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Genotype–phenotype correlation in a large group of Turkish patients with familial Mediterranean fever: evidence for mutation-independent amyloidosis

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Abstract

Objectives. Differences in clinical manifestations of familial Mediterranean fever (FMF) between different ethnic groups have been documented. The FMF gene was recently cloned and four missense mutations (Met694Val, Met680Ile, Val726Ala, and Met694Ile) that account for a large percentage of the patients were identified. The results of initial mutation studies have led to the hypothesis that phenotypic variation of the disease may be attributable to the existence of some of these mutations. The purpose of this study was to evaluate whether this phenotypic variation is associated with the existence of particular mutations in Turkish FMF patients living in Turkey.

Methods. Four missense mutations and genotype–phenotype correlation were investigated in 167 Turkish FMF patients. The patients were grouped according to the presence of the Met694Val and the Met680Ile mutations, and 12 clinical parameters were compared between the groups.

Results. The presence of the Met694Val mutation was not found to be associated with a severe form of the disease or the development of amyloidosis. Arthritis frequency was found to be lower in the patients with homozygous Met680Ile mutation.

Conclusions. None of the four missense mutations is associated with a severe disease or the development of amyloidosis in Turkish FMF patients living in Turkey. The influence of unknown environmental factors and/or the presence of other genetic changes are necessary to explain the phenotypic variation of the disease and the development of amyloidosis.

KEY WORDS: Familial Mediterranean fever, Turks, Genotype–phenotype, Amyloidosis.

Familial Mediterranean fever (FMF) is a genetic disease with autosomal recessive inheritance. The disease most commonly occurs in Jews, Turks, Armenians, and Arabs. FMF is characterized by recurrent and self-limited attacks of fever accompanied by peritonitis, pleuritis, synovitis, or erysipelas-like erythema. Amyloidosis is the most important complication of the disease that determines the prognosis [1]. Phenotypic variation between different ethnic groups has already been documented [1, 2]. Several authors have emphasized that Turks have a severe disease with a relatively higher incidence of amyloidosis as compared with the other ethnic groups [3–6]. The incidence of amyloidosis in patients with FMF appears to be markedly influenced by ethnic background. In early reports, an incidence of 60% has been reported in Turks, 27% in Jews, and lower rates in Armenians and Arabs [1, 4, 5, 7, 8].

In 1997, the International FMF Consortium and the French FMF Consortium independently cloned the gene for the disease on the short arm of chromosome 16 and identified four missense mutations on FMF carrier chromosomes [9, 10]. The French FMF Consortium reported that four disease-associated mutations (Met694Val, Met680Ile, Val726Ala and Met694Ile) accounted for 85% of the carrier chromosomes in their study group [10]. The results of these initial studies have led to the hypothesis that phenotypic variation of the disease may be attributable to the existence of particular mutations [9, 10]. The Met694Val mutation is also known as the Mediterranean mutation since it was demonstrated in FMF patients from different ethnic backgrounds including non-Ashkenazi Jewish, Turkish, Armenian, and Arab. The haplotype analyses implied that all chromosomes carrying this mutation may have been derived

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from a single ancestral chromosome, which originated probably more than 2000 yr ago [11]. The Met694Val mutation was found in 97% of the North African, 30% of the Iraqi Jewish, and about 25% of the Armenian carrier chromosomes [12, 13]. As this is the most common mutation in populations with a high incidence of amyloidosis, the possible explanation was that the Met694Val homozygotes would be prone to the development of amyloidosis [9, 13]. In contrast to the above findings, the Val726Ala mutation was described in somewhat different ethnic groups, including Ashkenazi Jews, Druzes, Armenians, and Iraqi Jews in which amyloidosis is seen at lower frequencies. This suggested that the Val726Ala mutation might be protective for amyloidosis [9, 13]. However, recently our group has shown that the development of amyloidosis is not prevented by the presence of the Val726Ala mutation [14]. Some preliminary studies covering mostly North African Jewish FMF patients claimed that there is a relationship between the severity of the disease and Met694Val homozygosity [13, 15]. Recent reports from Israel demonstrated a high frequency of the Met694Val mutation among people suffering from amyloidosis and supported this idea [16, 17]. In addition, the Met680Ile mutation, commonly seen in Armenians was suggested to be associated with a milder phenotype of the disease and lower frequency of amyloidosis [7, 18]. These exciting data from the literature encouraged us to gather a large amount of data on Turkish FMF patients to study the genotype–phenotype correlation. In this respect, by this study we intended to evaluate the existence of four mutations and to correlate the phenotypic features with the co-existing type of mutation.

Materials and methods

The ethics committee approved the study encompassing 238 Turkish FMF patients from 167 unrelated families. Informed consent was obtained from each patient or parent. Nevertheless, the study was limited to 167 index cases to prevent the influence of background genes in multiplex families. The diagnosis of FMF was established according to previously described criteria [19]. As the patients had been referred from a number of medical centres throughout Turkey, all of them were interviewed directly by one of the clinicians with identical clinical forms. A questionnaire including the following information: sex, age of onset, age at diagnosis, the presence of fever, peritonitis, pleuritis, arthritis, erysipelas-like lesion, the severity score of the disease, the development of amyloidosis, and the presence of a family history of FMF and amyloidosis, was developed. The severity score of the disease (manifested by an earlier age of onset, an increase in frequency and severity of joint involvement, a higher incidence of erysipelas-like erythema and requirement of a higher dose of colchicine to control symptoms) was calculated according to the previously described criteria [20]. Patients with amyloidosis presented with persistent proteinuria or nephrotic syndrome and the diagnosis was confirmed by the presence of amyloid depositions in renal biopsy specimens in all of them. The genetic data covering the mutation results were kept by another investigator coded for each patient as a blind trial to be evaluated later.

Mutation analysis

The four mutations (Met694Val, Met680Ile, Val726Ala, and Met694Ile) were systematically investigated in the patients. DNA was extracted from peripheral blood lymphocytes according to standard procedures. Mutation identification was performed according to previously described techniques. Exon 10 of the gene was amplified with the polymerase chain reaction (PCR) with specific forward 5′-GAGGTGGAGGTTGGAGACAA-3′ and reverse 5′-TCCTCCTCTGAAATCCATGG-3′ primers [9]. For the identification of the Met680Ile and Val726Ala mutations, *Hin*fI (Promega, Madison, USA) and *Alu*I (Fermentas, Lithuania) restriction endonucleases were used, respectively. A *Hin*fI site was destroyed in the first case; an *Alu*I site was created in the second case. The PCR products were analysed on 2% agarose and 10% polyacrylamide gels, respectively [10].

The mutations were assessed by amplifying the genomic DNA template with three sets of normal and mutant-specific amplification refractory mutation system (ARMS) primers designed to selectively amplify the normal or altered sequence of each of the FMF gene mutations: Met694Val, Val726Ala and Met694Ile. Each set of primers consisted of three oligonucleotides, their sequences were as follows: Met694Val common: 5′-TATCATTGTTCTGGGCTC-3′, mutant: 5′-TGGTACTCATTTTCCTTCAC-3′, normal: 5′-TGGTACTCATTTTCCTTCAT-3′ [21]. Val726Ala common: 5′-TGGAGGTTGGAGACAAGACAGCATGGATCC-3′, mutant 5′-TGGGATCTGGCTGTCACATTGTAAAAGGAGATGCTTCCTG-3′, normal: 5′-TGGGATCTGGCTGTCACATTGTAAAAGGAGATGCTTCCTA-3′ [21]. Met694Ile common: 5′-TATCATTGTTCTGGGCTC-3′, normal: 5′-CTGGTACTCATTTTCCTTC-3′, mutant: 5′-CTGGTACTCATTTTCCTTT-3′ [10]. For the analysis of Val726Ala both ARMS and restriction endonuclease (RE)/*Alu*I methods were used.

PCR amplification was performed in a final volume of 100 μl containing 100 ng of purified genomic DNA, 0.2 U Taq polymerase (Promega) and its 10× PCR buffer (containing 15 mM $MgCl_2$), 0.2 mM dNTP mix (Promega) and 1 pmol of each primer. The PCR amplification conditions were kept the same for all of the ARMS tests and the reaction was carried out as follows: heating of the reaction to 94°C for 5 min for denaturation, followed by 35 cycles with denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 2 min, followed by 7 min final extension at 72°C. The amplified products were separated by electrophoresis on a 2% agarose gel. Ethidium bromide staining of the agarose gel was used to detect the amplified fragments [21].

Statistical analysis

Categorical variables were compared by χ^2 test or Fisher's exact test where applicable. For the Met694Val mutation, differences between the three groups for age of onset, age at diagnosis and disease severity score were evaluated using one-way ANOVA after log transformation. When there was a significant difference between the groups, Tukey's Honestly Significant Difference (HSD) test was used for multiple comparisons. For the Met680Ile mutation, differences between the three groups in the above parameters were evaluated by Kruskall–Wallis variance analysis. The results are reported as mean $\pm$ standard deviation (S.D.). For all tests a two-tailed P value of <0.05 was considered as significant.

Results

Clinical data

Table 1 summarizes the phenotypic features of the patients. Eighty-four of the 167 patients were male and 83 were female. The age of onset ranged from 1 month to 40 yr with a mean of 6 yr. The common clinical features were abdominal pain and fever, followed by arthritis and chest pain. Four patients had protracted arthritis, one of whom had been reported as an unusual complication of FMF [22]. Renal biopsy revealed amyloidosis in 25 (15%) patients.

Genetic data

The mutation frequencies of the carrier chromosomes are shown in Table 2, Met694Val being the most common mutation, found in 41% of the chromosomes studied. The Met694Ile mutation, which was exceedingly rare in other than Arabic FMF patients, was found in 17% of the carrier chromosomes in our patient population. The Met680Ile and Val726Ala mutations were found in 16 and 14%, respectively. Fifty-two (31%) of the 167 patients were homozygotes, 78 (47%) were compound heterozygotes with two of the four mutations. Thirty-three (20%) patients had only a single mutation and no mutation was identified on either of the alleles in four (2%). These results show that 88% of the studied chromosomes were carriers of the mutations and clinical diagnosis of FMF was confirmed by molecular study in 78% of the patients in our study group.

TABLE 1. Phenotypic features of the patients

n	167
Male/female	84/83
Age at onset (yr $\pm$ S.D.)	6.30 $\pm$ 6.85
Age at diagnosis (yr $\pm$ S.D.)	11.97 $\pm$ 9.61
Abdominal pain	91%
Fever	90%
Arthritis	46%
Chest pain	45%
Erysipelas-like erythema	23%
Amyloidosis	15%
Disease severity score	8.26 $\pm$ 2.46
Family history of FMF	67%
Family history of amyloidosis	20%

FMF, familial Mediterranean fever.

TABLE 2. Mutation distribution of 167 patients (a total of 334 alleles)

Mutation	*n*	
Met694Val	137	41%
Met694Ile	55	17%
Met680Ile	53	16%
Val726Ala	48	14%
Unidentified mutations	41	12%

Genotype–phenotype correlation

In order to demonstrate whether there is a mutation-specific difference in the phenotypic expression of the disease, the patients were pooled according to the presence of the two mutations: Met694Val and Met680Ile, the former previously defined as severe and the latter as milder.

For evaluation of the Met694Val mutation, 167 patients were divided into three groups according to the presence of the Met694Val mutation on both of the alleles (homozygotes), on only one allele (heterozygotes), and on none of the alleles. Twelve clinical parameters of the disease were compared between the 41 homozygous and 91 compound heterozygous patients for the Met694Val mutation, and 35 patients who did not have this mutation (Table 3). The age of onset was higher in the homozygous patients as compared with the patients of the other groups ($P < 0.05$). No statistically significant differences were found between these groups for the other phenotypic features, including the frequency of amyloidosis.

Similarly, the patients were divided into three groups according to the presence of the Met680Ile mutation (Table 4). The frequency of arthritis was significantly lower in the patients who were homozygous for the Met680Ile mutation when compared with the other two groups. There were no statistically significant differences for the other parameters between the patients homozygous or heterozygous for the Met680Ile mutation, or the patients without this mutation.

Table 5 shows the frequencies of all observed genotypes and the frequencies of amyloidosis in these genotypes. The mutation frequencies in the patients with amyloidosis are shown in Table 6.

Discussion

In this study we present a mutation analysis of a large group of Turkish patients suffering from FMF. Our study confirms the mutational heterogeneity of FMF in a Turkish population. The Met694Val mutation, which is found in about 90% of North African Jews, was found in about 40% of carrier chromosomes in Turkish FMF patients. The Met680Ile mutation, which is known to be common in Armenians, was found in 14% of the carrier chromosomes of our patients. Recently, three mutations (Met694Val, Met680Ile and Val726Ala) were studied in a panel of 21 Turkish FMF patients and 85% of the chromosomes were found to be the carrier for

Table 3. Phenotypic features according to the Met694Val mutation

	Met694Val–Met694Val	Met694Val–other[a]	Other[a]–other[a]
n	41	91	35
Male/female	20/21	42/49	22/13
Age of onset (yr)	10.33 ± 10.45[b]	5.21 ± 4.79	5.33 ± 5.91
Age at diagnosis (yr)	13.37 ± 12.03	11.50 ± 8.97	11.82 ± 8.74
Fever	85%	87%	97%
Abdominal pain	87%	90%	100%
Chest pain	46%	42%	46%
Arthritis	39%	47%	51%
Erysipelas-like erythema	15%	25%	34%
Amyloidosis	10%	15%	20%
Disease severity score	7.78 ± 1.78	8.24 ± 2.42	8.63 ± 2.29
Family history of FMF	49%	71%	74%
Family history of amyloidosis	15%	24%	20%

FMF, familial Mediterranean fever.
[a]FMF chromosomes with either one of the three other mutations (Met680Ile, Val726Ala, Met694Ile) or with other unidentified mutations.
[b]$P < 0.05$ *vs* Met694Val–other and Other–other.

Table 4. Phenotypic features according to the Met680Ile mutation

	Met680Ile–Met680Ile	Met680Ile–other[a]	Other[a]–other[a]
n	11	31	125
Male/female	9/2	12/19	63/62
Age of onset (yr)	9.0 ± 10.83	5.33 ± 3.95	6.39 ± 7.16
Age at diagnosis (yr)	15.44 ± 13.52	10.75 ± 8.96	11.97 ± 9.42
Fever	90%	97%	88%
Abdominal pain	100%	94%	91%
Chest pain	55%	45%	44%
Arthritis	0%[b]	42%	50%
Erysipelas-like erythema	0%	19%	27%
Amyloidosis	18%	13%	15%
Disease severity score	6.75 ± 1.83	7.70 ± 2.00	8.53 ± 2.32
Family history of FMF	73%	74%	64%
Family history of amyloidosis	0%	16%	23%

FMF, familial Mediterranean fever.
[a]FMF chromosomes with either one of the three other mutations (Met694Val, Val726Ala, Met694Ile) or with other unidentified mutations.
[b]$P < 0.05$ *vs* Met680Ile–other and Other–other.

one of the three mutations [23]. Our data, which included 167 patients along with the above findings, confirm that these four mutations can be used to identify the molecular defect in most Turkish FMF patients. However, it should be noted that we included only typical FMF patients in this study. Therefore, it would not be surprising if these four mutations accounted for a smaller percentage in a broader sample. This phenomenon was observed in the National Institutes of Health experience in which three mutations were found in 85% of FMF patients initially, yet 16 mutations are detected in only 79% of the patients currently [11]. Since the disease is clearly autosomal recessive, our patients with only one mutation most probably have one of the other mutations that were not studied here. Similarly, four patients who did not have the four mutations studied probably have other mutations because they have classical features of FMF.

Two preliminary studies from Israel and France have emphasized the importance of the Met694Val mutation. Their results indicate that Met694Val homozygotes generally do have more severe disease and a higher risk of developing amyloidosis than those who are not carriers of this mutation [13, 15]. It was also suggested that it is essential to detect asymptomatic patients who are homozygous for the Met694Val mutation and treat them in order to prevent amyloidosis [15, 18]. However, none of the 83 patients from Israel showed the Met680Ile or Met694Ile mutations. Moreover, 80 of them had the Met694Val mutation on at least one allele and 70 patients were homozygotes for this mutation, including 12 with amyloidosis [13]. The data from the French group included only three patients with amyloidosis, all were homozygous for the Met694Val mutation in their 109 Jewish FMF patients [15]. Recently three reports have supported these preliminary studies. Brik *et al.* [24] reported the mutation analysis of a total of 70 patients with FMF from mainly North African Jews and Moslem Arabs. Although they found an association between severe disease and homozygosity of the Met694Val mutation, their study did not include any patients with amyloidosis. In Shohat *et al.*'s [17] report, amyloidosis was present in 18 of 87 patients who were homozygous for the Met694Val mutation and two of 41 who were compound heterozygous. They could not find a patient with amyloidosis without the Met694Val mutation. Livneh *et al.* [16] reported the mutation analysis of 178 non-Ashkenazi Jewish patients which included

TABLE 5. Distribution of the major genotypes among all familial Mediterranean fever (FMF) patients in comparison with the patients with amyloidosis

	Met680Ile–Met680Ile	Met680Ile–Met694Val	Met680Ile–Met694Ile	Met680Ile–Val726Ala	Met680Ile ?	Met694Val–Met694Val	Met694Val–Met694Ile	Met694Val–Val726Ala	Met694Val ?	Met694Ile–Val726Ala	Met694Ile ?	Val726Ala ?	? ?
(a)	11 (6.6%)	24 (14.4%)	2 (1.2%)	4 (2.4%)	1 (0.5%)	41 (24.6%)	10 (6%)	36 (21.6%)	21 (12.6%)	2 (1.2%)	5 (3%)	6 (3.6%)	4 (2.4%)
(b)	2/11 (18%)	1/24 (4%)	1/2 (50%)	2/4 (50%)	–	4/41 (10%)	1/10 (10%)	7/36 (19%)	5/21 (24%)	–	1/5 (20%)	1/6 (17%)	–

?: other unidentified mutation.
(a) Number and (percentage) of patients with this genotype.
(b) Number and (percentage) of patients with amyloidosis in FMF patients with this particular genotype.

TABLE 6. Mutation distribution in 25 patients with amyloidosis (a total of 50 alleles)

Mutation	*n*	
Met694Val	22	44%
Met680Ile	8	16%
Val726Ala	10	20%
Met694Ile	3	6%
Unidentified mutations	7	14%

30 patients with amyloidosis. Twenty-seven of their patients with amyloidosis were homozygous for the Met694Val mutation. However, their report also included two patients who were homozygous for the Val726Ala mutation. In another recent report, the Met694Val mutation was found to be uncommon in an Ashkenazi Jewish population in which amyloidosis was also uncommon [11]. Based on these reports, one should expect to observe a high frequency of the Met694Val mutation with an association of amyloidosis in Turkish patients, since Turks have severe disease and a high frequency of amyloidosis. However, we could not find any evidence that supports this claim for Turkish FMF patients. It should be noted that the FMF patients in this study had a broad spectrum of mutation combinations, which might reflect the inter-cultural interactions of ancient ethnic groups that lived in Anatolia. Moreover, certain environmental factors might play roles in the development of amyloidosis. Our results show that the presence or homozygosity of the Met694Val mutation is associated with neither a severe form of the disease nor the risk of developing amyloidosis in this Turkish FMF population living in Turkey.

Previous reports indicate that the Armenian population tends to have a less severe disease and a low incidence of amyloidosis that may be due to the high occurrence of the Met680Ile mutation in this ethnic group [7, 18]. When we compared the clinical features, only the frequency of arthritis was found to be lower in the patients with homozygous Met680Ile mutation. Interestingly, none of our 11 patients with homozygous Met680Ile mutation had a history of arthritis during their febrile attacks. The frequency of amyloidosis did not differ with homozygosity, heterozygosity or the absence of this mutation.

The relationship between FMF and amyloidosis is still obscure. It is known that the development of amyloidosis is not a complication of a more severe disease, but rather an associated condition, because there are some patients who present with amyloidosis with very mild or no inflammatory symptoms. In our study, considerable variations in the phenotypic features of the disease were noted in all groups, including the patients who were homozygous for the Met694Val mutation. Unlike the French and Israeli experience, our data indicate that specific FMF mutations are not the sole determinants of disease severity and the development of amyloidosis. Furthermore, we also noted that 25 patients with amyloidosis showed all four missense mutations studied. Two recent reports from the USA and the UK

support our results in a completely different group of patients with a diverse spectrum of ethnic backgrounds. In 100 patients from the USA, there were only two patients with amyloidosis and none of them were homozygous for the Met694Val mutation [25]. In the study from the UK, nine patients out of 27 had amyloidosis, of whom only one was homozygous and three were compound heterozygotes with the Met694Val mutation, whereas the remaining five were without the Met694Val mutation [26]. These results provide additional evidence that the patients with mutations other than Met694Val are also at risk for the development of amyloidosis. Ethnic background is one of the major contributors to the risk of amyloidosis, but the possibility of an environmental influence cannot be ignored by the observation that amyloidosis was not found in Armenians living in the USA, but in 25% of Armenian FMF patients living in Armenia [7].

As our study group represents one of the largest samples of FMF patients published so far with four missense mutations including 25 patients with amyloidosis, we came to the conclusion that the risk of amyloidosis is not as simple as explained by the presence of the Met694Val mutation. We suggest that the influence of unknown environmental factors and/or the presence of other involved genetic changes are necessary to explain the association of FMF and amyloidosis. In order to solve this mysterious relationship, more population-based, epidemiological, well-collected data in world-wide collaborative studies are warranted.

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