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Analysis of common *MDR1* (*ABCB1*) gene C1236T and C3435T polymorphisms in Turkish patients with familial Mediterranean fever

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ABSTRACT. The multidrug resistance (MDR1) gene encodes a P-glycoprotein that plays a key role in drug bioavailability and response to drugs in different human populations. More than 50 SNPs have been described for the MDR1 gene. Familial Mediterranean fever (FMF) is considered an autosomal recessive hereditary disease, associated with a single gene named the Mediterranean fever gene (MEFV). However, about one-third of FMF patients have only one mutated allele, suggesting that this disease is expressed as an autosomal dominant trait with partial penetration or an additional gene might be responsible for the disease. We made genotype and haplotype analyses of the MDR1 gene in 142 FMF patients and 130 unrelated Turkish subjects; two MDR-1 genetic markers (C1236T and C3435T) were analyzed by PCR-RFLP analysis. FMF patients had a significantly higher frequency of the 3435 CT genotype compared with the control group (59.9% in FMF patients versus 44.6% in controls; odds ratio [OR] = 1.85; 95% confidence interval [CI] = 1.14-3.00). Based on

haplotype analysis, the T-C shift was significantly more frequent in controls (14.4% versus 7.1% in FMF patients). This haplotype could be protective for FMF disease (OR = 0.45; 95%CI = 0.25-0.84). The frequency of CC-CT (1236-3435) binary genotype was significantly higher in FMF patients (14.79% versus 4.61% in controls; OR = 3.59; 95%CI = 1.40-9.20).

Key words: FMF; MDR1; Polymorphism; Haplotype; Turkey

INTRODUCTION

Familial Mediterranean fever (FMF) is an autosomal recessive disorder characterized by recurrent inflammatory attacks affected by typical symptoms of different autoinflammatory disorders; such as peritonitis, pleuritis, arthritis with fever and erythemas (Pras, 1998). The first case for FMF was described in 1908 by Janeway and Mosenthal, but the first series of affected patients was reported in 1945 (Siegal, 1945). FMF is associated with a single gene named MEditerranean FeVer (*MEFV*), which was defined in 1997. The *MEFV* gene is located on chromosome 16 (16p13) (Pras, 1998) and consists of 10 exons encoding a 781 amino acid protein called Pyrin. The *MEFV* gene is expressed in polymorphonuclear cells, cytokine activated monocytes, dendritic cells, and synovial fibroblasts (Centola et al., 2000). Mutations in the *MEFV* gene have been shown to be correlated with FMF (Akarsu et al., 1997; Ben-Chetrit and Levy, 1998; Bakkaloglu, 2003). To date, more than 199 mutations have been identified in the *MEFV* gene and 84 of these mutations are known to be associated with the FMF phenotype (http://fmf.igh.cnrs.fr/infevers; INFEVERS, 2011). However, about 75% of FMF patients show a single or no mutation (Fonnesu et al., 2009). Since about one-third of FMF patients bear a single mutation on one allele, it is thought that the disease might be transferred as an autosomal dominant trait with partial penetration. Alternatively, an additional gene might be responsible for the disease in cases with a single allele mutation (Akarsu, 1997; Ben-Chetrit and Levy, 1998; Ozdemir et al., 2011).

P-glycoprotein (P-gp) is a member of the adenosine triphosphate (ATP)-binding cassette (ABC) transporter of the MDR/TAP subfamily and is also called ATP-binding cassette sub-family B member 1 (*ABCB1*), *MDR1*, and *PGY1*. P-gp, which was first isolated from colchicine-resistant Chinese hamster ovary cells (Juliano and Ling, 1976), and functions as a transmembrane efflux pump moving drugs from the intracellular to the extracellular compartment (Higgins and Gottesman, 1992). P-gp is encoded by the human *ABCB1* gene, also called *MDR1* (multidrug resistance). The gene extends over more than 100 kb containing 28 introns, 26 of which interrupt the protein-coding sequence (Annese et al., 2006).

In addition to being expressed in drug resistance cancer cells, P-gp is also expressed in various normal tissues such as liver, kidney, brain, ovaries, testes, gastrointestinal tract, hematopoietic stem cells, peripheral blood mononuclear cells, mature macrophage, natural killer cells, antigen presenting dendritic cells, and T and B-lymphocytes (Annese et al., 2006). Alterations in P-gp expression and function potentially depend on variations of the *MDR1* nucleotide sequence. MDR1 expression is highly variable between subjects. This variability shows that the interethnic diversity and genetic polymorphism of the MDR1 gene is associated with a variation in expression level (Hoffmeyer et al., 2000; Taniguchi et al., 2003; Meissner

et al., 2004). To date, genetic variations of the human *MDR1* gene have been extensively studied and 76 SNPs (single nucleotide polymorphisms) in the coding region have been reported, of which 47 SNPs changed the amino acid composition of P-gp and 29 are known to be silent (www.ncbi.nlm.nih.gov=SNP=snp_ref.cgi?chooseRs¼coding&locusId¼5243&mrna; Anonymous, 2011). The effect of most of these polymorphisms on P-gp function or their clinical impact is in most cases unknown, but some of the SNPs are known to be of functional relevance and have been shown to alter the pharmacokinetics of substrate drugs. The most commonly reported *MDR1* SNPs in P-gp are the synonymous 1236 (exon 12, C>T, Gly 412 Gly) and 3435 (exon 26, C>T, Ile 1145 Ile), and the nonsynonymous 2677 (exon 21, G>T/A, Ala 893 Ser/Thr) polymorphisms. It is known that the studying the genetic variations of the *MDR1* gene in different diseases is import for a better understanding of the functional consequences of these polymorphisms in P-gp function and etiology of diseases.

The aim of the present study was to perform, for the first time, the frequency distribution of functional *MDR1* gene C1236T (rs 1128503) and C3435T (rs 1045642) polymorphisms and their haplotypes in FMF patients.

MATERIAL AND METHODS

This study was carried out in the Molecular Biology Laboratory of the Medical Biology Department at Gaziosmanpaşa University, Tokat, Turkey. The study was performed in accordance with the Declaration of Helsinki and was approved by the ethics committee of Gaziomanpasa University (approval # 09-GEKTIP-031). In the current survey, 142 FMF patients (68M/74F) and 130 (73M/57F) healthy control subjects were enrolled after receiving their informed consent (Table 1). Patients and controls were recruited from the Department of Internal Medicine, School of Medicine, Gaziomanpasa University. Patients who fulfilled the international criteria for FMF diagnosis (Pras, 1998; Fonnesu et al., 2009) were included in this study and their mutation analysis supported the diagnosis of FMF. Clinical evaluation was conducted by the Internal Medicine Department. The control group was selected at random from healthy subjects.

Table 1. Demographic features of study groups.

	FMF	Control
No of individuals	142	130
Mean age of first symptoms (SD)	10.6 (7.1)	-
Gender		
Male	68 (47.89%)	73 (56.15%)
Female	74 (52.11%)	57 (43.85%)

Blood samples were collected from each subject and DNA was extracted from blood samples using a PureLink™ Genomic DNA Kit (Invitrogene) according to the manufacturer's instructions. Detection of C1236T and C3435T SNPs was carried out by the PCR-RFLP method. Details with regard to primers, PCR conditions, restriction enzymes and digestion conditions are given in Table 2. Genotypes were determined by agarose gel electrophoresis of restriction digests on 3% Nusieve GTG agarose (Invitrogene) gel containing5 µg/mL ethidium bromide.

Table 2. Primers, PCR conditions, restriction enzymes and digestion conditions used for genotyping analysis of two MDR1 SNPs.

SNPs	Primers	PCR conditions	Restriction enzymes	Digestion conditions
C1236T	F: 5'-TAT CCT GTG TCT GTG AAT TGC C-3' R: 5'-CCT GAC TCA CCA CAC CAA TG-3'	94°C 2 min (initial denaturation) 94°C 30 s; 60°C 30 s; 72°C 30 s	*Hae*III	37°C 16 h
C3435T	F: 5'-TGT TTT CAG CTG CTT GAT GG-3' R: 5'-CCT GAC TCA CCA CAC CAA TG-3'	35 cycles 72°C 7 min (final extension)	*Sau*3AI	37°C 16 h

The Arlequin software, version 3.1.1, was used for all of the genetic data analyses. The genotype frequencies for each SNP in the study group for deviation from Hardy-Weinberg equilibrium (HWE) were tested by Fisher's exact test. The haplotype frequencies were estimated based on the expectation-maximization algorithm (Excoffier and Slatkin, 1995). Linkage disequilibrium (LD) between SNP pairs was estimated by Lewontin's coefficient (D') and Pearson's correlation (r^2) (Lewontin and Kojima, 1960; Lewontin, 1964). Gene diversity ($\hat{H}$) was calculated using the following formula: $\hat{H} = \frac{n}{n-1}(1- \sum_{i=1}^{k} p_i^2)$, where n is the number of gene copies in the sample, k is the number of haplotypes, and pi is the sample frequency of the i^{th} haplotype. Standard deviation of $\hat{H}$ was computed as follows: SD ($\hat{H}$) = $\sqrt{V(\hat{H})}$, where V is the sampling variance of $\hat{H}$ (Nei, 1987). Fisher's exact test was used for pairwise comparisons of the allele, genotype and haplotype frequencies belonging to the patient and control groups. P values less than 0.05 were considered statistically significant.

RESULTS

We genotyped 142 patients with FMF and 130 healthy control subjects at nucleotide positions 1236 and 3435 of the *MDR1* gene to assess its potential association with FMF. Demographic features of both patient and control groups are summarized in Table 1. As can be seen from Table 3, frequencies of the C and T alleles of the 1236 locus were found to be 53.5 and 46.5% in the FMF patient group, similar to the 46.5 and 53.5% found in the healthy control group, respectively. The C allele was detected more frequently in the patient group, although the difference was not significant (P = 0.062). The frequencies of *MDR1* 1236 CC, CT and TT genotypes were 29.6% (N = 42), 47.9% (N = 68) and 22.5% (N = 32) in the patient group; those in the control group were 20.8% (N = 27), 51.5% (N = 67) and 27.7% (N = 36), consecutively. However, the observed genotype frequencies did not show significant difference in either group (P > 0.05). Genotype distribution of the 1236 locus in both patient and control groups were found to be in HWE (P > 0.05) (Table 3).

As with the locus 3435, allele frequencies were found as follows: C-46.8 and T-53.2% for the patient group, and C-47.7 and T-52.3% for the control group. The observed frequencies of the *MDR1* 3435 CC, CT and TT genotypes were 16.9, 59.9 and 23.2% for FMF patients; those in the healthy subjects were 26, 44 and 30%, respectively. The observed frequency of the 3435 CT genotype was found to be statistically significant and higher in the patient group (P = 0.008), and the OR was calculated to be 1.85 (95%CI = 1.14-300). Genotype frequency distribution of the 3435 locus was in HWE in the control group; however, it showed significant deviation from HWE in FMF patients (Table 3).

Table 3. Allele and genotype frequencies for MDR1 C1236T and C3435T loci in FMF patients and controls.

		FMF patients		Healthy Controls			FMF patients					Healthy controls					
SNP	Allele	n = 284 chromosomes	Freq.	n = 260 chromosomes	Freq.	Genotype	N = 142 individuals	Freq.		Heterozygosity		P for HWE	N = 130 individuals	Freq.		Heterozygosity		P for HWE
								Obs.	Exp.	Obs.	Exp.			Obs.	Exp.	Obs.	Exp.	
C1236T	**C**	152	0.535	121	0.465	**CC**	42	0.296	0.286				27	0.208	0.216			
	T	132	0.465	139	0.535	**CT**	68	0.479	0.498	0.479	0.499	0.735	67	0.515	0.498	0.515	0.500	0.730
						TT	32	0.225	0.216				36	0.277	0.286			
P value		0.062				**P value; OR (95%CI)**	**CC** - 0.063; 1.60 (0.92-2.80) **CT** - 0.316; 0.86 (0.54-1.39) **TT** - 0.200; 0.76 (0.44-1.32)											
C3435T	**C**	133	0.468	124	0.477	**CC**	24	0.169	0.219				33	0.254	0.228			
	T	151	0.532	136	0.523	**CT**	85	0.599	0.498	0.599	0.500	***0.019**	58	0.446	0.499	0.446	0.501	0.221
						TT	33	0.232	0.283				39	0.3	0.273			
P value		0.299				**P value; OR (95%CI)**	**CC** - 0.058; 0.60 (0.33-1.08) **CT - *0.008; 1.85 (1.14-3.00)** **TT** - 0.130; 0.71 (0.41-1.21)											

n = chromosome number; N = subject number; HWE = Hardy-Weinberg equilibrium. *P < 0.05.

The haplotype frequencies concerning the 1236 and 3435 loci are presented in Table 4. Each of the four possible haplotypes was noted in both patient and control groups. The C-C haplotype was the most frequent haplotype in FMF patients; however, in healthy control subjects, the T-T haplotype has been estimated to be the most common. When the frequency distributions of estimated haplotypes were compared between the patient and control group, the frequency of the T-C haplotype was found to be significantly higher in the control group than in FMF patients 14.4 *vs* 7.1%, OR = 0.45, P = 0.008).

Table 4. Comparison analysis of observed haplotypes MDR1 C1236T-C3435T loci between FMF patients and controls.

Haplotype	FMF (N = 284)		Control (N = 260)		Odds ratio (95%CI)	P value
	n	*F*	*n*	*F*		
C-C	116	0.3969	92	0.3326	1.26 (0.89-1.78)	0.111
C-T	36	0.1383	29	0.1328	1.16 (0.69-1.95)	0.340
T-C	17	0.0714	32	0.1444	0.45 (0.25-0.84)	*0.008
T-T	115	0.3934	107	0.3902	0.97 (0.69-1.37)	0.472
	D' = 0.677 r^2 = 0.350 χ^2 = 99.41 P = 0.0000		D' = 0.447 r^2 = 0.191 χ^2 = 49.60 P = 0.0000			

*P < 0.05.

In this current survey, binary genotypic analysis was carried out for the nine potential combinations from the two loci (Table 5). In both patient and control groups CT-CT and TT-TT were detected as the two most common genotype combinations with frequencies of 38.7 and 15.5% for the patient group, and 33.1 and 16.2% for the control group, correspondingly. On the other hand, the TT-CC genotype was overrepresented in controls, showing significant difference from FMF patiets 4.62% *vs* 0.70%; P = 0.047), but 95% confidence interval of odds ratio, was detected outside the statistically significance range (OR = 0.12; 95%CI = 0.02-1.23) (Table 5). However, the CC-CT binary genotype frequency in FMF patients was observed to be significantly higher than in controls(14.79 *vs* 4.61%; P = 0.004; OR = 3.59; 95%CI = 1.40-9.20) (Table 5).

Table 5. Comparison analysis of observed binary genotypes MDR1 C1236T-C3435T loci between FMF patients and controls.

Genotype	FMF (N = 142)		Control (N = 130)		Odds ratio (95%CI)	P value
	n	*F*	*n*	*F*		
CC-CC	17	0.1197	16	0.1231	0.97 (0.47-2.01)	0.539
CC-CT	21	0.1479	6	0.0461	3.59 (1.40-9.20)	0.004*
CC-TT	4	0.0282	5	0.0385	0.73 (0.19-2.76)	0.445
CT-CC	6	0.0423	11	0.0846	0.48 (0.17-1.33)	0.117
CT-CT	55	0.3873	43	0.3308	1.28 (0.78-2.10)	0.199
CT-TT	7	0.0493	13	0.1000	0.47 (0.18-1.21)	0.085
TT-CC	1	0.0070	6	0.0462	0.15 (0.02-1.23)	0.047*
TT-CT	9	0.0634	9	0.0692	0.91 (0.35-2.37)	0.519
TT-TT	22	0.1549	21	0.1615	0.95 (0.50-1.83)	0.506

*P < 0.05.

DISCUSSION

FMF has been proposed as a prototype of autoinflammatory syndromes and has a wide ranging clinical spectrum (Fonnesu et al., 2009). Attacks can be triggered by different types of factors such as stress, fat-rich meals, certain drugs, menstrual cycle, etc. (Fonnesu et al., 2009; Ben-Chetrit et al., 2009). In FMF patients, gastrointestinal amyloidosis, inflammatory bowel disease (IBD) and vasculitis can be the cause of abdominal pain (Fonnesu et al., 2009).

Many of the genetic and non-genetic risk factors can be related to FMF. The genotype to phenotype correlation is very complex for this disease and the ethnic and environmental factors also play a role in the clinical outcome. The disease mainly affects populations of Mediterranean ancestry including Sephardic Jews, Turks, Armenians, Arabs and Italians. The *MEFV* gene is a main genetic factor for FMF. However, the *MEFV* gene may not be the sole genetic factor, because approximately 75% of FMF patients have a single or no mutations and genetic testing for the *MEFV* gene has a 75% positive predictive value (Goulielmos et al., 2006; Fonnesu et al., 2009). In addition, FMF may present distinct genetic and clinical heterogeneity. Many of the genetic factors are linked to modifier genes (e.g., *SAA*- Seroamyloid A gene; *MICA*- MHC class I chain-related A gene) and can play a role in FMF phenotypes (Ben-Chetrit et al., 2009; Papadopoulos et al., 2010).

The P-gp, encoded by the *MDR1* gene, is an ATP-dependent efflux pump that transports inflammatory material and xenobiotic toxins from the intracellular to extracellular region. Polymorphisms of the *MDR1* gene have been shown to change both the expression level and function of P-gp. *MDR1* gene polymorphisms and their haplotypes are associated not only with multidrug resistance phenotype in cancer but also with many other disease conditions including lung cancer, AML, colorectal cancer, esophageal cancer, inflammatory bowel disease (IBW), Parkinson disease, and male infertility (Tan et al., 2005; Gervasini et al., 2006; Kim et al., 2006; Komoto et al., 2006; Annese et al., 2006; Tufan et al., 2007; Hueneber, 2009; Drozdzik et al., 2009; Balcerczak et al., 2010). However the results of these studies regarding the effects of MDR1 gene polymorphisms on different disease conditions are controversial. It has been suggested that studying each MDR1 SNP individually is the main cause of these discrepancies. Actually most of the SNPs in the MDR1 gene, especially C1236T, G2677T/A, and C3435T, are found in linkage disequilibrium and are part of a common haplotype. Recent studies suggest that grouping these SNPs into haplotypes may provide a better understanding of the observed inconsistencies and serve as a useful predictor of the functional consequences of MDR1 polymorphisms (Tang et al., 2002; Sai et al., 2003; Kimchi-Sarfaty et al., 2007; Gumus-Akay, 2010).

The effects of MDR1 SNPs have not been surveyed in the etiology of FMF yet. However, the role of the different MDR1 SNPs have been reported in inflammatory bowel disease (IBD) and Behcet's disease, which shows similarities with FMF in respect of its inflammatory nature. Sapmaz et al. (2008) investigated the association of MDR1 gene G2677T/A polymorphism and IBD and reported no statistically significant difference between IBD patients and the healthy control group. In the meta-analysis reported by Annese et al. (2006) MDR1 C3435T polymorphism was associated with ulcerative colitis. In the study by Huebner et al. (2009), MDR1 gene polymorphisms were studied in ulcerative colitis patients, and the frequency of the heterozygote individuals were found to be statistically different from control

subjects, which is in accordance with our results. In that study heterozygous CT and GT genotypes in loci C1236T and G2677T/A respectively were reported as a protective factor for UC (Huebner et al., 2009).

Tufan et al. investigated the clinical effects of MDR1 gene C3435T polymorphism on colchicine efficacy in FMF patients, which is the only published study on the clinical relevance between FMF and MDR1 gene polymorphism. In that study, association between C3435T polymorphism and colchicine drug response in FMF patients was observed (Tufan et al., 2007).

In a recent study by Saricaoglu et al. (2011), the effect of MDR1 C3435T polymorphism was analyzed in Behçet's disease. Results of this study show that C3435T polymorphism is not associated with the Behcet's disease and the drug response (Saricaoglu et al., 2011).

In our study we investigated an association between FMF and MDR1 gene C1236T and C3435T polymorphisms, their haplotypes and binary genotypes. For C1236T polymorphism, no association was detected in FMF patients. However, the CC genotype was detected with higher frequency in FMF patients than ontrols (29.6 *vs* 20.8%) although this did not reach the threshold of statistical significance(P = 0.063; OR = 1.6; 95%CI = 0.92-2.80) (Table 3). Similarly, the C allele was found to be higher in FMF patients than ontrols (53.5 *vs* 46.5%), although it is not statistically significant (P = 0.062) (Table 3). All of these results might be caused by the relatively small sample size of this study and large-scale studies are warranted to appropriately investigate this possibility.

Allelic frequencies of the C3435T polymorphism were found to be similar in patient and control groups. On the other hand, the heterozygous CT genotype has been more frequently observed in FMF patiets (P = 0.08; OR = 1.85; 95%CI = 1.14-3.00) (Table 4). Our results suggest that the CT genotype might be a susceptibility factor for the disease phenotype. In contrast to our results, the 3435 CT genotype has been shown to be a protective factor for male infertility (Drozdzik et al., 2009), and 1236 CT and 2677 GT genotypes for ulcerative colitis (Hueneber, 2009). As far as we know, the current survey is the first study showing the role of the heterozygote genotype in disease susceptibility. In addition, homozygous genotypes, especially the CC genotype, were detected less frequently in FMF patients than in the control group (16.9 *vs* 25.4%), but the difference did not reach the significance level (P = 0.058) (Table 4).

In various studies, the polymorphisms of the MDR1 gene and their haplotypes have been shown to affect the expression levels of the P-gp (Hoffmeyer et al., 2000; Kroetz et al., 2003; Meissner et al., 2004). Our result shows that the T-C haplotype may be a protective factor for the FMF phenotype, because this haplotype was found to be statistically lower in FMF patients than in cotrol groups (7.1 *vs* 144%; P = 0.08; OR = 0.45; 95%CI = 0.25-0.84) (Table 4). Other data supporting this hypothesis emerges when we look at binary genotypes. The TT-CC binary genotype in FMF patients was found to be lower than i the controls (0.7 *vs* 4.6%; P = 0.047). However, the risk confidence interval was not within ignificantlimits (OR = 0.15; 95%CI = 0.02-1.23) (Table 5).

Although FMF disease has been accepted as a single gene disorder, nowadays it is discussed whether it is a syndrome rather than a disease. At least, with the environmental factors many other genetic factors that influence the course and the emergence of the disease are considered. According to the results of this study, we believe that, the MDR1 gene may have certain effects on FMF disease etiology. This finding is more prominent when both SNPs are evaluated as haplotypes and binary genotypes. Because the present survey is the first study

reporting the association of MDR1 genotypes/haplotypes with FMF, our results needs to be verified with further studies with a larger sample size.

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