Chapter 7

The Prototype of Hereditary Periodic Fevers: Familial Mediterranean Fever

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Additional information is available at the end of the chapter

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1. Introduction

Autoinflammatory disorders are multisystem periodic fever syndromes, and characterized with recurrent unprovoked inflammation of the serosal membranes. Unlike autoimmune disorders, autoinflammatory disorders lack the production of high-titer autoantibodies or antigen-specific T cells. These diseases primarily include hereditary syndromes (**Table 1**); Familial Mediterrenean fever (FMF), TNF receptor-associated periodic fever syndrome (TRAPS), hyperimmunoglobulinaemia D and periodic fever syndrome (HIDS), and the cryopyrin-associated periodic syndrome (CAPS) which involves familial cold autoinflammatory syndrome (FCAS), Muckle–Wells syndrome (MWS) and neonatal onset multi-system inflammatory disease (NOMID)/chronic infantile neurological cutaneous and articular syndrome (CINCA). Familial mediterrenean fever has been considered as the most prevalent of innate immune system disorders involving systemic autoinflammatory reaction effecting joints, skin, bones and the kidney. Systemic amyloidosis is the most severe manifestation of the disease, commonly effecting the kidneys (11% of cases), and sometimes the adrenals, intestine, spleen, lung, and testis (1). As an innate immune system disorder, FMF is characterized by recurrent episodes of unseemingly unprovoked inflammation and fever with lasting 1- to 3-day attacks accompanied by sterile peritonitis, pleurisy, rash, arthritis, and in some cases amyloidosis leading to renal failure. this (Sohar et al., 1967). Apart from the typical implications of the disease, there is increasing evidence about the expanding clinical spectrum of FMF that embraces unusual clinical characters (2-4). These are the rare presentations of the disease and therefore undescores the role of molecular analysis in particular for the suspicious and probable cases.

FMF is classically transmitted with autosomal recessive inheritance, and has been common among Mediterranean populations; however, previous reports have confirmed its presence worldwide. It has been described in Mediterranean populations, including

Italian, Spanish (5), Portuguese, French, and Greek, as well as in patients from Northern Europe and Japan. Nevertheless, only rare occurrences have been reported throughout the general population because of the low frequency of the causative alleles (6). Among susceptible ethnic groups, FMF prevalence is between 1/500-1/2000, and the carrier rate is between 16-22%. Contrary to the traditionally known monogenic inheritance of the disease, it has been previously evidenced that there have been a number of patients who have the typical FMF phenotype or FMF related symptoms with only one MEFV heterozygous mutation and/or even without any MEFV mutations (5-7), indicating the presence of clinical phenotype not only in homozygous patients, but also similarly in the heterozygous patients with mild disease.

Syndrome (MIM)	**State of Inheritance**	**Gene (GenBank no)**	**Protein**	**Age at disease onset**
FMF (249100)	Autosomal Recessive (dominant forms are rarely presented)	MEFV (NM_000243) Ch-16	Pyrin (marenostrin)	Childhood
HIDS (260920; 251170)	Autosomal Recessive	MVK (M88468) Ch-12	Mevalonate kinase	Infancy
TRAPS (142680; 191190)	Autosomal Dominant	TNFRSF1A (NM_001065) Ch-12	TNF-receptor type I (p55)	Childhood
CAPS (606416) - MWS - FCAS - CINCA/ NOMID	Autosomal Dominant	NLRP3 Ch-1	Cryopyrin	-Childhood -Infancy -Neonatal
PAPA Syndrome	Autosomal Dominant	PSTPIP1 Ch-15	PSTPIP1	Childhood
Blau Syndrome	Autosomal Dominant	NOD2/CARD15 Ch-16	NOD2/CARD15	Childhood

*CINCA, chronic infantile neurological, cutaneous, and articular syndrome; FCAS, familial cold autoinflammatory syndrome; FMF, familial Mediterranean fever; MWS, Muckle-Wells syndrome; NOMID, neonatal onset multisystem inflammatory disease; PAPA, pyogenic sterile arthritis, pyoderma gangrenosum, and acne; CAPS, cryopyrin-associated periodic syndrome; TRAPS, tumour necrosis factor receptor-associated periodic syndrome.

Table 1. Hereditary autoinflammatory syndromes with identified gene loci (adapted from Lachmann and Hawkins, 2009: 36).

16p13.3 chromosomally located MEFV (Mediterranean Fever) gene has been found responsible for FMF disease, and the protein product, Pyrin, is a 781-amino-acid protein (8-11). Evolutionary conserved domains of pyrin protein involves N-terminal pyrin domain, a B-box zinc-finger, a coiled coil and a C-terminal B30.2 PrySpry domains. Pyrin protein has been reported as a component of the inflammasome complex with both pro-inflammatory and anti-inflammatory role in the cytokine regulation (10-13). Thus, a proapoptotic or antiapoptotic role have been still not precise for the pyrin protein in NF-kB activation and apoptosis (11-16). By means of its PYD and B30.2 interacting domains, pyrin has been shown to bind different proteins of autoinflammatory disease genes. Each interacting protein that binds through the pyrin domains (PYD) consists of PSTPIP1 (17), 14-3-3 (18), Caspase-1 (19), ASC (20), and Siva (21).

In 1997, The International FMF Consortium and The French FMF Consortium reported four missense disease associated mutations in the MEFV gene involving M694V, M680I, V726A, and M694I. Major and minor mutations of MEFV gene are well documented in INFEVERS, the database of hereditary autoinflammatory disorder mutations, and exons 2 and 10 comprises the hot-spots (22). To date, mutations have been mostly identified in exons 2, 3, 5, and 10 of the MEFV gene. According to previous reports by Touitou I. (2001), and by the Turkish FMF study group (2005), the most common MEFV mutation in Turkey is M694V (57.0 and 51.4%, respectively), followed by M680I (16.5 and 14.4%, respectively), and V726A (13.9 and 8.6%, respectively). Moreover, no correlation has been reported between various MEFV gene mutations and the severity of the phenotype in various populations supporting the genotypic and phenotypic heterogeneity present for FMF (5, 23-25).

According to INFEVERS (22), the database of hereditary autoinflammatory disorder mutations, To date, approximately 222 sequence variants including both missense mutations (only one nonsense mutation; Y688X) and polymorphisms have been defined in the FMF gene (MEFV), INFEVERS, 100 of them was clinically associated with the phenotype, 33 of them was not associated with the disease and the remaining was of uncertain pathogenicity. The remarkably wide clinical variability of the disease, as indicated by previous reports, has been linked to the MEFV allelic heterogeneity that underlies genotypic and phenotypic heterogeneity (23, 26, 27), and this has made detailed mutation screening critically important. In particular, Turkish FMF patients are characterized by an increased genetic heterogeneity due to various mutation frequencies from different regions, explained by the intrapopulation differentiation.

With respect to our mutation screenings, a previous comprehensive study was performed with 3430 Turkish individuals from all regions of Turkey (ages range from 2 months to 67 years; 2101 females and 1329 males) including first and second-degree relatives of individuals with FMF clinical diagnoses (including suspicious, possible, and definitive cases) who referred to the Molecular Medicine Laboratory for genetic diagnosis between years May, 2005 and December, 2010. The Tel-Hashomer and Livneh criteria were used for the clinical diagnosis of FMF based on the model of major, minor, and supportive criteria, which stipulates the presence of either 1 major or 2 minor criteria or 1 minor and 5 supportive criteria for a diagnosis. A simple set of criteria for the diagnosis of FMF required

1 or more major and/or 2 or more minor criteria (28). None of the patients with FMF had an immunological disorder or another rheumatic disease. Active clinical presentations (fever, abdominal pain, arthritis, and myalgia) and laboratory parameters (high levels of serum amyloid A [SAA], C-reactive protein [CRP], fibrinogen, white blood cell [WBC] counts and erythrocyte sedimentation rates [ESR]) were determined for each patient. For the detection of all coding and non-coding sequence variations along the MEFV gene, we performed bidirectional DNA sequencing analysis in all 10 coding exons and exon-intron boundaries of the respective gene, and reported frequencies of common and rare nucleotide substitutions and synonymous and non-synonimous single nucleotide polymorphisms obtained in the Turkish population (7).

2. Methods

2 ml peripheral blood was collected into ethylenediaminetetraacetic acid (EDTA)-anticoagulated tubes by the standard venipuncture method and DNA was extracted using the QIAamp DNA Blood Isolation kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. The extracted DNA concentration was determined using a Thermo Scientific NanoDrop spectrophotometer (Wilmington, USA). The quality assessment of the extracted DNA was determined by 2% agarose gel electrophoresis.

2.1. FMF strip asay - Reverse hybridization multiplex PCR

Reverse hybridization assay (FMF StripAssay, Viennalab Labordiagnostika GmbH) was used to investigate the mutations. According to the manufacturer's instructions, in a first step multiplex PCR was performed using biotinylated primers for exons 2, 3, 5, 10 amplification. PCR products were selectively hybridized to a test strip presenting a paralel array of allele-specific oligonucleotide probes which includes 12 MEFV mutations [E148Q, P369S, F479L, M680I (G/C), M680I (G/A), I692del, M694V, M694I, K695R, V726A, A744S, R761H]. Hybridizations were illuminated by the reaction of streptavidin-alkaline phosphatase and color substrate.

2.2. DNA sequencing strategy

Hot-spots, exons 10, and 2; with 3 and 5, and when necessary exons 1, 4, 6, 7, 8 and 9 of the MEFV gene were analyzed for MEFV mutations by PCR amplification followed by automated DNA sequence analysis. One microliter (100 ng) of genomic DNA was added to Polymerase Chain Reaction (PCR) amplification buffer containing 20 mM Tris (pH 8.3); 50 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM each of dATP, 2′-deoxycytidine 5′-triphosphate, dGTP, and 2′-deoxythymidine 5′-triphosphate; 10 pmol each of reverse and forward primers provided by Invitrogen; and 1.0 U of PlatiniumTaq DNA Polymerase (Invitrogen, Carlsbad, CA) in a total volume of 25 μl. The cycling conditions included a hot-start denaturation step at 95˚C for 10 min, followed by 35 amplification cycles of denaturation at 95˚C for 30 s, annealing at 61˚C for exon 10, 58˚C for exons 2 and 3, or 57˚C for exon 5 for 40 s, and elongation at 72˚C for 45 s; a final extension was performed at 72˚C for 7 min (the

oligonucleotide sequences are available upon request). Prior to sequencing, PCR products were purified using an ExoSAP-IT PCR Product Clean-Up kit. BigDye Terminatorv3.1 Cycle Sequencing Kit (Applied Biosystems, San Diego, CA, USA) was used in cycle sequencing reactions. Cycle sequencing PCR products followed purification with the BigDyeXT kit(Applied Biosystems,) and the data were analyzed using an ABI3130xl Genetic Analyzer (Applied Biosystems). DNA sequencing was performed in both directions, initiated from the forward and reverse primers that were used in the initial PCR reaction. SeqScape 2.0 sequence analysis software (Applied Biosystems, San Diego, CA, USA) was employed for sequence evaluation.

2.3. Restriction fragment length polymorphism analysis (RFLP)

A RFLP was identified in the mutation site and was utilized for mutation detection. Amplicons encompassing exon 5 were digested with the restriction enzyme Tsp509I, and electrophoresed on a 1% agarose gel.

3. Results

We found that M694V accounted for the majority of FMF chromosomes (44%), followed by E148Q (19%), V726A (10%), M680I (10%), P369S (4%), R408Q (3%), K695R (2%), M694I and R761H (1.6%), A744S (1.4%), and F479L (0.09%) (**Tables 2, 3**). Missense disease-causing mutations and synonymous polymorphisms accounted for 38% and 54% of MEFV chromosomes, respectively. Among the Turkish general population, the most frequent healthy heterozygous carrier mutation was found E148Q (6.9%), and the carrier rate was found 16%, with a mutation frequency of 8% (Berdeli et al., 2011). Except for the known major FMF mutations, by DNA sequencing, we frequently detect additional rare and novel mutations and critical SNPs about which we have only limited information in Turkish FMF patients. Remarkable consequences of sequencing analysis have been found relative to mutation-SNP combination underlying the combined existence of nucleotide variations in the same haplotype.

For patients whose MEFV gene does not contain mutations of exons 2, 3, 5, and 10, we performed bidirectional DNA sequencing also in exons 1, 4, 6, 7, 8, and 9. However, we could not find any disease related mutation except for an exon 9 homozygous SNP, P588P, which is thought to be symptomatic with disease relation. This SNP was always in homozygous state and was not seen in combination with any of the major and minor mutations or any of the SNPs in the entire coding and non-coding regions of the gene. Relative to our experiences, this SNP has a disease relation to a minor degree, however possible validation of other autoinflammatory disease gene mutations should need to be considered. Single P588P SNP was associated with continuously high SAA levels and musculoskeletal complications which has a good response to colchicine in a three-member family who did not have any sequence variations along other coding and non-coding regions of the MEFV gene.

Genotype					
MEFV Mutation				Number of Patients	Genotype Frequency
Exon 2	Exon 3	Exon 5	Exon 10	No	(%)
			M680IG-C/Wt	69	5.24
			M680IG-A/Wt	5	0.37
			M680IG-C/M680I	12	0.91
			M680IG-C/V726A	23	1.74
			M680IG-C/ M694V	42	3.19
E230K			M680IG-C/ M694V	2	0.15
			M680IG-C/A744S	1	0.12
			M680IG-C/ R761H	2	0.15
E148Q			M680IG-C	4	0.3
E167D		F479L	M680IG-C	1	0.07
E167D		F479L		2	0.15
		F479L/Wt		1	0.12
			M694V/Wt	322	24.4
			M694V/M694V	91	6.91
			M694V/V722M*	1	0.12
			M694V/V726A	42	3.19
			M694V/R761H	5	0.37
			M694V/K695R	2	0.15
			M694V/A744S	1	0.12
R241K			M694V/	1	0.12
E230K			M694V/	3	0.22
E148Q	P369S		M694V	1	0.12
E148Q/S179N*			M694V	1	0.12
E148Q/Wt				237	18
E148Q/E148Q				19	1.44
E148Q			M694V	42	3.19
E148Q			L709R	1	0.12
E148Q	P369S			4	0.3
E148Q			V726A	7	0.53
E148Q			A744S	1	0.12
E148Q			M694I	7	0.53
E148Q			K695N	1	0.12
E148Q			R761H	4	0.3
E148Q			I72OM	2	0.15
E148Q/L110P				6	0.45
E148Q/R151S				1	0.12

Genotype					
MEFV Mutation				**Number of Patients**	**Genotype Frequency**
Exon 2	**Exon 3**	**Exon 5**	**Exon 10**	**No**	**(%)**
E148Q			K695R	2	0.15
E148Q/T267M				1	0.12
E148Q/E230K				4	0.3
E148Q/T267I				1	0.12
E148Q/L110P			M694I	1	0.12
E148Q	P369S/R408Q			18	1.36
E148Q	P369S/R408Q		M680I	1	0.12
			M694I/A744S	1	0.12
			V726A/Wt	111	8.43
			V726A/V726A	4	0.3
E167D			V726A	3	0.22
			V726A/M694I	2	0.15
			V726A/R761H	3	0.22
			V726A/R761H/ M680IG-C	1	0.12
			V726A/K695R	1	0.12
		F479L	V726A	3	0.22
			K695R/Wt	38	2.88
			A744S/Wt	19	1.44
	P369S/Wt			7	0.53
	P369S/R408Q			40	3.03
	P369S/R408Q		M694V	4	0.3
			M694I/Wt	10	0.75
			R761H/Wt	31	2.35
			R761H/ A744S	1	0.12
			R653H/Wt	1	0.12
			E685K/E685K	1	0.12
L110P/L1010P				1	0.12
E230K/E230K				1	0.12
E230K/ Wt				1	0.12
T267M/Wt				3	0.22
R241K/R241K				1	0.12
E148V/Wt				5	0.37
E148L/Wt				2	0.15
E167D/Wt				2	0.15
	P350R/Wt			1	0.12
	P350R		A744S	2	0.15

Genotype					
MEFV Mutation				**Number of Patients**	**Genotype Frequency**
Exon 2	**Exon 3**	**Exon 5**	**Exon 10**	**No**	**(%)**
		G456A/Wt		1	0.12
		S503C/Wt		2	0.15
		I506V/Wt		1	0.12
		Y471X/Wt		1	0.12
	G340R/Wt			1	0.12
S141I/Wt				3	0.22
S166L/Wt				2	0.15
		A511V/Wt		1	0.12
	R354W/Wt			1	0.12
	S339F/Wt			4	0.3
	R329H/Wt			3	0.22
	R329H/		M694V	1	0.12
E148Q	R329H/			1	0.12
Heterozygotes				885	67.2
Compound heterozygotes				271	20.5
Homozygotes				130	9.87
Complex genotypes				30	2.27
Total number of patients with mutations				1316	38.36
No mutation or SNPs identified				231	6.7
Total number of patients with only SNPs (**+R202Q**)				1883	54.8
Total number of patients				3430	100

*, novel mutations

Table 2. DNA sequencing results of MEFV genotyping among 3430 Turkish patients.

Mutation	Number of Alleles (No)	Allelic Frequency (%)
M694V	908	44.7
E148Q	386	19
V726A	204	10
M680IG-C	170	8.3
P369S	75	3.69
R408Q	63	3.1
K695R	43	2.11
M694I	21	1
R761H	47	2.31
A744S	26	1.28
E148V	5	0.24
E167D	8	0,39
T267M	4	0.19
L110P	8	0.39
R241K	3	0.14
I720M	2	0.09
E230K	12	0.59
M680IG-A	5	0.24
E148L	2	0.09
F479L	7	0.34
E685K	2	0.09
R653H	1	0.04
T267I	1	0.04
V722M	1	0.04
S141I	3	0.14
S339F	4	0.19
R151S	1	0.04
I506V	1	0.04
S503C	2	0.09
L709R	1	0.04
K695N	1	0.04
P350R	3	0.14
G340R	1	0.04
G456A	1	0.04
Y471X	1	0.04
R329H	5	0.24
S166L	2	0.09
S179N	1	0.04
A511V	1	0.04
R354W	1	0.04
Total	**2033**	**100**

Table 3. Allelic frequencies of totally 40 MEFV mutations involving major, rare and, novel sequence changes among the detected mutations in 1316 mutation positive patients group (mutation frequency for the studied mutations; complex alleles excluded).

Additionally, sequence analysis revealed that there was a single FMF-associated mutation in the MEFV coding region of 76% of the Turkish individuals studied, and 80% of these individuals initiated colchicine treatment following molecular diagnosis. The prevalence of a single mutation in patients experiencing a pathogenic effect in Turkey (76%) is contrary to the expected pattern of autosomal recessive inheritance and does not support the ''heterozygous advantage'' selection theory. However, the expression of the FMF phenotype may be influenced by other candidate modifier gene loci, autoinflammatory pathway genes or FMF-like diseases (29-31). For this reason, genome-wide association studies involving more patients should be performed and the data included in future investigations covering critical coding and noncoding gene SNPs for Turkish FMF patients.

As an ancestral population of FMF, Turkey was one of the regions which involves most of the rare and novel mutations. As referenced in INVEFERS, most of the rare mutations in view of the ethnic origins were found to be symptomatic. Novel Y471X mutation found in the present study was the second nonsense mutation in FMF era. Among the newly identified mutations, involving R151S, S166N, S179N, and G340R; P350R, G456A, Y471X, S503C, I506V, L709R and K695N; Y471X, R151S, L709R, and K695N were observed as pathogenic reflecting the typical FMF character. The main clinical characteristics of the patients were as follows: abdominal pain (92.1%), fever (93.9%), thoracic pain (59%), myalgia (67.8%), arthritis (55.1%), erysipelas like erythema (ELE) (21.8%). None of the patients developed amyloidosis. This finding verifies the importance of molecular diagnosis and detailed sequencing which is recommended to perform in particular for the ancestral populations of FMF.

In this report, from a large scaled heterogeneous group of patients, we describe a 44-year-old Turkish patient from Western Turkey with clinical diagnosis of periodic fever. The case presented here is a 44-year-old Turkish woman, from western Turkey. The course of the patient includes short and rare episodes of fever, ongoing abdominal pain, temporary myalgia and arthralgia since her childhood. Physical examination revealed no pathology except for arthritis on the right knee. Her weight, height, and blood pressure were normal. Primarily, she had diagnosed as having conditions secondary to FMF. Although family and relatives screening are of great importance, her family (parents are dead in an accident) and past history were noncontributory and unhappy. She had undergone antibiotherapy, steroid treatment and appendectomy. Laboratory tests revealed the acute phase reactants as follows; ESR 81 mm/h, SAA 76 mg/dl, CRP 3.46 mg/dl, and fibrinogen 526 mg/dl. Renal function tests and other biochemical parameters were normal. No molecular genetic diagnosis was done except for Strip Assay in other centers. The clinical figure associated with her was not much contributed to the start of colchicine not fulfilling most of the clinical criteria, so in our laboratory, FMF strip assay was used as the first stage of mutation detection method involving 12 common mutations. However, no particular mutation was identified. Thereafter, DNA sequence analysis revealed the responsible nonsense mutation, p.Y471X, in MEFV gene (**Figure 1**). By means of the molecular diagnosis, colchicine therapy (1.5 mg/day) was started properly. She had no symptoms after the colchicine therapy and had a good response to 1,5 mg/d, and the acute phase reactants were completely normal in

the last 3 years. So, other autoinflammatory genes, MVK, TNFRSF1A, CIAS1, were not considered to evaluate as the suspicious genes in this case and were not evaluated as molecular diagnostics.

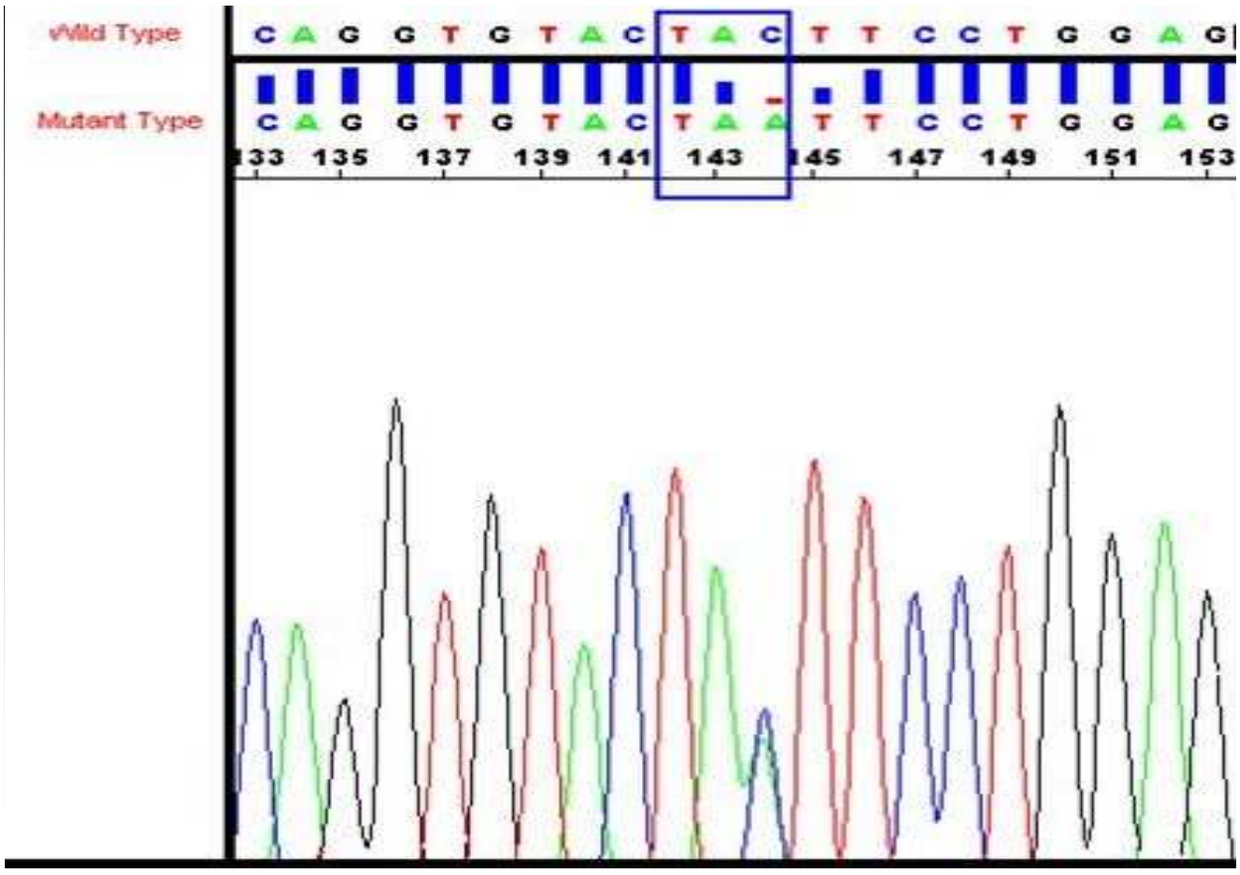

Figure 1. Electropherogram of the p.Y471X nonsense mutation in the MEFV gene revealed by DNA Sequencing analysis in the Turkish patient.

The case presented here was one of the patients who had misdiagnosis in particularly during the childhood losing time by unnecessary processes and treatments. Therefore, certain diagnosis determined by detailed DNA sequence analysis is essential for suspicious and undefined cases, and for cases disestablished by other limited screening methods. In the molecular analysis of Mediterranean fever gene, c.1413C>A nucleotide change in exon 5 resulting in p.Tyr471X nonsense mutation was determined (Figure I). We also exploited the fact that the p.Y471X creates a novel recognition site for the Tsp509I restriction enzyme to develop a PCR-RFLP assay in order to screen the affected families and healthy controls for the mutation.

Y471X nonsense mutation in MEFV gene is the first noted in Turkish FMF patients (7), and the second nonsense mutation of FMF mutation database worldwide. Inherited missens mutations reported in the 5th exon of MEFV gene in FMF patients are very rare. Though the fifth exon of the gene could not called as a critical region carrying the mutational hotspots, the result could demonstrate there is still way to walk on the road through the hidden side of FMF. Novel Y471X mutation in exon 5 of the MEFV gene located in the coiled coil domain

of pyrin protein is implicated in association with actin binding interacting selectively with monomeric or multimeric forms of actin. Since effects of nonsense mutations in the amino acids are known damaging and pathogenic, we did not use the PolyPhen software (32) in order to evaluate the potential pathogenicity of this newly found amino acid substitution which we carry out regularly in our laboratory. Nevertheless, expression studies will be required.

Due to the abundance of mutations in exon 10 and clinical heterogeneity of the disease, different screening methods have been developed. As long been known the majority of FMF patients in classically affected populations were screened by routine methods for only common mutations, which primarily targets only the most prevalent MEFV mutations in a specific population; thus, rare or novel mutations can be overlooked. The first nonsense mutation in FMF era, Y688X, was evaluated by Touitou I. (5), and was suggested to have a location between two well-known hotspots for FMF mutations (codons 680 and 694) in exon 10. This finding contributed to the critical role of exon 10 for the MEFV function as an hotspot. Here, it is discussed that, the newly found Y471X nonsense mutation has a great significance in screening asymptomatic individuals since it was not found in one of the hot-spots of MEFV gene.

Autoinflammatory diseases are heterogeneous group of disorders, thus FMF like phenotypes and related genes most likely exists (33-36). In some cases, the causal genes may not only be the unique causes of the diseases. It is well known that Mendelian disorders caused by the dysfunction of a single gene have a wide heterogeneity of disease phenotypes (37). FMF has both genetic and phenotypic heterogeneity and mutations within a single gene are known to cause different clinical phenotypes in Turkey. Thus, all MEFV gene sequence variations found in symptomatic cases should not be considered as causative pathogenic disease mutations. In particular, FMF related Turkish patients with no MEFV mutation or with only single MEFV mutations may not actually reflect the phenotype seen in FMF.

Another point is subclinical inflammation concerning asymptomatic heterozygous patients without a second mutation mostly continues with the typical disease characteristics possibly due to the presence of other modifier genes and/or environmental factors. Therefore, factors other than casual MEFV gene and other pyrin-dependent effects should be contributing to the sustainable systemic inflammation that is sufficient for the occurrence of the symptomatic FMF related phenotype. Previously, MICA, TLR2 and SAA loci were shown as modifying alleles in FMF (5, 38). Synonimous or non-synonimous sequence variations of MEFV relevant genes involving SAA and TLR2 were previously considered as critical factors for the course of the disease. Both SAA1 locus and Arg753Gln TLR2 polymorphism were implied as genetic susceptible loci for a risk factor of developing secondary amyloidosis in different ethnic populations of FMF patients (26, 27, 30). Against the traditionally considered monogenic inheritance pattern, compound heterozygotes of 2 autoinflammatory disease genes were also reported describing patients who were found to have 2 or more reduced penetrance mutations, involving E148Q in MEFV, R92Q or P46L in

TNFRSF1A, V377I in MVK, and V198M in CIAS1 (29, 34, 35). For the purpose of screening mutations in other known autoinflammatory genes for typical FMF patients carrying 1 single heterozygous MEFV mutation, Booty et al screened 6 candidate genes that encode proteins known to interact with pyrin or genes functioning in IL-1B pathway involving ASC/PYCARD, SIVA, CASP1, PSTPIP1, POP1, and POP2 (6). A novel PSTPIP1 nucleotide mutation, two novel substitutions in ASC/PYCARD and SIVA genes were identified while Casp1, POP1, and POP2 were mutation negative. In a Jewish patient with FMF, novel W171X (513G>A) mutation was identified which is presumed as a stop codon, to remove the last 2 of the 6 helices in the CARD domain of ASC/PYCARD. In FMF patients with only 1 MEFV mutation, including milder FMF-associated mutations, 1 Turkish patient was identified as a carrier of W171X (6). To date, SNPs in ASC/PYCARD gene were identified in 5′/3′ region, exon 1, intron 1, exon 3 coding region involving rs79351176, rs8056505, rs11648861, rs79464842, rs73532217, rs75471387, rs11867108, rs61086377, rs76878620, and rs75216100. In the ASC/PYCARD protein, the conserved PyD domain is 91 aa in lenght (1-91) and CARD domain is 89 aa in lenght (107-195). The previously reported W171X (513G>A) mutation (31) corresponds to the exon 3 coding region of the ASC/PYCARD gene and results with a stop codon. Thus, in our sequencing analysis, we also searched the presence of mutations in the ASC/PYCARD gene in our entire patients group. However, this sequence was not mutated, and we have neither identified the above substitutions along the entire coding regions and flanking segments of ASC/PYCARD gene (unpublished data).

For investigating of mutations in other periodic fever disease genes, in a study of our group, a total of 75 Turkish patients and 25 ethnically matched healthy control individuals diagnosed with periodic fever was molecularly diagnosed for having mutations in causative disease genes (apart from the present patients group; unpublished data). Mutation screening of coding and noncoding regions of MVK, TNFRSF1A, and NLRP3/CIAS1 genes were carried out for different group of patients according to their clinical implications.

MVK gene transcript variant 1 (12q24; NM_000431.2→NP_000422.1) was fully sequenced in 25 periodic fever patients. Molecular diagnosis revealed the following results: p.Ser52Asn missense mutation was identified in 6 patients. In addition, p.Asp170Asp and p.Ser135Ser synonimous aminoacid mutations and IVS6-18 A>G, homozygous IVS9+24 G>A, and IVS 4+8 C/T intronic nucleotide substitutions were observed in the remaining patients group.

NLRP3 gene (CIAS1; 1q44; NM_004895.4→NP_004886.3) NACHT, LRR and PYD domains-containing protein 3 isoform a was fully sequenced in 25 periodic fever patients. Molecular diagnosis revealed the following nucleotide substitutions in the screened gene region: K608fsX611 frameshift mutation, p.Ser726Gly and p.Gln703Lys missense mutations, together with Ser34Ser, Ala242Ala, Arg260Arg, Thr219Thr ve Leu411Leu synonimous aminoacid mutations.

TNFRSF1A gene (12p13.2; NM_001065.3→NP_001056.1) tumor necrosis factor receptor superfamily member 1A precursor form was fully sequenced in 25 periodic fever patients. Molecular diagnosis revealed the following nucleotide substitutions in the screened gene

region: p. Arg92Gln and p. Ala301Thr missense mutations with IVS6+10 A>G and IVS8-23 T>C intronic nucleotide substitutions.

Intronic nucleotide substitutions and synonimous aminoacid mutations of all the screened gene regions were also observed in the 25 ethnically matched healthy control individuals. Mutation frequency was 4% (1/25), 32% (8/25), and 40% (n:10/25) in TRAPS, HIDS, and CAPS patients.

Nonetheless, finding of symptomatic rare MEFV mutations in particular for at-risk populations and the individuals who have been asymptomatic and negative for common mutations makes detailed mutation screening critically important in FMF. It has been previously evidenced that there have been a number of patients who have typical FMF phenotype or FMF related symptoms with only one MEFV heterozygous mutation and/or even without any MEFV mutations (6, 7).

The majority of FMF patients in classically affected populations are screened by routine methods that are limited to the detection of common mutations. These tests primarily target the most prevalent MEFV mutations to rule out asymptomatic cases in at-risk populations. Therefore, while searching for the common mutations that underlie typical FMF symptoms, we should primarily consider the entire coding sequence of the MEFV gene before analyzing other recurrent fever genes. Patients with no mutation or with only single pyrin mutations may not actually reflect the phenotype seen in FMF. Compound heterozygotes of 2 autoinflammatory disease genes involving MEFV, TNFRSF1A, CIAS1, and MVK were reported (29, 34, 35). Thus, screening of other autoinflammatory disease genes, e.g. CIAS, were considered for the MEFV gene mutation/SNP negative FMF patients. In conclusion, by using sequencing analysis, we can prevent less common, population-restricted, novel sequence variants from being overlooked. This has implications for the characterization of typical and atypical FMF; screening for the most common mutations by routine methods is sufficient for the initial laboratory diagnosis of FMF in Turkish patients; however, the results should be confirmed by specific DNA sequencing of all coding exons and exon-intron flanking regions.

4. Conclusions

Among the newly identified mutations in this comprehensive study, Y471X, R151S, L709R, and K695N were observed as pathogenic reflecting the typical FMF character involving abdominal pain, fever, thoracic pain, myalgia, arthritis, and erysipelas like erythema. Rare mutations and SNPs have great importance for FMF pathogenesis. For this periodic fever disorder, heterogeneity is present in phases of allelic, frequency and critical locations of mutant alleles, and clinical appearance. Therefore, in particular for the suspicious cases; possible presence of other autoinflammatory disease gene mutations as we outlined above and rare mutations and SNP variations in the MEFV gene, molecular techniques, sample sizes, ethnic origins, and regions in the ancestral countries should be regarded as critical and determinative keys in FMF clinical and molecular diagnosis.

Sequencing analysis not only the common major mutations but also the detection of rare mutations can be carried out which have great importance in particular for at-risk populations. By means of sequencing analysis, we could prevent the missing of less common rare variants that might be restricted to the populations by routine techniques. The majority of FMF patients in classically affected populations are screened by routine methods that are limited to the detection of common mutations. These tests primarily target the most prevalent MEFV mutations to rule out asymptomatic cases in at-risk populations. Therefore, while searching for the common mutations that underlie typical FMF symptoms, we should primarily consider the entire coding sequence of the MEFV gene before analyzing other recurrent fever genes. In conclusion, by using sequencing analysis, we can prevent less common, population-restricted, novel sequence variants from being overlooked. This has implications for the characterization of typical and atypical FMF; screening for the most common mutations by routine methods is sufficient for the initial laboratory diagnosis of FMF in Turkish patients; however, the results should be confirmed by specific DNA sequencing of all coding exons and exon-intron flanking regions. We should consider gene mutation screening in early diagnosis and the follow-up of the clinical course in particular for the asymptomatic cases. Early determination of the disease causing mutation will be favorable in order to prevent abundant treatments in newly diagnosed patients.

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