Rheumatol Int (2007) 27:545–551
DOI 10.1007/s00296-006-0255-8

ORIGINAL ARTICLE

The contribution of genotypes at the MICA gene triplet repeat polymorphisms and MEFV mutations to amyloidosis and course of the disease in the patients with familial Mediterranean fever

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Received: 6 March 2006 / Accepted: 6 October 2006 / Published online: 11 November 2006

Abstract
Objective To evaluate the effects of MEFV genotypes and the major histocompatibility complex class I chain-related gene A (MICA) triplet repeat polymorphism on the severity and clinical features of familial Mediterranean fever (FMF) and amyloidosis in a group of Turkish FMF patients.
Methods We evaluated 105 adult FMF patients (with or without amyloidosis, 33 and 72, respectively) along with 107 healthy controls who were neither related to the patients nor had a family history of FMF or Behcet's disease. After recording the demographic and clinical data, the predominant mutations in the MEFV gene locus (M694V, M680I, V726A, M694I, and E148Q) were investigated by direct sequencing. MICA transmembrane polymorphisms in exon 5 were studied by vertical gel electrophoresis and fragment analysis of the amplicons obtained from MICA locus with appropriate primers.
Results Earlier age at onset, increased frequency of attacks, arthritis attacks, erysipelas-like erythema, increased severity scores and amyloidosis were significantly more common in M694V homozygous patients compared to the patients not M694V homozygous ($P = 0.005$, OR 4.55; $P = 0.001$, OR 7.60; $P = 0.003$, OR 4.57; $P = 0.002$, OR 7.58; $P = 0.004$, OR 5.15 and $P = 0.018$, OR 3.33, respectively). We did not detect any modifying effects of MICA alleles as an independently risk factor on the amyloidosis development. However, when we examined the effects of MICA alleles on the course of the disease and development of amyloidosis in the M694V homozygous patients, A5 allele had a protective effect against the development of amyloidosis ($P = 0.038$, OR_{adj} 0.26 with A5 and $P = 0.009$, OR_{adj} 4.42 without A5).
Conclusion Though the effects of the MEFV genotypes seem clear, there are definitely other modifying factors or genes on the development of amyloidosis and on the course of the disease. For example, some MICA alleles have a protective effect on the prognostic factors in FMF.

Keywords Familial Mediterranean fever · Amyloidosis · MICA · MEFV

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Introduction

Familial Mediterranean fever (FMF) is an autosomal recessive disease characterized by recurrent attacks of fever, peritonitis, pleuritis, arthritis and erysipelas-like skin erythema. Renal failure due to amyloidosis is the most severe manifestation and the disease mainly affects the population of the Mediterranean ancestry including

Sephardic Jews, Armenians, Turks, Arabs and Italians. FMF is caused by mutations in MEFV gene [1]. The MEFV gene is located on the short arm of chromosome 16 and codes for a 781-amino acid-protein called pyrin or marenostrin expressed mainly in neutrophils and myeloid bone marrow precursors. This protein is localized with both microtubules and the actin filaments. It is proposed that pyrin regulates the inflammatory responses at the level of leukocyte cytoskeletal organization [2, 3]. MEFV includes 10 exons and 83 MEFV gene variants have been detected so far, but half of them are non-pathogenic. However, exon-10 (M694V, M694I, V726A, and M680I) and exon-2 (E148Q) include the predominant mutations in most of FMF patients [1, 4].

The associations of M694V homozygosity with a more severe form of the disease and with a higher frequency of amyloidosis have been reported although genotype–phenotype correlation is not well established [5, 6]. Nevertheless, in several other studies, the association of M694V mutation was not prevalent with all of the FMF patients, especially in Turks [7–12]. A recently reported multicenter Turkish study revealed that the most common genotype was M694V homozygosity, which was associated with an earlier onset and higher frequencies of arthritis and arthralgia but not erysipelas-like erythema and amyloidosis. Severity scores were not calculated and frequencies of attacks per year were not compared to the M694 homogosity in this study [13]. Unidentified genetic factors might have a protective or deteriorating effect on renal amyloidosis. The serum amyloid A (SAA) 1 gene polymorphisms have been shown to play a role as modifiers in FMF by Gershoni-Baruch et al's studies [14].

The major histocompatibility complex class I chain-related gene A (MICA) was suggested as another modifier gene in FMF. MICA gene is highly polymorphic and it is expressed on fibroblasts, keratinocytes, monocytes, endothelial cells and gastrointestinal epithelium. It can be recognized by the δγ T cells and natural killer (NK) receptor G2D (NKG2D) on the NK cells [15–17]. MICA has recently been found to be associated with a number of inflammatory and autoimmune diseases, such as acute anterior uveitis, psoriatic arthritis, celiac disease, Addison's disease, type 1 diabetes mellitus, mixed connective tissue disease, Behçet's disease and FMF [18–30]. There have been only two studies regarding the association of the MICA with FMF so far. One of them is Touitou et al's [29] study showing the modifying effect of MICA on FMF. Medlej-Hashim et al. [30] could not find any modifying effect of this locus on amyloidosis development.

The aim of this study was to determine the effects of MEFV genotypes and MICA genes on the course of the disease and amyloidosis in the Turkish FMF patients.

Materials and methods

Patients and controls

The FMF patients were collected from the Outpatient Clinic of Clinical Immunology and Rheumatology Department, Ibni Sina Hospital, School of Medicine, Ankara University. The patients with renal amyloidosis were referred from the Department of Nephrology in the same hospital. We evaluated 105 consecutive adult FMF patients who fulfilled the criteria for FMF diagnosis [31]. The control group consisted of 107 healthy adult subjects who had no history of FMF or Behçet's disease in their families and who were not related to the patients. Both the patients and the controls were of Turkish origin. The demographic and clinical data were recorded on a standardized form. The presence of amyloid depositions was confirmed by the tissue biopsies (rectal, duodenal, renal, gastric, intestinal and labial salivary gland) and subcutaneous fat aspiration. In the FMF patients without proteinuria, at least two tissue biopsies were performed in order to show that the patients didn't have amyloidosis. The severity score of the disease for each patient was calculated from the Tel-Hoshemer key. The Severity score was created by following information: age of onset, number of attacks per month, presence of arthritic attacks, erysipelas like erythema and amyloidosis and the dose of colchicine required the control symptoms) [32]. The informed consents were obtained from the patients and the controls before the study.

This study was approved by the Ethical Committee, School of Medicine, Ankara University, Ankara, Turkey (July 11, 2002 and No: I-10/18460).

DNA analysis

Genomic DNA was extracted from 0.5 ml, peripheral blood by spin colon based DNA extraction kit (CLP, USA).

MEFV mutation analysis

Five predominant mutations in exons 2 and 10 of the MEFV gene (M694V, M680I, M694I, V726A and E148Q) were investigated by amplifying the genomic DNA samples of the patients with normal and mutant-specific ARMS primers designed to selectively amplify the normal or altered sequence of each MEFV mutations

according to the previously described techniques [33]. The rare mutations in exons 3 and 5 were not systemically searched for or reported.

Triplet repeat polymorphism of MICA gene

The polymorphic transmembrane region of MICA gene was amplified using polymerase chain reaction (PCR) primers (F:5′ TGCCCTCTGGTGAGCCTA 3′, labeled at the 5′ end with Cy5.5; R:5′ CCTTACCATCTCCAGAAACTGC 3′). The amplifications were carried out in a total volume of 25 µl. The amplification mixture contained 50 ng genomic DNA, 18.6 µl dH_2O, 2.5 µl 10× PCR buffer, 1.5 µl 25 mM $MgCl_2$, 0.3 µl 10 mM, dNTPs, 0.2 µl PCR primers each and 0.2 µl 5 U/µl Taq DNA polymerase (DNAamp, England). Genomic DNA was amplified in a thermal cycler (ABI 9600, USA). The PCR amplification was performed beginning with an initial denaturation step at 94°C for 5 min then doing 32 cycles at 94°C for 30 s, at 62°C for 1 min, and at 72°C for 1 min, and finalizing with an extension step at 72°C for 5 min. The amplification products were mixed with size markers (225, 259 and 313 bp) and formamide-containing stop buffer. The mixtures were denaturized at 94°C for 2 min and subjected to vertical electrophoresis (53°C, 1,300 V, 32 min) on 2% polyacrylamide gel in an automated DNA sequencer (Visible Genetics, Canada). Respective alleles were determined as amplification products of sizes 275 bp (A4), 278 bp (A5), 279 bp (A5.1), 281 bp (A6), 290 bp (A9) and 293 bp (A10). Alleles were blindly and independently read by two different investigators. The five common alleles (A4, A5, A5.1, A6 and A9) and 15 genotypes (A4A4, A4A5, A4A5.1, A4A6, A4A9, A5A5, A5A5.1, A5A6, A5A9, A5.1A5.1, A5.1A6, A5.1A9, A6A6, A6A9 and A9A9) of the MICA microsatellite in the transmembrane region were found both in the patients and in the controls (26).

Statistical analyses

The associations of the clinical features and amyloidosis with MEFV and MICA genes were tested on 103 patients (phenotype I) and the associations between MICA and MEFV genes were tested on 105 patients (phenotype I and II). The statistical significance of differences between the groups was calculated by either chi-square test or Fisher's exact test for the categorical data and the *t* test for quantitative data. When necessary, a correction for multiple tests was made and corrected *P* (P_{corr}) values calculated by Bonferroni method. All the statistical tests were two-sided and the statistical significance was assigned to *P* values less than or equal to 0.05. Odds ratios (OR) and their 95% confidence intervals (95% CIs) were calculated. To make a parametric test, cutoff values were determined for severity scores, the age at onset and the frequency of attacks. The range severity of the scores was between 2 and 15 and a cutoff value for this parameter over seven was considered high and that of seven and less was considered low. The unit for the age at onset was determined as 'years' and the cutoff value was accepted 10 years of age. According to this, the patients were categorized into two groups as FMF symptoms beginning at 10 years of age and before, and those beginning after 10 years of age (29). The frequency of attacks was given as the number of the FMF attacks per year '*n*/year' and the cutoff value was determined as 12; 12 and less than 12/year was low and more than 12/year was high.

The relative importance of the predictive variables of severity was tested through a multivariate analysis consisting of a stepwise logistic regression and adjusted Odds ratios (OR_{adj}) and their 95% confidence intervals (95% CIs) were calculated.

Results

Demographic and clinical characteristics of the patients

One hundred and three patients were symptomatic for FMF at the time of diagnosis (Phenotype I). Two patients were asymptomatic (Phenotype II) and they had positive family histories of FMF and AA type amyloidosis in their renal biopsies. Their mutation analysis supported the diagnosis of FMF. A total of 105 patients (64 F, 41 M, mean age = 37.08 year) and 107 controls (62 F, 45 M, mean age = 34.78 year) were included in the study. All the symptomatic patients had fever and abdominal pain during their attacks. Of the patients, 33 had amyloidosis (phenotype I, $n = 31$ and phenotype II, $n = 2$) and 72 did not have amyloidosis. Fifty-eight FMF patients had familial histories of the disease. A familial relation was noted in 25 patients. Of the patients, 46 had undergone one or more abdominal operations in which appendectomy ($n = 41$) was the most common. All erysipelas-like erythemas were accompanied by arthritis attacks.

Associations of MEFV genotypes

The distribution of the clinical features and the presence or absence of amyloidosis in the patients according to MEFV genotypes were presented in Table 1.

Table 1 The distribution of the clinical characteristics according to MEFV genotypes

MEFV genotypes/*n*	Age at onset [a]		Frequency of attacks [b]		Severity scores[c]		Arthritis attacks[d] *n* = 40	Erysipelas-like erythema[e] *n* = 13	Pleuritis[f] *n* = 64	Amyloidosis[g] *n* = 31
	≤10 year	>10 year	<12/year	≥12/year	≤7	>7				
M694V homozygous *n* = 19	14	5	11	8	5	14	13	7	12	10
Not M694V homozygous *n* = 84	32	52	76	8	50	34	27	6	52	21

One hundred and three FMF patients (Phenotype I)

[a] $P = 0.005$, [b] $P = 0.001$, [c] $P = 0.004$, [d] $P = 0.003$, [e] $P = 0.002$, [f] $P > 0.05$, [g] $P = 0.018$

The major MEFV genotypes were M694V homozygous ($n = 20$; 19 phenotype I, 1 phenotype II), M694V/M680I ($n = 19$), M694V/not identified (NI), ($n = 19$), M694V/V726A ($n = 12$), M694V/E148Q ($n = 5$), M680I/V726A ($n = 7$), M680I/M680I ($n = 5$; 4 phenotype I, 1 phenotype II), V726/NI) ($n = 4$), M680I/NI ($n = 3$), E148Q/NI ($n = 3$), E148Q/E148Q, M694V/NI ($n = 2$), M694V/M694I ($n = 1$), E148Q/E148Q ($n = 1$) and M694V/M680I-V726A (complex allele, $n = 1$) and none of the investigated mutations were found in three patients.

In this study, it was confirmed once more that M694V homozygosity was related to a severe form of FMF. M694V homozygous FMF patients had an earlier age at onset ($P = 0.005$, OR = 4.55, 95%CI = 1.5–13.83), more frequent arthritis attacks ($P = 0.003$, OR = 4.57, 95%CI = 1.57–13.34), more frequent FMF attacks ($P = 0.001$, OR = 7.6, 95%CI = 2.33–24.76), higher severity scores ($P = 0.008$, OR = 5.15, 95%CI = 1.56–16.98), more erysipelas-like erythema ($P = 0.002$, OR = 7.58, 95%CI = 2.18–26.43) and more amyloidosis ($P = 0.018$, OR = 3.33, 95%CI = 1.19–9.31) compared with the patients who did not have M694V homozygosity, but there was not such a relation with pleuritis ($P > 0.05$) (Table 2).

Associations of MICA alleles and genotypes

No significant differences in MICA alleles and genotypes were detected between the patient group and the controls ($P > 0.05$). The relation of A5A5 genotype to arthritis was significant and it may be worth reporting that all patients with A5A5 had arthritis attacks ($P_{corr} = 0.003$, OR = 23.93, 95%CI = 1.31–437.57), but the number of the patients with this genotype was small ($n = 6$). The pleuritis, the age at onset, the frequency of attacks, erysipelas-like erythema, amyloidosis and severity scores were not different in the distribution of MICA alleles and genotypes ($P > 0.05$). Since the number of patients in each MICA genotype was insufficient, MICA genotypes could not be evaluated when corrected *P* values were calculated.

We evaluated the possible effect of MEFV and MICA in the course of the disease, severity scores and development of amyloidosis. The impact of M694V homozygosity on the development of amyloidosis was higher ($P = 0.009$, OR_{adj} 4.42, 95%CI 1.44–13.55 with or without M694V homozygosity). However, the presence of A5 allele showed a protective effect for amyloidosis in FMF patients ($P = 0.038$, OR_{adj} 0.26, 95%CI 0.074–0.93 with or without A5, Fig. 1). However, a similar association between M694V homozygous and MICA alleles on other parameters was not detected.

Table 2 The frequency of A5 allele and M694V genotype status in the amyloid and non amyloid FMF patients

	Amyloidosis ($n = 31$)	Nonamyloidosis ($n = 72$)
A5 (+) *n* = 25	4	21
A5(-) *n* = 78	27	51
M694V homozygous *n* = 19	10	9
Not M694V homozygous *n* = 84	21	63

Discussion

Familial Mediterranean fever is an auto-inflammatory disease with phenotypic differences due to some genetic factors. It is suggested that MEFV, SAA and MICA genes have important roles on different clinical presentations of FMF [5, 6, 13, 27–29].

The overall risk of amyloidosis in FMF is the product of a complex interaction of factors that beside the MEFV genotype include a positive family history for amyloidosis, delay in diagnosis and treatment, male gender the SAA1 genotype, and colchicine noncompliance. However, in most of the studies the risk of amyloidosis is associated with the M694V/M694V genotype [5, 6, 14]. In this study, it once more was shown that genotypes of M694V in homozygosity were independently

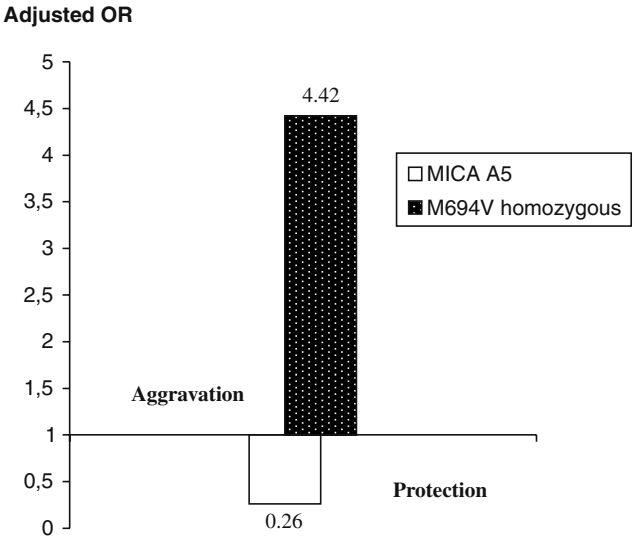

Fig. 1 Relative contributions of MEFV homozygosity and MICA-A5 allele on the development of amyloidosis ($P = 0.038$, OR_{adj} 0.26, 95%CI 0.074–0.93 with A5 and $P = 0.009$, OR_{adj} 4.42, 95%CI 1.44–13.55 without A5)

predisposed to arthritis attacks, earlier age at onset, higher severity scores, frequency of attacks, the presence of erysipelas-like erythema and amyloidosis. Both the national Turkish study and our study revealed that the most common genotype was M694V homozygosity, which was associated with earlier onsets and higher frequencies of arthritis. In our study we also found an association between M694V homozygosity and erysipelas-like erythema and amyloidosis; however, in the national Turkish study there was not an association like that. The national multi-center Turkish study was conducted on child and adult patients (mean age = 23 years) and the patients were from different regions of Turkey (13), but our study was conducted only on adult patients (mean age = 37 years) and the patients were from Ankara, a single center and they were from different socioeconomic classes. Although there are certain sub ethnic groups in Turkey, these groups are generally mixed and it is hard to specify, but the intensity level of these subgroups may be different from one region to another. And it may explain the differences between the two groups. An increased risk of amyloidosis among M694V homozygotes was also reported by two other Turkish studies [34, 35].

The frequency of FMF attacks may be precipitated by tiring physical activity, emotional stress and infections. MICA protein has recently been shown to activate γδ T cells and NK cells through a surface receptor, NKG2D, and a cell adaptor protein, DAP10. Although the function of the MICA molecule is not yet fully understood, MICA genes are considered to be a stress-inducible surface molecule because of its up regulated expression upon heat shock protein (HSP 70) or oxidative stress protein [16, 17]. In the cases which have different phenotypic features, although they bear the same MEFV genotype, it is assumed that environmental factors, particularly stress and some other genetic factors may affect this difference. MICA genes may arrange the synthesis of HSP or oxidative stress proteins and therefore they may be responsible for the differences on the phenotype of the disease. Recent studies have shown that some MICA alleles may be over represented in inflammatory diseases, that is, A9 in psoriatic arthritis and diabetes, A6 in Behçet's disease, A5.1 in Addison's disease and celiac disease, A4 in acute anterior uveitis, and both A4 and A5.1 in mixed connective tissue disease [18–23, 26–28]. Nevertheless, MICA-A6 was protective in diabetes and MICA-A4 was protective in Kawasaki disease [24, 25].

Polymorphisms at the MICA gene were also found to play a modifying role in FMF. Touitou et al. [29] reported that MICA-A9 had an aggravating effect on an earlier age at onset of the disease in homozygous patients for M694V. On the contrary, MICA-A4 dramatically reduced the frequency of the attacks. In their study, the effect of MICA alleles on the development of amyloidosis was not searched. Medlej-Hashim et al. [30] on the other hand, reported the absence of a MICA-modifying effect on the development of amyloidosis.

In this study, when we looked at the effect of MICA alleles on the course of the disease and on the development of amyloidosis in the M694V homozygous patients, A5 allele appeared to have a decreasing effect on the development of amyloidosis. When we used multivariate analysis, we could not statistically evaluate each of the MICA genotypes due to the small number of the patients in MICA genotypes. The reason may be stated due to difference in the composition of the patient population between the two studies. Touitou at al. had significant results in their series of 150 patients but we had significant results in our series 103 patients. Their patients consisted of a high number of non-Ashkenazi Jews (NAJs) and a small number of Turks, Arabs, Armenians and Southern Europeans but in our group all the patients were Turks. However, the structure of Medlej-Hashim et al's population was not revealed in details in their paper and the number of the patients was smaller than Touitou's and ours [29, 30].

It is possible to suggest the existence of other modifying factors or genes on the development of amyloidosis and on the course of the disease although the effects of the MEFV genotypes on them are clear. Some MICA alleles, especially A5, seem to exert a protective effect on the development of amyloidosis. Furthermore,

the encouraging results will require more studies with larger groups from different populations.

Acknowledgment This study is supported by the Foundation of Academic Research Projects of Ankara University (No: 2003-0809093).

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