Original Article
Association between myeloperoxidase gene polymorphism and familial mediterranean fever in Turkish Children

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Abstract: Background: Familial Mediterranean Fever (FMF) is the most common autoinflammatory disease. Autoinflammatory disorders are characterized by exaggerated immune system responses. Neutrophils and their byproduct, myeloperoxidase, are important components of the innate immune system. In the present study, we searched for myeloperoxidase gene polymorphisms in FMF patients. Methodology/Principal Findings: We evaluated 83 children diagnosed with FMF by their physicians and 93 controls without any family history of FMF. MPO gene polymorphisms were detected using polymerase chain reaction (PCR)-based methods. We genotyped all samples in terms of the -463G/A single-nucleotide polymorphism, the most extensively studied MPO polymorphism. Allelic and genotypic frequencies were calculated, and possible associations with FMF explored. The frequencies of MPO polymorphisms differed significantly between the study and control groups (P = 0.003). The AA and AG gene polymorphisms were more prevalent in the FMF group than in the controls. The A allele was more prevalent in the FMF group (P = 0.001), and the frequency of the G allele was similar between the two groups (P = 0.128). Conclusion: MPO gene polymorphisms and allelic differences may be important in the pathogenesis of FMF.

Keywords: Familial mediterranean fever, MEFV gene mutation, myeloperoxidase, oxidative stress, gene polymorphism

Introduction

Familial Mediterranean Fever (FMF) is an autosomal, recessive autoinflammatory disorder characterized by recurrent attacks of fever, serositis, and arthritis/arthralgia and massive influxes of polymorphonuclear neutrophils. FMF is the most common autoinflammatory disease worldwide, and is particularly prevalent in countries surrounding the Mediterranean Sea, affecting principally Turks, Arabs, Jews, and Armenians [1-4]. Autoinflammatory diseases are characterized by exaggerated activation of innate immunity in response to exogenous or endogenous stimuli. The MEFV gene (encoding pyrin) is the gene involved in FMF; pyrin is predominantly expressed by the innate immune system. Pyrin binds to the protein NLRP3 to form the inflammasome complex, and mutations in MEFV can cause excessive inflammation [3, 5].

Although the detailed pathogenesis of FMF has been intensely studied, the etiology of the disease and diversity in the clinical course of disease remain incompletely understood. Many studies have suggested that increased oxidative stress, generation of reactive oxygen species (ROS), and/or impairment of the oxidant/ antioxidant balance may play important roles in disease pathogenesis. Neutrophils are an important component of the innate immune system and are also a significant source of free radicals; these cells play a key role in FMF pathogenesis. Massive neutrophil influx into the serous membranes, accompanied by increased cytokine levels and elevated concentrations of acute-phase reactants, are evident

Table 1. Comparison of MPO G-463/A genotype distributions and allele frequencies between FMF and control subjects

		Study (n = 83)	Control (n = 98)	P
		n (%)	n (%)	
MPO Genotype	AA	16 (19.3)	10 (10.2)	[b]0.003**
	AG	45 (54.2)	38 (38.8)	
	GG	22 (26.5)	50 (51.0)	
	G allele	67 (80.7)	88 (89.8)	[c]0.128
	A allele	61 (73.5)	48 (49.0)	[c]0.001**

[b]Pearson's chi-squared test; [c]Yates' continuity correction test; **P < 0.01.

during inflammatory episodes [6, 7]. The acute febrile attacks are only the tip of the iceberg; subclinical inflammation, accompanied by elevated levels of acute-phase proteins and cytokines, persists during attack-free periods [4, 6, 7]. Myeloperoxidase (MPO), which is present in neutrophils, is an important oxidative enzyme, being an essential component of the antimicrobial system; the enzyme is also involved in regulation of inflammation [8, 9]. MPO catalyzes conversion of H_2O_2 to hypochlorous acid (HOCl), which is a potent microbiocide and a toxic oxidant impairing DNA repair and mononuclear cell responsiveness. An increase in the levels of MPO and its reactive byproducts at a site of inflammation may cause tissue damage [10, 11].

Although it is known that oxidative stress plays a crucial role in FMF, no work has yet explored whether MPO polymorphisms play a role in FMF. We hypothesized that the MPO G-463A polymorphism might be important in FMF pathogenesis. In the present study, we evaluated the frequency of this polymorphism in FMF patients.

Material methods

Study population

This was a case-control study that included 83 children with FMF (40 girls, 43 boys; mean age 9.86±3.63 years, range 2-18 years) who were admitted to the Pediatric Nephrology Unit, Medical Faculty, BezmialemVakif University, and 98 controls (50 girls, 48 boys; mean age 11.13±2.61 years, range 5-16 years) who visited the pediatric outpatient clinic of the same hospital for routine checkups. FMF was diagnosed by physicians using the Tel-Hashomer

criteria [12]. Disease severity was assessed using the modified scoring system of Pras et al. [13]. No child in the study group had any other chronic or autoinflammatory disorder. No control or study patient had any acute or chronic illness, and no control child had a family history of FMF.

DNA isolation: Blood specimens were collected into tubes containing EDTA, and DNA was extracted from whole blood using a salting-out procedure [14].

Identification of the MPO-463 G/A polymorphism: The polymorphic site at position -463 on the MPO gene was amplified using forward (5'-CGG TAT AGG CAC ACA ATG GTG AG-3') and reverse primers (5'-GCA ATG GTT CAA GCG ATT CTT C-3') (Invitrogen), as earlier described [15]. Polymerase chain reaction (PCR) was performed with the aid of Taq polymerase (Invitrogen); the cycling conditions were 95°C for 2 min; followed by 35 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s. The PCR product was 350 bp in length. Forty microliters of each PCR product was digested with *Aci*I (Thermo Scientific) at 37°C overnight, and the fragments were separated on a 2% (w/v) agarose gel. Three possible genotypes were defined by three distinct banding patterns: A/A: 289- and 61-bp fragments; A/G: 289-, 169-, 120-, and 61-bp fragments; and G/G: 169-, 120-, and 61-bp fragments [15].

Statistical analysis

Statistical analysis was performed with the aid of Number Cruncher Statistical System software (2007 version) (Kaysville, UT, USA). Numerical parameters are presented as median with range or mean with standard deviation. The distributions of categorical measurements are presented as frequencies with percentages. Comparisons were performed using the Student's t-test, Kruskal-Wallis test, and Mann-Whitney U-test. Categorical data were evaluated using the chi-squared test, Pearson's chi-squared test, Fisher-Freeman-Halton test, Fisher's exact test, and the Yates' continuity correction test. A *p*-value < 0.05 was considered to reflect statistical significance.

Results

The study and control group did not differ in terms of gender ratio (P = 0.705). However, the age distribution differed between the groups (P
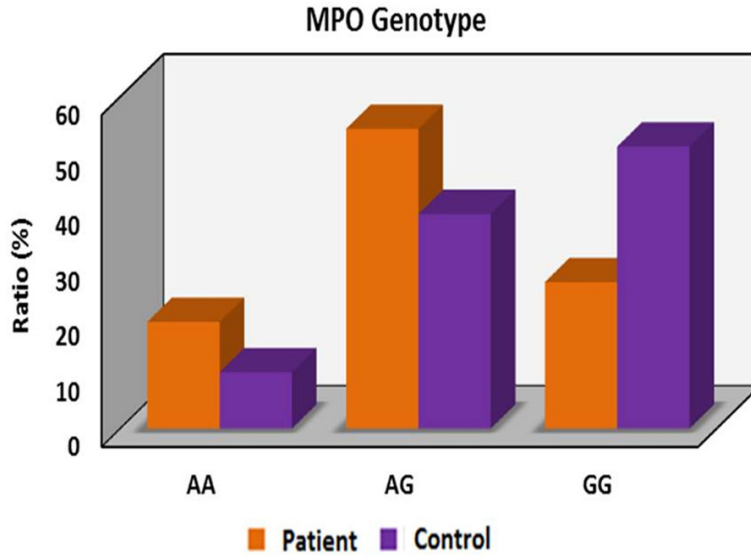
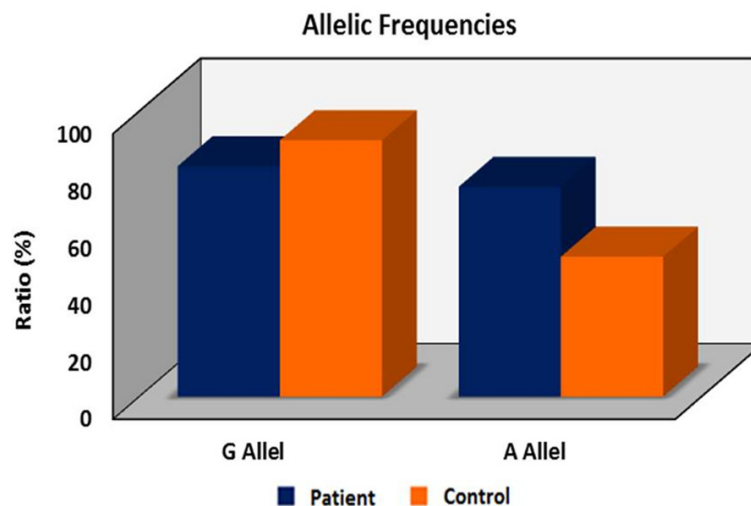Figure 1. MPO genotype distributions in the study and control groups.

Figure 2. Comparison of allelic frequencies between the study and control groups.

= 0.008). As we were concerned with genetic polymorphisms, we suggest that age distribution is not important when comparing groups, as polymorphisms are age independent.

The distribution of the MPO G-463/A genotype differed significantly between the study and control groups (**Table 1**, P = 0.003).

In the study group, the AA and AG genotypes were significantly more frequent than they were in the control group (**Figure 1**).

The frequency of the G allele did not differ significantly between the study and control groups (P > 0.05), but the A allele was significantly more frequent in the study group (P = 0.001) (**Figure 2**).

Children with the AA+AG genotype were at a 2.89-fold greater risk of disease than were those with the GG genotype (odds ratio 2.888 [95% confidence interval [CI] 1.541-5.412]). Also, A-allele-positive children were at a 1.85-fold greater risk of disease than were those with the G allele (odds ratio: 1.851 [95% CI: 1.207-2.838]) (**Table 2**).

When we grouped FMF patients according to MPO genotype (AA, AG, and GG), we found that the drug response (P = 0.988) and disease severity scores (P = 0.313) did not differ significantly among the groups. Also, the clinical findings were similar in terms of recurrent fever, abdominal pain, chest pain, and arthralgia (all *p*-values > 0.05) (**Table 3**).

When we compared FMF subgroups in terms of G allele presence/absence, neither the drug response (P = 1.000) nor the disease severity score (0.453) differed significantly between the groups. Also, the clinical findings were similar in terms of recurrent fever, abdominal pain, chest pain, and arthralgia (all *p*-values >0.05) (**Table 4**).

When we compared FMF subgroups in terms of A allele presence/absence, neither the drug response (P = 1.000) nor the disease severity score (0.294) differed significantly between the groups. Also, the clinical findings were similar in terms of recurrent fever, abdominal pain, chest pain, and arthralgia (all *p*-values > 0.05) (**Table 5**).

Table 2. Distribution of *MPO G-463/A* gene polymorphisms and allele frequencies in the FMF and control groups

	MPO G-463/A genotype; n (%)			Allele n (%)	
	AA	AG	GG	A	G
Patients (n = 83)	16 (19.3)	45 (54.2)	22 (26.5)	77 (43.7)	99 (56.3)
Homozygotes (n = 27)	8 (29.6)	14 (51.9)	5 (18.5)	30 (55.6)	24 (44.4)
Heterozygotes (n = 29)	5 (17.2)	15 (51.7)	9 (31)	25 (43.1)	33 (56.9)
Compound heterozygotes (n = 20)	3 (15)	11 (55)	6 (30)	17 (42.5)	23 (57.5)
No mutation (n = 7)	0 (0)	5 (71.4)	2 (28.6)	5 (35.7)	9 (64.3)
Controls (n = 98)	10 (10.2)	38 (38.8)	50 (51)	58 (29.6)	138 (70.4)
	GG	AA+AG		A	G
Odds ratio	0.346	2.888		1.851	0.540
95% CI	0.185-0.649	1.541-5.412		1.207-2.838	0.352-0.829
P	c0.001**			b0.005**	

cYates' continuity correction test; bPearson's chi-squared test; **$P < 0.01$.

Table 3. The clinical features of FMF patients by *MPO G-463/A* genotype

		MPO G-463 A genotype			
		AA (n = 16)	AG (n = 45)	GG (n = 22)	p
		n (%)	n (%)	n (%)	
Drug response	No response	0 (0)	2 (4.4)	1 (4.5)	e0.988
	Partial response	4 (25.0)	13 (28.9)	6 (27.3)	
	Complete response	12 (75.0)	30 (66.7)	15 (68.2)	
Severity Score	Min-max (median)	4-11 (9)	4-12 (8)	6-12 (8)	d0.313
	Mean ± SD	8.69±1.74	8.22±1.92	8.91±1.60	
Recurrent fever	No	3 (18.8)	12 (26.7)	5 (22.7)	b0.804
	Yes	13 (81.3)	33 (73.3)	17 (77.3)	
Abdominal pain	No	2 (12.5)	8 (17.8)	5 (22.7)	e0.801
	Yes	14 (87.5)	37 (82.2)	17 (77.3)	
Chest pain	No	7 (43.8)	22 (48.9)	11 (50.0)	b0.921
	Yes	9 (56.3)	23 (51.1)	11 (50.0)	
Arthralgia	No	2 (12.5)	7 (15.6)	1 (4.5)	e0.436
	Yes	14 (87.5)	38 (84.4)	21 (95.5)	

bPearson's chi-squared test; eFisher-Freeman-Halton test; dKruskal Wallis Test.

We also compared patients by MEFV gene status. Disease severity scores were significantly higher in the MEFV mutation-positive than in the MEFV mutation-negative group (P = 0.07). The severity scores are compared in **Figure 3**.

However, the drug response scores and the incidences of recurrent fever, abdominal pain, chest pain, and arthralgia were similar between the groups (all *p*-values > 0.05). Children who were M694V homozygotes had higher disease severity scores than did others. Also, the severity scores of M694V heterozygotes were higher than those of M694V-negative children (P = 0.012). The other clinical characteristics were similar between the groups (all *p*-values > 0.05).

Discussion

FMF is an inherited autoinflammatory disease characterized by recurrent attacks of synovial and serosal membranes inflammation accompanied by elevated levels of acute-phase reactants. Although the gene responsible (MEFV) has been identified, genotype-phenotype correlations remain unclear. Many environmental factors and other modifying genes may play roles in disease pathogenesis [2, 6]. Previous studies have revealed roles for oxidative stress

Table 4. Comparison of the clinical features of FMF patients by G allele status

		G allele		
		Negative (n = 16)	Positive (n = 67)	P
		n (%)	n (%)	
Drug response	No response	0 (0)	3 (4.5)	e1.000
	Partial Response	4 (25.0)	19 (28.4)	
	Complete Response	12 (75.0)	45 (67.2)	
Severity Score	Min-max (median)	4-11 (9)	4-12 (8)	a0.453
	Mean ± SD	8.69±1.74	8.45±1.84	
Recurrent fever	No	3 (18.8)	17 (25.4)	f0.750
	Yes	13 (81.3)	50 (74.6)	
Abdominal pain	No	2 (12.5)	13 (19.4)	f0.724
	Yes	14 (87.5)	54 (80.6)	
Chest pain	No	7 (43.8)	33 (49.3)	c0.907
	Yes	9 (56.3)	34 (50.7)	
Arthralgia	No	2 (12.5)	8 (11.9)	f1.000
	Yes	14 (87.5)	59 (88.1)	

aMann-Whitney U-Test; cYates' continuity correction test; eFisher-Freeman-Halton test; fFisher's exact test.

Table 5. Comparison of the clinical features of FMF patients by A allele status

		A Allele		
		Negative (n = 22)	Positive (n = 61)	P
		n (%)	n (%)	
Drug response	No response	1 (4.5)	2 (3.3)	e1.000
	Incomplete response	6 (27.3)	17 (27.9)	
	Complete Response	15 (68.2)	42 (68.9)	
Recurrent fever	No	5 (22.7)	15 (24.6)	c1.000
	Yes	17 (77.3)	46 (75.4)	
Abdominal pain	No	5 (22.7)	10 (16.4)	f0.528
	Yes	17 (77.3)	51 (83.6)	
Chest pain	No	11 (50)	29 (47.5)	c1.000
	Yes	11 (50)	32 (52.5)	
Arthralgia	No	1 (4.5)	9 (14.8)	f0.277
	Yes	21 (95.5)	52 (85.2)	

cYates' continuity correction test; eFisher-Freeman-Halton test; fFisher's exact test.

and oxidant/antioxidant imbalance in FMF pathogenesis. Also, inflammation remains elevated even during attack-free periods, suggesting that genes related to oxidative stress may play roles in disease pathogenesis [16-20].

MPO, an oxidative enzyme, is a component of the oxygen-dependent intracellular microbio-cidal system of phagocytes. MPO is secreted by reactive neutrophils during phagocytosis [21]. Recent reports have shown that neutrophil MPO activity is associated with individual genetic features. The -463G/A single-nucleotide polymorphism in the promoter region is the most extensively studied MPO polymorphism. Transition from the G to the A allele reduces mRNA expression and thus MPO transcriptional activity. The allele is associated with reduced production of MPO by both neutrophils and monocytes/macrophages. The GG (wild type) genotype is associated with higher levels of MPO and ROS [22, 23]. Several studies have suggested that MPO directly or indirectly causes neoplasia. MPO not only generates the potent oxidant hypochlorous acid during infection but also metabolically activates a number of pro-carcinogenesis genes. The MPO A allele has been shown to protect against breast cancer, lung cancer, hepatoblastoma, and acute promyelocytic leukemia [21, 24, 25]. Thus, we suggest that the G allele and the GG genotype, which are associated with elevated ROS production, predispose various diseases. The A allele and the AA and AG genotypes may protect against the development and reduce the severity of many diseases. However, some studies have suggested that the A allele exerts negative effects in certain diseases including cervical cancer [26], coronary artery disease [27], pelvic inflammatory disease [28], esophageal squamous cell dysplasia [29], and gastric cancer [30]. In the present study, we explored the relationships between FMF and MPO gene polymorphisms, given the important role of MPO in the maintenance of oxidative balance. To the best of our knowledge, this is the first study to explore the effects of MPO polymorphisms on FMF. We found that the A allele frequency was
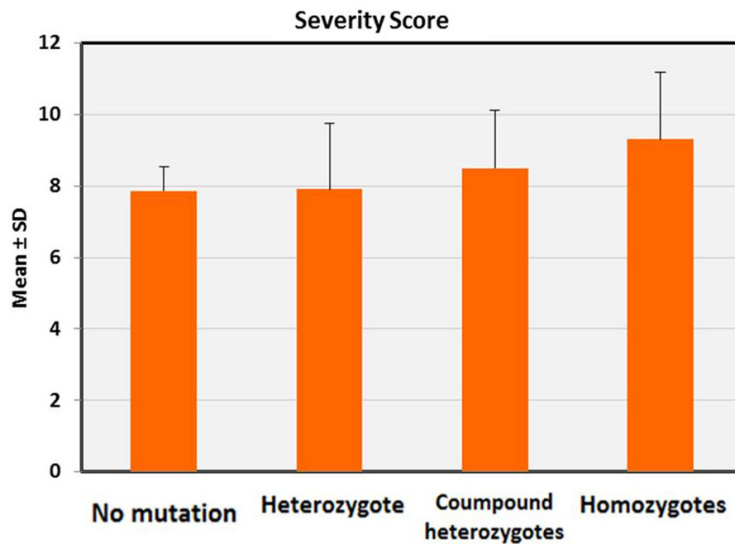
Figure 3. Disease severity scores of FMF patients by MEFV mutation status.

higher in children with FMF than in healthy controls (OR: 1.85). Also, the distributions of the AA, AG, and GG genotypes differed significantly between controls and FMF patients. The AA+AG genotypic combination was 2.89-fold more frequent in FMF patients.

How might the A allele affect FMF pathogenesis? The literature offers useful clues. A recent study on FMF-associated arthritis involved sampling synovial tissue from inflamed hip joints. Infiltrating cells were examined immunohistologically, and the in situ expression levels of plausible contributors (MPO and lysozyme) to FMF-associated synovitis were measured. MPO and lysozyme, both of which occur at high levels in myeloid granules, were deficient in neutrophils of patient with FMF-associated arthritis compared to that of patient with septic gonarthritis [31]. These results are similar to ours.

Endo et al., in an animal study, showed that MPO negatively regulated the expression of proinflammatory cytokines and chemokines (including IL-1α, IL-1β, and TNF-α) by neutrophils [32]. Accumulating evidence indicates that cytokine activation is continuous in patients with FMF, occurring during both attacks and attack-free periods. The levels of sIL-2r, IL-6, and TNF-alpha were all elevated in FMF patients [33-35]. Thus, we suggest that the A allele and the AA and AG genotypes may decrease MPO levels, thereby reducing the cytokine and che-

mokine responses of FMF patients. Further studies exploring the relationships among MPO polymorphisms, MPO concentrations, and chemokine/cytokine levels are required to explore this possibility further.

MPO-deficient neutrophils kill many species of microorganisms more slowly than do normal neutrophils, thus dysregulating inflammation [8, 9, 36].

FMF patients have increased levels of small molecules originating from microbes (SMOMs) [37]. Yalcinkaya et al. found that the antistreptolysin O (ASO) and anti-DNase-B responses of FMF patients were significantly higher than those of healthy controls after infection with group A beta-hemolytic streptococci, which causes pharyngitis. It was concluded that FMF patients might be more prone to late complications of streptococcal infections. The higher frequency of acute rheumatic fever in FMF patients supports this suggestion [38]. We suggest that the AA and AG *MPO* genotypes may trigger MPO deficiency and impaired defenses against infectious agents in FMF patients.

The most common *MEFV* mutation we found was *M694V*, and the disease severity score was higher in *M694V* carriers compared to that in subjects with other mutations or no mutation. This is in agreement with previous findings [39-42]. However, we found no significant differences between patients differing in *MEFV* mutation or *MPO* genotype.

Our study has certain limitations. First, we did not measure MPO activities or levels. Also, we did not explore the effects of *MPO* polymorphisms on chemokine or cytokine levels. Additionally, our sample size was small, limiting our ability to determine the effects of single-gene polymorphisms on disease pathogenesis.

In conclusion, MPO plays important roles in the host defenses against infection and inflammation. We found an association between the *MPO*-463G > A gene polymorphism and FMF. The A allele was more common in FMF patients

than in healthy controls. Also, the AA+AG genotype was 2.89-fold more common in FMF patients. However, we found no relationship between this polymorphism and the disease severity or *MEFV* mutational status. Further studies with larger numbers of subjects should be performed to explore the relationships among genotypes, MPO enzyme levels, oxidative status, and inflammatory cytokine levels. Such work would determine the clinical significance of the *MPO*-463/A genetic polymorphism in terms of FMF pathogenesis.

Acknowledgements

Disclosure of conflict of interest

None.

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