haplotype	**T**	**A**	**C**	**G**	**C**	**G**	**A**	**T**	**C**	**A**	**A**
M694del haplotype	T	A	C	G	C/T	A/G	A/G	C/T	C/T	A/T	A/G
Patient 407											
M694del	T	A	C	G	C/T	A/G	A/G	C/T	C/T	A/T	A/G
Second haplotype	**T**	**G**	**C**	**G**	**C/T**	**A/G**	**A/G**	**C/T**	**C/T**	**A/T**	**A/G**
Patient 408											
M694del	T	A	C	G	C/T	A/G	A/G	C/T	C/T	A/T	A/G
Second haplotype	**C**	**G**	**A**	**A**	**C/T**	**A/G**	**A/G**	**C/T**	**C/T**	**A/T**	**A/G**
Patient 1803											
M694del	T	A	C	G	C/T	A/G	A/G	C/T	C/T	A/T	A/G
Second haplotype	**C**	**G**	**A**	**G**	**C/T**	**A/G**	**A/G**	**C/T**	**C/T**	**A/T**	**A/G**
Patient 1804											
M694del	T	A	C	G	C/T	A/G	A/G	C/T	C/T	A/T	A/G
Second haplotype	**T**	**A**	**C**	**G**	**C/T**	**A/G**	**A/G**	**C/T**	**C/T**	**A/T**	**A/G**
R653H haplotype	T	A	C	G	C	G	A	C	C	A	A
Patient 742											
R653H	T	A	C	G	C	G	A	C	C	A	A
Second haplotype	**T**	**A**	**C**	**G**	**C**	**G**	**A**	**C**	**C**	**A**	**A**
Patient 367											
R653H	T	A	C	G	C	G	A	C	C	A	A
Second haplotype	**T**	**A**	**C**	**G**	**T**	**A**	**G**	**T**	**T**	**T**	**G**

Patients 367 and 742 were sibling carriers with the rare mutation R653H and were of mixed European ancestry. Although many of the patients included in this study were of Middle Eastern background, about half of the patients had an atypical ancestry, either European or mixed Middle Eastern/European. We have also identified the first reported African American patient with FMF; this patient possessed the common FMF mutation M694V.

Findings of *MEFV* mutation screening. Using standard sequencing, 46 FMF patients with 1 *MEFV* mutation were screened for a second disease-associated mutation in all 10 exons of *MEFV,* including the exon-flanking regions. Thirty of these patients possessed a mutation affecting residue M694 (M694V, M694I, M694del), and 7 patients were carriers of another common FMF-associated mutation, V726A. Of the remaining 9 patients, 3 had the R653H mutation, 1 patient each was a carrier of R761H and S702C, and 4 patients were carriers of the complex allele V726A–E148Q, as shown by family studies. In addition, the *MEFV* coding region was sequenced in 13 patients with FMF-like symptoms who carried milder FMF-associated mutations/functional polymorphisms, such as E148Q and K695R. No new mutation was identified in any of the patients analyzed.

A subset of 10 DNA samples was selected for additional sequencing of the entire 15-kb *MEFV* genomic region using hybridization-based chip technology. This method is capable of detecting single-nucleotide variations as well as small deletions or insertions (24). All but 3 of the 46 study patients were found to be heterozygous for at least 1 of the many known single-nucleotide polymorphisms (SNPs) in the *MEFV* genomic region, which is evidence against the presence of large genomic deletions. In addition, the *MEFV* genomic region was amplified through a set of overlapping PCR fragments, and based on the size of these fragments, no intragenic genomic deletions were identified.

A novel heterozygous mutation was identified in the putative regulatory region 1 kb upstream of the start codon at position c.–888G>A in a North American patient (patient 407). This heterozygous change was determined to be a SNP based on its allele frequency of 4% in a panel of 350 Caucasian controls and 3% in a panel of 378 Ashkenazi Jewish controls.

Findings of haplotype analysis. Haplotype analysis was performed in patients who had affected first-

degree relatives. First, a haplotype associated with the disease-associated mutation within a family was deduced. For example, in a Jewish family with M694V (patients 116 and 139), the A (also known as Med) haplotype, a previously reported founder haplotype associated with the M694V mutation, was identified (Table 2) (3,25). The patients, a mother and daughter, shared the same second haplotype, which could be consistent with the existence of a common mutation on the second allele, or it could simply reflect a conserved haplotype in the population. In a second family, 2 affected siblings who were carriers of the R653H mutation (patients 367 and 742) did not entirely share the same haplotype on the second chromosome. The intragenic haplotype analyses were done with other sporadic cases of FMF in patients who carried the M694V mutation, but none shared a common haplotype that could be associated with a mutation in the second allele. Interestingly, the first and only African American patient with FMF described thus far had the same Med (A) haplotype associated with M694V as patients in other Mediterranean populations.

In 2 families with 4 patients who were carriers of M694del (patients 1803 and 1804 and patients 407 and 408), an unambiguous common intragenic M694del-associated haplotype was identified only at the 5′ end of the gene. The 5′ end haplotype (T-A-C-G) based on 4 SNPs within exon 2 appeared to be a relatively common haplotype in the general population (Table 2). All 4 patients were heterozygous for the same alleles in SNPs residing at the 3′ end of the gene. Under the recessive model of inheritance, there was no common haplotype within each family that could be associated with a putative mutation on the second allele.

***MEFV* expression.** Analysis of allele expression was performed to determine whether both *MEFV* transcripts were expressed in 8 of the FMF patients. The RNA was converted into cDNA, and the entire transcript was sequenced with 5 overlapping amplicons. All patients were carriers of at least 1 heterozygous mutation, indicating that both alleles were expressed. In addition, no transcript size mutations were identified in any of the 8 patients.

Quantitative RT-PCR was then used to compare the expression levels of *MEFV* in patients with 1 or 2 mutations and healthy controls. *MEFV* expression levels were measured with primers directed at exon junction 9–10, and β_2m levels were measured as an internal control. Due to the large degree of intragroup variability and the relatively small number of samples examined, a significant difference in the expression level of *MEFV* was not demonstrated in any of the 3 groups (Figure 1). In contrast to previous findings (26,27), there was a slight trend toward higher *MEFV* expression in the patients as compared with the healthy controls.

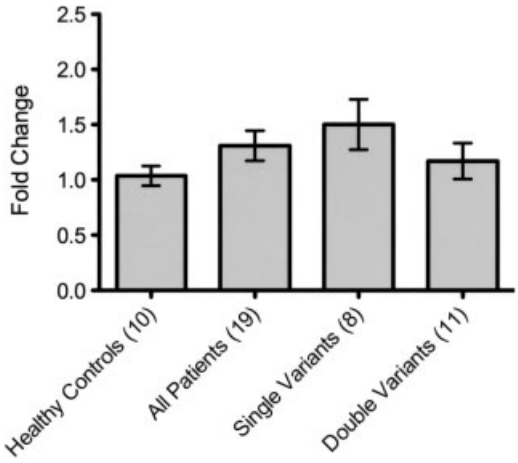

Figure 1. Mean relative expression levels of *MEFV* in familial Mediterranean fever (FMF) patients with 1 *MEFV* mutation, FMF patients with 2 *MEFV* mutations, and healthy control subjects. Data are expressed as the fold change relative to the mean expression in the healthy control group and were generated using β_2-microglobulin as the endogenous control. Numbers in parentheses are the number of subjects. Values are the mean ± SEM. No significant differences in expression levels between any of the groups were observed.

Pyrin expression in FMF patients. To date, only 1 study has examined pyrin protein expression in the cells of FMF patients (28). To further investigate the observed trend toward higher *MEFV* gene expression, Western blot analysis using a polyclonal antipyrin antibody was performed on cell lysates isolated from PBMCs. Due to the presence of multiple bands, these blots proved difficult to analyze. This experiment was then repeated with lysates isolated from polymorphonuclear cells (PMN), which highly express pyrin in its native state (Figure 2A). The Western blots were clear, and showed a trend toward higher pyrin levels in FMF patients as compared with healthy controls. Although there was variability among the samples in each group, we did not observe a statistically significant difference between patients with 1 or 2 disease mutations. However, there was a significant difference in pyrin levels between FMF patients and healthy controls ($P = 0.007$) (Figure 2B).

To investigate whether the higher pyrin expression was specific to FMF patients, we compared pyrin levels in FMF patients with those in non-FMF patients with active inflammation. We observed significantly higher pyrin expression in FMF patients whose disease

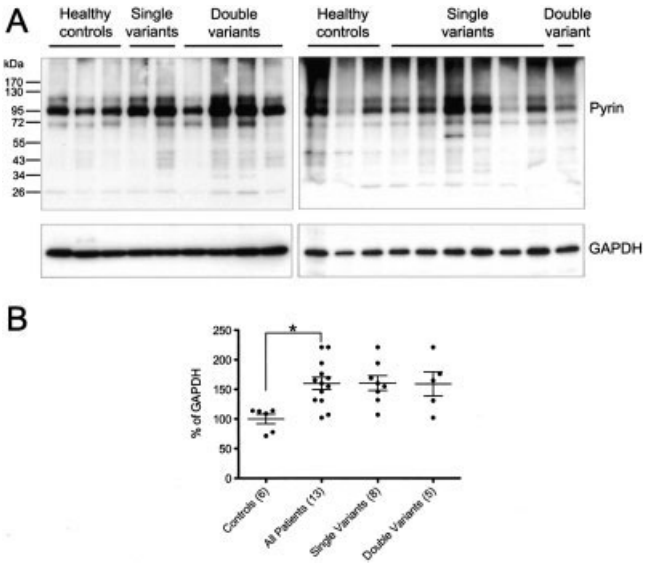

Figure 2. A, Western blots of granulocyte lysates derived from familial Mediterranean fever (FMF) patients with 1 (n = 8) or 2 (n = 5) *MEFV* mutations and healthy control subjects (n = 6). Lysates were probed with antibodies to pyrin (95 kd) or GAPDH. **B,** Densitometry analysis of pyrin bands in the Western blots. Data are expressed as the percentage of GAPDH in each study subject and are normalized to the mean pyrin expression in the healthy control group within each individual blot. Numbers in parentheses are the number of subjects. Horizontal bars show the mean ± SEM. FMF patients exhibited significantly higher levels of pyrin as compared with controls (P = 0.007 by Mann-Whitney U test). There was no significant difference in pyrin levels between patients with 1 or 2 *MEFV* mutations.

was in remission relative to that in patients with non–FMF-related active inflammatory disease (inflammatory bowel disease and chronic granulomatous disease) as well as that in healthy controls (Figures 3A and B).

Screening for mutations in other known autoinflammatory genes. Although these patients did not appear to have symptoms associated with other autoinflammatory diseases, we considered the possibility that they may have had mutations in other known autoinflammatory genes. Fourteen patients were screened for the tumor necrosis factor receptor–associated periodic syndrome (TRAPS)–associated mutations in *TNFRSF1A*, and only 1 patient (patient 1454) was found to carry the R92Q reduced-penetrance mutation. The same 14 patients tested negative for the 2 most common hyperimmunoglobulinemia D with periodic fever syndrome (HIDS)–associated mutations (I268T and V377I).

Digenic model of inheritance. Oligogenic and digenic modes of inheritance have recently been described in the genetics of several diseases that were initially characterized as monogenic disorders. Bardet-Biedl syndrome and deafness are examples of such diseases that were initially thought to be recessively inherited (29). Thus, we considered the possibility that FMF patients with only 1 *MEFV* mutation might have a second disease-associated mutation in a gene that acts in concert with *MEFV* to produce a disease phenotype. Likely candidates for this second mutation are genes that encode proteins known to interact with pyrin or genes that have some function in regulating the IL-1β pathway.

Several genes that encode proteins fitting this description were chosen for analysis in 10 patients: *ASC*, *SIVA*, *CASP1*, *PSTPIP1*, *POP1*, and *POP2*. In a single FMF patient, a novel *PSTPIP1* nucleotide mutation was identified at position c.540G>A, which did not change the amino acid at position K181. Two novel missense substitutions were identified in the genes encoding ASC

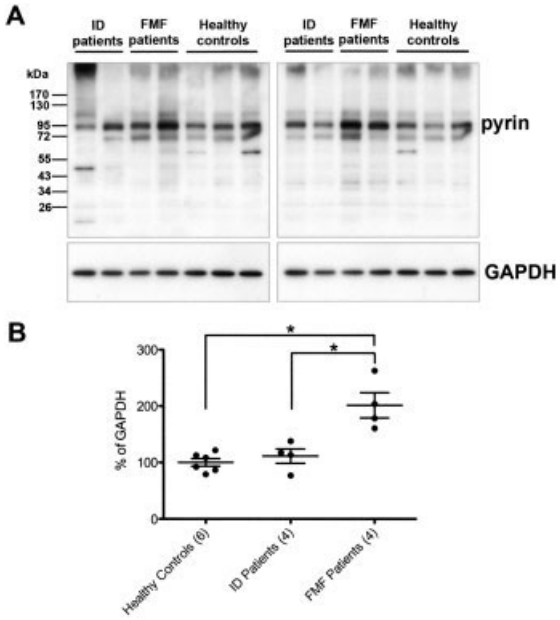

Figure 3. A, Western blots of granulocyte lysates derived from non–familial Mediterranean fever (non-FMF) patients with active inflammation (ID; n = 4), FMF patients (n = 4), and healthy control subjects (n = 6). Lysates were probed with antibodies to pyrin (95 kd) or GAPDH. **B,** Densitometry analysis of pyrin bands in the Western blots. Data are expressed as the percentage of GAPDH in each study subject and are normalized to the mean pyrin expression in the healthy control group within each individual blot. Numbers in parentheses are the number of subjects. Horizontal bars show the mean ± SEM. FMF patients exhibited significantly higher levels of pyrin as compared with patients with active inflammation and with controls (P = 0.0286 and P = 0.0095, respectively, by Mann-Whitney U test). There was no significant difference in pyrin levels between patients with active inflammation and controls.

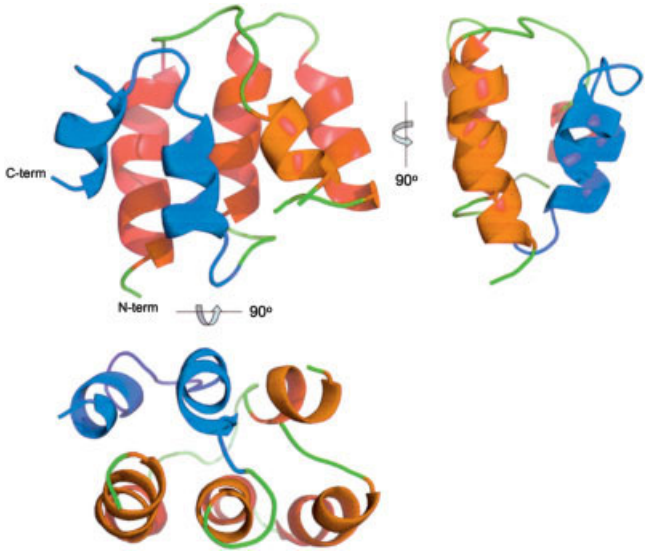

Figure 4. A modeled structure for the caspase activation and recruitment domain (CARD) domain of ASC (residues 109–195). This homology model, based on the CARD domain structures of RAIDD and Apaf-1, shows the ASC CARD domain as a 6-helix structure adopting a Greek key fold. The mutation W171X would truncate the final 2 α-helices from the C-terminus, and these are highlighted in blue.

and the apoptosis regulatory protein Siva (also known as CD27BP), while the genes encoding IL-1β–converting enzyme (CASP-1; also known as ICE), pyrin only protein 1 (POP-1), and POP-2 were mutation negative. In exon 3 of *SIVA*, the novel substitution 416G>A was identified at codon 120, replacing a valine with a methionine (V120M). We screened 374 Caucasian control samples for this allele, as well as 382 Jewish control samples to match the ancestry of the original carrier. Two carriers were identified in the Caucasian panel and 12 in the Jewish panel, thus qualifying V120M as a novel polymorphism (see Supplementary Table 1, available on the *Arthritis & Rheumatism* Web site at http://www3.interscience.wiley.com/journal/76509746/home).

In contrast to most other genes associated with autoinflammatory pathways, *ASC* contains no known sequence mutations. We identified in a Jewish patient with FMF a novel mutation, W171X (513G>A), that introduces a premature stop codon that could delete the last 24 amino acids of the protein. At a structural level, a homology model for the CARD domain of ASC was generated, and it was observed that this premature stop codon removed the last 2 of the 6 helices in the CARD domain of ASC (Figure 4). Only a single carrier of W171X was identified in a panel of 371 Caucasian DNA samples, and 2 carriers were found in 382 Jewish DNA samples. The combined carrier frequency of W171X in both panels of control samples was 0.004 (see Supplementary Table 1, available on the *Arthritis & Rheumatism* Web site at http://www3.interscience.wiley.com/journal/76509746/home). We also screened a panel of 70 FMF patients with only 1 *MEFV* mutation, including milder FMF-associated mutations. In this cohort, 1 additional patient of Turkish background was identified as a carrier of W171X.

DISCUSSION

In this study, we searched for a second disease-associated mutation in 46 FMF patients who had only 1 documented *MEFV* mutation. To date, this is the most comprehensive search for a second disease-associated mutation. Two different sequencing techniques were applied in this endeavor, and both failed to identify a second mutation in the *MEFV* gene.

MEFV transcript analysis established the presence of both alleles in a subset of 8 patients. In this same cohort of 8 patients, relative gene expression analysis via quantitative RT-PCR did not show a significant difference in the *MEFV* expression level among FMF patients with 1 mutation or 2 mutations. These data contrast with those from 2 previous studies showing that FMF patients with 1 or 2 *MEFV* mutations appeared to express lower levels of *MEFV* as compared with healthy controls (26,27). Like these previous studies, our experiments were performed with total RNA derived from PBMCs; however, our study differs in terms of the time frame in which samples were processed, the real-time PCR platform used, the reaction chemistry and primer sets used, and the manner in which the data were analyzed.

In order to study this question at the level of expressed protein, we examined pyrin levels in the granulocytes from patients with FMF. Granulocytes are perhaps the most relevant cells to study in FMF patients since the symptoms of disease are caused by a massive influx of granulocytes into affected areas. This study is the first to investigate this question in unstimulated cells from FMF patients. Samples were collected from 13 FMF patients and 6 healthy controls. We observed no significant difference in pyrin expression between patients harboring 1 versus 2 mutations. We did observe a significant increase ($P = 0.007$) in pyrin expression in FMF patients as compared with controls, consistent with our observed trend toward increased mRNA expression in FMF patients. The experiments shown in Figures 3A and B suggest that the increase in pyrin expression is not merely a consequence of inflammation and that it ap-

pears to be specific for FMF patients. This intriguing result should be corroborated with studies of pyrin expression in additional FMF patients and in patients with active disease.

There are multiple explanations for the apparent divergence from the typical paradigm of recessive inheritance seen in FMF. Against the hypothesis of pseudodominant inheritance, there is the fact that in this study, a number of FMF patients with a single *MEFV* mutation had atypical ancestry; thus, the possibility of having the second FMF-associated mutation in 2 successive generations is highly unlikely. Previous studies of FMF families of Spanish and British ancestry also failed to identify the common haplotype that should be associated with the transmission of the second *MEFV* mutation within a pedigree (15,16). In the present study, we were unable to identify 1 or 2 common haplotypes that cosegregated with the second disease-associated mutation both in familial and in sporadic cases.

Although the possibility that these patients have other periodic fevers, such as TRAPS, HIDS, or cryopyrin-associated periodic syndromes (CAPS), cannot be completely excluded, it is highly unlikely for several reasons: the patients clinically appeared to have FMF, significant numbers of patients responded to colchicine therapy, and other periodic fevers are typically uncommon in patients from the Middle East. Nevertheless, we screened 14 FMF patients for TRAPS-associated and HIDS-associated mutations. One patient of mixed European ancestry was identified as a carrier of the TNFRSF1A R92Q mutation, which has a carrier frequency of 2–5% in Caucasians, depending on the population. Our most recent data indicate that the R92Q carrier frequency in North American Caucasian control samples is 0.038 (see Supplementary Table 1, available on the *Arthritis & Rheumatism* Web site at http://www3.interscience.wiley.com/journal/76509746/home).

One other explanation is that having only 1 *MEFV* mutation may give rise to an FMF phenotype in the presence of 1 or more modifying alleles in other related genes or in the presence of other environmental factors, such as stress. Asymptomatic carriers of 1 FMF mutation have biochemical evidence of subclinical inflammation (30,31), and a recent study found a higher frequency of carriers of highly penetrant FMF mutations among patients with systemic inflammatory response syndrome (SIRS) and sepsis (32). Further supporting this hypothesis is the observation that patients who carry complex *MEFV* alleles appear to have more severe disease (33). Previous studies in FMF patients showed that the presence of modifying alleles in *MICA* and *SAA* are associated with a severe FMF phenotype and susceptibility to amyloidosis (34,35). Therefore, modifying alleles could contribute to an inflammation dosage threshold that is necessary for the development of systemic inflammation and symptomatic FMF.

Although hereditary recurrent fevers are considered monogenic diseases, a few recent reports have described patients who are compound heterozygotes for mutations in 2 known recurrent fever genes (36–38). These patients were found to have 2 or more reduced-penetrance mutations, such as E148Q in *MEFV*, R92Q or P46L in *TNFRSF1A*, V377I in *MVK,* and V198M in *CIAS1*. In some cases, patients presented with symptoms of both diseases or with a more severe disease, and their treatment was also compromised due to unknown gene interactions among mutations in the known recurrent fever genes (39). Considering that these mutations have carrier frequencies close to or higher than 1% in control populations, it is likely that compound heterozygotes will be identified. The carrier frequencies of these mutations generated in our laboratory using Caucasian and Jewish control DNA samples are summarized elsewhere (see Supplementary Table 1, available on the *Arthritis & Rheumatism* Web site at http://www3.interscience.wiley.com/journal/76509746/home). Finally, as a major referral laboratory for patients with recurrent fevers, we have tested samples from more than 1,900 patients, and the majority of them have been negative for mutations in known recurrent fever genes, suggesting that there are additional recurrent fever genes yet to be identified. Thus, the interactions between mutations and modifying alleles among known and unknown recurrent fever genes could give rise to a range of inflammatory phenotypes.

Under the hypothesis of digenic inheritance, we screened 6 candidate genes for mutations. Two missense mutations were identified, 1 in *SIVA* and 1 in *ASC*. Although the *SIVA* mutation appears to be a polymorphism, it could still have potential consequences in the pathogenesis of FMF. Both the mutation in *SIVA* and the mutation in *ASC* are currently under investigation. While our candidate gene approach was limited, it is the first attempt to investigate polygenic inheritance in FMF. Ideally, this question should be interrogated using a genome-wide association study similar to those conducted for complex diseases. This approach would require setting up a large international collaborative project, which would include FMF patients in whom only 1 highly penetrant *MEFV* mutation is documented

and in whom the presence of the second disease-associated mutation has been completely ruled out.

This study has shown for the first time that pyrin expression appears to be higher in granulocytes from FMF patients as compared with those from controls. The relevance of this finding is difficult to interpret, since there is some controversy regarding the function of pyrin. Depending on the experimental model used, pyrin has been shown to both activate and inhibit the caspase 1/IL-1β signaling pathway (7–9,40,41). An explanation for the higher pyrin levels in FMF patient granulocytes would greatly depend on the function of pyrin in the cell and on whether the mutations associated with FMF are gain or loss of function mutations. Increased levels of pyrin could lead to an increase in the caspase 1/IL-1β signaling pathway and would explain the apparent dominant inheritance of FMF in some patients. Alternatively, FMF mutations could cause a loss of function, and the observed increase in pyrin expression may be a compensatory mechanism for recovering this deficit.

Pyrin likely regulates the NF-κB pathway, apoptosis, and possibly other aspects of inflammation independently of IL-1β, so it is reasonable to assume that the role of pyrin in the modulation of inflammation may be more complex than has previously been hypothesized. Given the high carrier frequency of FMF mutations and the lower than expected prevalence of the disease, it seems possible that other alleles could modify inflammatory signals initiated by mutant pyrin. Thus, FMF may not be a simple monogenic inflammatory disease, and the FMF phenotype may occur in patients with only 1 *MEFV* mutation in the presence of other permissive alleles or environmental factors.

Our study has 2 important messages for the practitioner. First, screening for the set of most common mutations seems, in the presence of clinical symptoms, to be sufficient for the diagnosis of FMF and for the initiation of a trial of colchicine. Second, our data underscore the need for continued referral of single-mutation cases to research laboratories that are actively investing potential modifier genes, in order to facilitate the identification of new susceptibility loci.

ACKNOWLEDGMENTS

We would like to thank all of the physicians who referred their patients to us for molecular diagnostic testing. We would also like to acknowledge Dr. Peter Gregersen for sharing the New York Cancer Project control samples and Dr. Doug Kuhns and Ms Deborah Long Priel for their expertise and help in processing patient granulocytes.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Aksentijevich had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Booty, Chae, Remmers, Barron, Holland, Kastner, Aksentijevich.

Acquisition of data. Booty, Chae, Masters, Remmers, Barham, Le, Aksentijevich.

Analysis and interpretation of data. Booty, Chae, Masters, Remmers, Barron, Kastner, Aksentijevich.

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