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ORIGINAL ARTICLE

A comprehensive molecular analysis and genotype–phenotype correlation in patients with familial mediterranean fever

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Abstract
Familial Mediterranean fever is an auto inflammatory genetic disease involving especially Turks, Armenians, Arabs and non-Ashkenazi Jews and caused by variants in the MEFV gene. In this study, we aimed to evaluate the distribution and frequency of clinical, MEFV gene variants in FMF patients and the relationship between mutations in different exons and phenotype-genotype and clinical findings. 1028 patients diagnosed as FMF were included. The most common genotypes were M694V / R202Q heterozygous (10.4%), M694V homozygous (7.5%), M694V / E148Q / R202Q heterozygous (4.6%), V726A heterozygous (4.5%), M680I heterozygous (4.2%). c.1611–1 G > C, G152R, S104C, R116S, E336K, R461Q mutations were detected in the literature for the first time in FMF patients. We also divided the patients into 4 groups according to whether the MEFV mutations were exon 10 or non-exon 10. The first group consisted of non-exon 10 homozygous or compound heterozygous (n = 180) patients, Group 2 consisted of exon 10- non-exon 10 compound heterozygous (n = 318) patients, Group 3 consisted of exon 10 homozygous or compound heterozygous (n = 256) patients, while Group 4 consisted of heterozygous (n = 227) patients at any exon. There was no significant difference between the groups in terms of abdominal pain, arthritis, arthralgia, vomiting diarrhea, erysipelas like rash, amyloidosis, renal failure family history. There was no difference in fever between Group 1 (55.6%) and 2 (62.3%); however, these two groups were different from Group 3 (75.8%) and 4 (76.7%). Group 3 (18.8%) had the highest rate of appendectomy. In addition, allele frequencies of all mutations detected in the analyses were compared with allele frequencies of healthy people in the gnomad database. It is useful to analyse all exons in the MEFV gene with the next generation sequence analysis in the detection of FMF disease. S104C, R116S, G152R, E336K, R461Q, L508Q and c.1611–1 G > C mutations are also new variants in literature. c.1611–1 G > C is a possible pathogenic variant.

Keywords Familial mediterranean fever · FMF · MEFV · Autoinflammatory disease

Introduction

Familial Mediterranean fever (FMF, MIM # 248100) is an autosomal recessive inherited auto inflammatory genetic disease affecting especially Turks, Armenians, Arabs and non-Ashkenazi Jews [1]. Autosomal dominant inherited form of the disease has also been described [2]. Individuals with symptomatic heterozygous variants have also been reported [3]. The characteristic features of FMF are abdominal pain, synovitis, pleuritis and / or erysipelas-like rushes that accompany recurrent fever attacks. In addition, the major complication of FMF disease is renal failure secondary to renal AA amyloidosis. The form in which patients are asymptomatic and present with renal amyloidosis as the first finding is called Type 2 FMF.

This disease, which is rare in the European population, has a prevalence of approximately 1/1000; and carrier frequency is around 20% in Turkish population [4]. FMF is caused by variants in the *MEFV* gene localized on chromosome 16p13.3 [5]. The *MEFV* gene consists of 10 exons and encodes a protein of 781 aa called pyrin or meranostrein

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[5]. Pyrin regulates cytokine secretion, neutrophic activity, and cytoskeletal signalling [5, 6]. The innate immune system is responsible for the activation of inflammasomes and IL-1ß release from the myleoid series. To date, more than 300 variants have been identified in the *MEFV* gene [7]. Dundar et al. reported the most common pathogenic variants in Turkish population as M694V, E148Q, M680I and V726A [8]. In this study, we aimed to evaluate the distribution and frequency of clinical, *MEFV* gene variants in FMF patients in Central Anatolia and the relationship between variants in different exons and phenotype-genotype and clinical findings.

Material And Methods

This study was conducted in Kayseri Training and Research Hospital Medical Genetics Clinic between January 2013 and December 2016. FMF patients consisted of patients who were referred to Kayseri Training and Research Hospital by department of pediatric nephrology, rheumatology and patients who directly referred to department of medical genetics. 1028 patients diagnosed as FMF according to the Tel Hashomer and Livneh Criteria were included [9]. Patients who did not meet these criteria were excluded from the study. Age, gender, *MEFV* variants, abdominal pain, fever, joint pain, arthritis, colchicine use, pleuritis, erysipelas-like rash, development of amyloidosis, family history of renal failure, appendectomy, first-degree relative family history and comorbidities were questioned retrospectively from patient files. A significant number of patients were not using colchicine due to being newly diagnosed. On the other hand, 188 patients were on various doses of colchicine. However, a large number of patients have been using irregular colchicine for many years. In order to detect responsible variants in the *MEFV* gene; 2,3,5, and 10 exon sequence analyses were performed with Sanger sequence method in 628 of these patients. 1–10 exons next generation sequence analysis was performed on 400 patients.

In addition, we divided the patients into 4 groups according to whether the *MEFV* variants were exon 10 or non-exon 10. The first group consisted of non-exon 10 homozygous or compound heterozygous (n = 180) patients, Group 2 consisted of exon 10- non-exon 10 compound heterozygous (n = 318) patients, Group 3 consisted of exon 10 homozygous or compound heterozygous (n = 256) patients, while Group 4 consisted of heterozygous (n = 227) patients at any exon. These groups were compared in terms of abdominal pain, arthritis, joint swelling, erythema-like erysipelas, colchicine treatment, chest pain, vomiting, diarrhea, amyloidosis, renal failure family history, FMF family history, and appendectomy. In addition, allele frequencies of all variants detected in the analyses were compared with allele frequencies of healthy people in the gnomAD database.

The study was approved by Erciyes University Local Ethics Committee with the decision numbered 2017/273 and conducted in accordance with the Declaration of Helsinki and good clinical practice guidelines. All subjects or parents/legally authorized representative of the minor participants provided written informed consent prior to taking part in the study.

Molecular analysis

Sanger sequence analysis

Sanger sequence method was conducted on 628 patients. Genomic DNA was extracted from peripheral blood samples using the DNA isolation kit according to the manufacturer's instructions (Zinexts Life Science Corp., Taiwan). Exons 2, 3, 5, and 10 were sequenced using the GML SeqFinder Sequencing System *MEFV* kit and using the Sanger sequence method. PCR conditions were as follows: Initial denaturation at 94 °C for 5 min; 35 cycles at 94 °C for 30 s and 58 °C for 45 s; 72 °C for 1 min; and a final extension at 72 °C for 5 min. The PCR products were observed with 2% agarose gel electrophoresis. PCR products with enzyme transition were purified using the Exo-SAP kit (Exo SAP PCR purification kit, UAB Corporation, Cleveland, Ohio, USA). Cycle sequence was amplified using Big Dye Terminator, and extension products were purified using the Sephadex. The product was sequenced in both strands initiating from the forward and the reverse primers used in the initial PCR and analysed on an ABI 3500 Genetic Analyzer (Applied Biosystems, Hitachi, Japan). Bioinformatic analysis was conducted using the SeqScape v2.6 program. Mutations numbered according to NM_000243.2 (cDNA) and NP_000234.1.

Next generation sequence (NGS)

Next generation sequencing method (NGS) was conducted on 400 patients. Genomic DNA isolation was carried out from peripheral blood according to the manufacturer's instructions (Zinexts Life Science Corp., Taiwan). The NEXTflex Familial Mediterranean Fever Amplicon panel (Bioo scientific,Austin) kit was used for mutation analysis. This panel includes sequencing of 10 coding exons. There is a total of 61 pairs of primary which provide the amplification and sequencing of all exons coding *MEFV* locus. Total length of the related amplicons differs between 83 and 226 bp. The related reading areas of libraries and primary pad areas have an average size of 137–280 bp. This target area was amplified with PCR1. PCR1 steps were 2 min at

98 °C, then 20 s at 98 °C and 4 min at 64 °C 6 cycles, 20 s at 98 °C and 4 min at 62 °C 6 cycles again and finally 6 cycles 20 s at 98 °C and 4 min at 60 °C. This is followed by adaptor ligation and the area products related with PCR2 are enriched. PCR2 steps are 20 min at 65 °C, 2 min at 98 °C, 11 cycles 30 s at 98 °C, 30 s at 65 °C and 60 s at 72 °C, with 4 min at 72 °C in the last step. The products were uploaded to MiSeq Illumina (Illumina, San Diego, California). The data obtained were analysed with Integrative Genomics Viewer (IGV) software (version 2.3.98). Mutations numbered according to NM_000243.2 (cDNA) and NP_000234.1.

Statistical analysis

Statistical analyses were conducted using SPSS software (Statistical Package for the Social Sciences, version 21.0; SSPS Inc., Chicago, IL, USA). Normal distribution analysis was conducted using the Kolmogorov–Smirnov and Shapiro–Wilk test. Values were presented as mean ± standard deviation or median (minimum–maximum range). A Student's t test for parametric variables and Mann–Whitney U test for non-parametric variables were used to determine the difference between groups with regard to continuous variables. To compare the differences between more than two groups, one-way ANOVA was used for parametric data, and Kruskal–Wallis analysis of variance was used for nonparametric data. A post hoc Scheffe's test was used to demonstrate significant differences between groups. Categorical variables were compared with Chi-square test. A p value less than 0.05 was considered to be statistically significant.

Results

Mutations in *MEFV* gene

The study group consisted of 1028 patients. In this study, homozygous variant was detected in 167 (16.25%) patients, compound heterozygous variant was detected in 587 (57.1%) patients and heterozygous variant was detected in 227 (22%) patients. No mutation was detected in 47 patients (4.5%) (Table 1).

The most common genotypes were M694V/R202Q heterozygous (10.4%), M694V homozygous (7.5%), M694V/E148Q/R202Q heterozygous (4.6%), V726A heterozygous (4.5%), M680I heterozygous (4.2%), respectively. Other genotypes are listed in Table 1.

In addition, c.1611-1 G>C, G152R, S104C, R116S, E336K, R461Q variants were detected in the literature for the first time in FMF patients.

Table 1 Genotype distributions of FMF patients are summarized

Zygosity	Genotypes	n	%
Homozygous	Total	167	16.25
	M694V	78	7.59
	R202Q	33	3.21
	E148Q	20	1.95
	M680I	18	1.75
	V726A	6	0.58
	Other	12	1.17
Compound heterozygous	Total	587	57.1
	M694V / R202Q	107	10.41
	M694V / E148Q / R202Q*	48	4.67
	M694V / V726A / R202Q*	41	3.99
	M694V / M680I / R202Q*	34	3.31
	E148Q / P369S / R408Q/ R202Q*	32	3.11
	M680I / V726A	31	3.02
	P369S / R408Q	29	2.82
	V726A / R202Q	15	1.46
	E148Q / R202Q	15	1.46
	V726A / E148Q	13	1.26
	M680I / R202Q	12	1.17
	A744S / R202Q	10	0.97
	Other	200	19.46
Heterozygous	Total	227	22.08
	V726A	47	4.57
	M680I	44	4.28
	M694V	34	3.31
	E148Q	32	3.11
	R761H	15	1.46
	R202Q	12	1.17
	K695R	11	1.07
	Other	32	3.11
Normal		47	4.57

Most common genotypes are M694V/ R202Q heterozygous (10.4%), M694V homozygous (7.5%), M694V / E148Q / R202Q heterozygous (4.6%), V726A heterozygous (4.5%), M680I heterozygous (4.2%) respectively

*Cases with and without R202Q variant are summarized

Clinical features

Of the 1028 patients, 619 were female and 409 were male. The clinical findings of the patients were abdominal pain (88.2%), arthritis (77.7%), fever (68.6%), family history of FMF (60.3%), colchicine use (18.2%), pleuritis (17.8%), appendectomy (12.8%), erythema-like erysipelas (5.4%), family history of renal failure (4.1%), vomiting (2.7%), diarrhea (1.7%) and amyloidosis (0.7%), respectively (Table 2).

Table 2 Characteristic features of FMF patients (n, % and ± SD)

N	1028
Male/female	409/619
Age	27.9 ± 15.6
Abdominal pain	907 (88.2)
Fever	706 (68.6)
Arthritis	799 (77.7)
Joint swelling	145 (14.1)
Use of colchicine	188 (18.2)
Plevritis	184 (17.8)
Appendectomy	132 (12.8)
Vomiting	28 (2.7)
Diarrhea	18 (1.7)
Erysipelas-like erythema	56 (5.4)
Amyloidosis	8 (0.7)
Family history of renal failure	43 (4.1)
Family history of FMF	620 (60.3)

Genotype–phenotype correlation

In this study, we evaluated 4 groups by genotype–phenotype correlation. There was no significant difference between the groups in terms of abdominal pain, arthritis, vomiting diarrhea, erysipelas like rash, amyloidosis, renal failure family history. There was no difference in fever between Group 1 (55.6%) and 2 (62.3%); however, these two groups were different from Group 3 (75.8%) and 4 (76.7%). Patients receiving colchicine treatment were more likely to be in Group 3 (31.6%), in the group with exon 10 homozygous or compound heterozygous. There was a significant difference between the groups in terms of chest pain. This was due to the difference between Group 2 (12.6%) and 3 (23.4%). There was a significant difference between the groups in terms of appendectomy. Group 3 (18.8%) had the highest rate of appendectomy. There was a difference between the groups in terms of FMF family history. Group 1, 2 and 3 were different from each other. The highest FMF family history was found in Group 2 (74.8%) and later in Group3 (61.7%). Genotype phenotype correlation p values and Post hoc analyses for each finding are shown in Table 3.

In this study, for the first time in literature, we compared different *MEFV* variants with healthy human data in the gnomAD database. The most common variants AF in our study were R202Q (0.31), M694V (0.24), E148Q (0.10), V726A (0.09), M680I (0.09), P369S (0.037), R408Q (0.036), R761H (0.025), respectively. The rarest variants were E230K, E336K, E456D, G152R, G196W, G687D, Leu233dup, R116S, R151S, R42W, R461Q, R653H, R708C, S104C, S166L, and V722M variant.

Table 3 The comparison of clinical findings and different mutation groups in patients with FMF

No	Phenotype	Group 1 (Non-Exon 10 homozygous or compound heterozygous) n = 180	Group 2 (Exon 10 and non-Exon 10 compound heterozygous) n = 318	Group 3 (Exon 10 homozygous or compound heterozygous n = 256	Group 4 (Heterozygous) n = 227	p
1	Abdominal pain	158 (87.8)	272 (85.5)	226 (88.3)	211 (93)	0.066*
2	Fever	100 (55.6)[a]	198 (62.3)[a]	194 (75.8)[b]	174 (76.7)[b]	< 0.0001
3	Arthritis	140 (77.8)	239 (75.2)	192 (75)	187 (82.4)	0.174
4	Colchicine treatment	13 (7.2)[a]	33 (10.4)[a]	81 (31.6)[b]	30 (13.2)[a]	< 0.0001
5	Chest pain	24 (13.3)[a, b]	40 (12.6)[b]	60 (23.4)[a]	45 (19.8)[a, b]	0.02
6	Joint swelling	24 (13.3)	32 (10.1)	39 (15.2)	31 (13.7)	0.299
7	Vomiting	7 (3.9)	8 (2.5)	5 (2)	6 (2.6)	0.665
8	Proteinuria	0 (0)	5 (1.6)	3 (1.2)	4 (1.8)	0.376
9	Diarrhea	8 (4.4)	5 (1.6)	4 (1.6)	5 (2.2)	0.159
10	Erysipelas	6 (3.3)	15 (4.7)	18 (7)	10 (4.4)	0.32
11	Amyloidosis	0 (0)	3 (0.9)	4 (1,6)	0 (0)	0.124
12	Renal Failure (family history)	8 (4.4)	12 (3.8)	8 (3.1)	15 (6.6)	0.269
13	Appendectomy	20 (11.1)[a, b]	28 (8.8)[a]	48 (18.8)[b]	26 (11.5)[a, b]	0.003
14	FMF (family history)	84 (46.7)[a]	238 (74.8)[b]	158 (61.7)[c]	127 (55.9)[a, c]	< 0.0001

Data are given as n (%)

Each superscript letter ([a,b,c]) denotes a subset of Groups whose column proportions do not differ significantly from each other at the 0.05 level. Same letter means significantly different. Different letter means statistically significance

*While comparing all groups despite p is greater than 0.05, there is significant difference between Group 2 and 4 (p = 0.007181)

Table 4 The comparison of *MEFV* variants found in this study and gnomAD database

No	Variant	Exon	Present Study			gnomAD				p
			Heterozigot	Homozigot	AF	Heterozigot	Homozigot	Allele Number	AF	
1	p.R202Q	2	379	133	0.3137	44,305	8505	260498	0.235400	<0.0001
2	p.M694V	10	338	78	0.2403	75	1	282876	0.000272	<0.0001
3	p.E148Q	2	172	20	0.1031	13,184	2140	265578	0.065760	<0.0001
4	p.V726A	10	179	6	0.0929	543	9	282870	0.001983	<0.0001
5	p.M680I	10	152	18	0.0914	26	0	251474	0.000103	<0.0001
6	p.P369S	3	73	2	0.0375	3978	86	282228	0.014700	<0.0001
7	p.R408Q	3	71	2	0.0365	3665	53	282186	0.013360	<0.0001
8	p.R761H	10	43	5	0.0258	58	0	282828	0.000205	<0.0001
9	p.F479L	5	25	2	0.0141	12	0	282890	0.000042	<0.0001
10	p.A744S	10	28	0	0.0136	495	2	282842	0.001764	<0.0001
11	p.K695R	10	24	1	0.0126	1626	11	282878	0.005826	<0.0001
12	p.E167D	2	22	1	0.0117	10	0	213066	0.000047	<0.0001
13	p.T267I	2	15	0	0.0073	42	0	282848	0.000149	<0.0001
14	p.M694I	10	13	0	0.0063	36	0	282886	0.000127	<0.0001
15	*p.I591T*	*9*	*9*	*0*	*0.0113*	*3028*	*24*	*282450*	*0.010890*	*0.922**
16	*p.G304R*	*2*	*8*	*0*	*0.0039*	*1294*	*12*	*281346*	*0.004685*	*0.6*
17	*p.L110P*	*2*	*6*	*1*	*0.0039*	*1646*	*64*	*278654*	*0.006366*	*0.159*
18	p.D510N	5	5	0	0.0024	34	0	251458	0.000135	<0.0001
19	p.L508Q	5	5	0	0.0024	0	0	–	0.000000	-
20	p.M680V	10	4	0	0.0019	0	0	–	0.000000	-
21	p.Y471-	5	2	1	0.0019	1	0	251492	0.000004	<0.0001
22	p.E148V	2	3	0	0.0015	19	0	265594	0.000072	<0.0001
23	*p.R329H*	*3*	*3*	*0*	*0.0015*	*437*	*2*	*279644*	*0.001577*	*0.893*
24	*p.R42W*	*1*	*1*	*0*	*0.0012*	*111*	*1*	*282866*	*0.000400*	*0.844**
25	p.E148D	2	0	1	0.0010	0	0	–	0.000000	-
26	p.I259V	2	2	0	0.0010	8	0	251446	0.000032	<0.0001
27	p.I641F	10	2	0	0.0010	0	0	-	0.000000	-
28	p.S154P	2	2	0	0.0010	10	0	261288	0.000038	<0.0001
29	p.S273L	2	2	0	0.0010	0	0	-	0.000000	-
30	p.S339F	3	2	0	0.0010	50	0	279412	0.000179	0.008
31	c.1611-1G>C	Intron 6	1	0	0.0013	1	0	251264	0.000004	<0.0001*
32	*p.E230K*	*2*	*1*	*0*	*0.0005*	*155*	*1*	*249694*	*0.000629*	*0.797*
33	**p.E336K**	**3**	**1**	**0**	**0.0005**	**2**	**0**	**247796**	**0.000008**	**<0.0001**
34	p.E456D	5	1	0	0.0005	0	0	-	0.000000	-
35	p.G152R	2	1	0	0.0005	0	0	-	0.000000	-
36	*p.G196W*	*2*	*1*	*0*	*0.0005*	*386*	*2*	*234764*	*0.001661*	*0.191*
37	p.G687D	10	1	0	0.0005	1	0	251486	0.000004	<0.0001
38	p.Leu233dup	2	1	0	0.0005	0	0	-	0.000000	-
39	**p.R116S**	**2**	**1**	**0**	**0.0005**	**3**	**0**	**247592**	**0.000012**	**<0.0001**
40	p.R151S	2	1	0	0.0005	16	0	231854	0.000069	0.027
41	*p.R461Q*	*5*	*1*	*0*	*0.0005*	*82*	*0*	*282866*	*0.000290*	*0.603*
42	p.R653H	10	1	0	0.0005	10	0	282580	0.000035	0.001
43	p.R708C	10	1	0	0.0005	2	0	251490	0.000008	<0.0001
44	p.S104C	2	1	0	0.0005	4	0	246732	0.000016	<0.0001
45	p.S166L	2	1	0	0.0005	18	0	189158	0.000095	0.077
46	p.V722M	10	1	0	0.0005	4	0	282886	0.000014	<0.0001

Mutations numbered according to NM_000243.2 (cDNA) and NP_000234.1. Bold written variants are novel variants, first time found in patients with preliminary clinical FMF diagnosis

Chi square test p value of variants in italics lines is > 0.05, meaning frequency of these variants are same in FMF diagnosed population from this work and healthy considered population from gnomAD

*These variants are detected via NGS method and located on exon 1,9 and intron 6. So, “Allele Number” was considered 800 (400 patients were analysed via NGS method, 628 via Sanger method for exon 2, 3, 5 and 10), for the remaining variants which are located at exon 2, 3, 5 and 10, “Allele Number” was considered 2056 (1028 patients × 2)

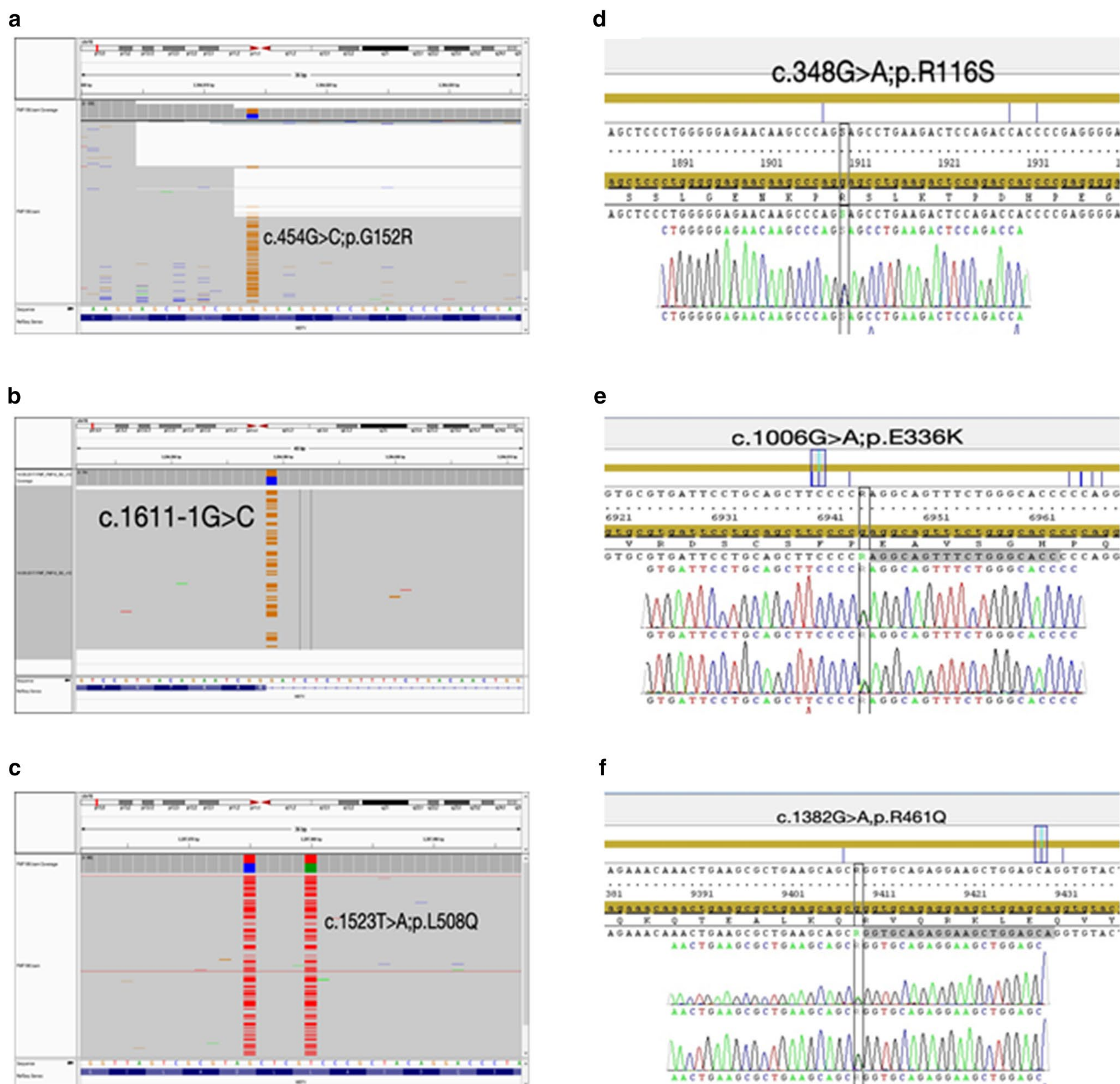

Fig. 1 Mutations numbered according to NM_000243.2 (cDNA) and NP_000234.1. The genotypes of new identified variants are shown as **a** c.454G>C, p.G152R; **b** c.1611-1G>C; **c** c.1523 > A,p.L508Q; **d** c.348G>A,p.R116S; **e** c.1006G>A,p.E336K; and **f** c.1382G > A,p.R461Q, respectively

There was a significant difference in terms of allele frequencies of all variant except for I591T, G304R, L110P, R329H, E230K, G196W, R42W, and R461Q variants shown in grey in Table 4 when compared with healthy individuals. In addition, we found that the allele frequency (0.31) of the R202Q exchange, known as polymorphism, was significantly different than that of healthy individuals (0.23). In addition, the variants S104C, R116S, G152R, E336K, R461Q, L508Q and c.1611–1 G > C were new variants which were not previously described in literature and which were not included in the HGMD and infevers database (Fig. 1).

Discussion

In this study, we identified *MEFV* gene variants, genotype distribution and allele frequencies in 1028 patients diagnosed with FMF. In addition, we made genotype–phenotype correlation in 4 groups formed according to the

presence of exon 10 and non-exon 10 mutations. The most common genotypes in this study were M694V heterozygous/R202Q heterozygous (10.4%), M694V homozygous (7.5%), M694V heterozygous/E148Q heterozygous/R202Q heterozygous (4.6%), V726A heterozygous/wt (4.5%) and M680 heterozygous/wt (4.3%), respectively. M694V heterozygous/wt (26.2%), E148Q heterozygous/wt (20%), M694V homozygous (8%), M680I heterozygous/wt (7.4%) were found in Coşkun et al.'s study [10]. M694V heterozygous/wt (20.8%), M694V heterozygous/M680I heterozygous (12.2%), E148Q heterozygous/wt (9.5%), M680I/wt heterozygous (7.2%) were found to be the most common genotypes in Dundar et al.'s study [8]. M694V homozygous (45%), M694V heterozygous/wt (12.7%), M694V heterozygous/M680I heterozygous (11.8%) were found in Dusunsel et al.'s study [11]. However, 12 variants were investigated by reverse dot blot method in the aforementioned studies. In our study, unlike the aforementioned studies, all 10 exons were sequenced by next generation sequence analysis method and exon 2, 3, 5, and 10 were sequenced by Sanger sequence method. Therefore, we demonstrated the presence of multiple complex genotypes with more than 2 variants in FMF patients (Table 1).

Mutations are seen intensively at exon 10 in *MEFV* gene. M694V has been found to cause early onset and more severe phenotype in homozygous patients [12]. However, V726A and E148Q have been reported to cause a milder disease [13]. In addition, M694V is the pathogenic variant most commonly associated with amyloidosis [14, 15]. In our study, no significant difference was found between the groups in terms of amyloidosis. However, the most common allele in patients with amyloidosis was M694V, which was in parallel with the literature.

Interestingly, appendectomy was found to be performed significantly more in group 3, the group with exon 10 homozygous or compound heterozygous group. This suggests that patients with more severe clinical findings may have undergone appendectomy during the attack. In case of FMF family history, Group 1,2, and 3 were different from each other. It was found to be the least in Group 1 and the most in Group 2. The proportion of patients receiving colchicine treatment was also significantly higher in Group 3.

In this study, no variant was detected in 47 patients (4.5%). This rate was 54.3% in Coskun et al.'s study and 49.6% in Dundar et al.'s study [10, 16]. This shows that researching exons 2, 3, 5 and 10 and all exons will be more useful in the diagnosis of FMF. In addition, we included patients who met FMF diagnostic criteria in this study. We excluded patients who did not meet the diagnostic criteria. The high difference when compared with other studies may be due to patient selection. In addition, other periodic fever syndromes, deep intronic variants and genetic heterogeneity may be implicated in these 47 patients.

Numerous studies have reported cases of no variant in patients with FMF clinic or cases with one variant [16, 17]. In our study, only 1 heterozygous variant was detected in 227 (22%) patients. Subclinical inflammation has been reported in patients with previous heterozygous variants [18, 19]. Therefore, we can classify FMF as a spectrum. It may be suggested that patients with heterozygous variants should be classified as mild FMF. Mutations in the *MEFV* gene, and therefore FMF disease, have been spread particularly in the Turkish population, as they do not cause death at an early age. With important advances in genetics, especially with the spread of exome sequence analysis, the importance of secondary findings (additional findings) has emerged in understanding different phenotypes in patients. In fact, other mutations to be detected in addition to *MEFV* variants in these patients may explain phenotypic diversity in patients, and some patients with a single variant showing signs. In addition, the severity of FMF disease and dietary habits, presence of additional disease, exposure to intestinal microbiota are potential areas of research and further research is needed.

In this study, we identified S104C, R116S, G152R, E336K, R461Q, L508Q and c.1611-1 G>C variants that were not previously reported in infevers, Clinvar, HGMD and literature. There were contradictory results in the insilico assessment we made using the Mutation tester, SIFT, and Polyphen2; one program said it was likely pathogenic, while the other said it was likely benign. As a result of our evaluation according to the ACMG criteria, we classified c.1611-1 G>C as likely pathogenic; R116S, G152R, L508Q exchanges as variant of insignificance; E336K and R461Q exchange as likely benign. However, the related patients were referred to our clinic with a preliminary diagnosis of FMF and met the diagnostic criteria themselves. Further studies are needed on the variant classification of these variants. Identification of founder variants in different population and region is a significantly important in genetic counselling. The fact that these mutations were not detected in previous studies makes us think that these are founder variants.

R202Q exchange is considered as polymorphism and has been reported to be quite common in Turkish population [20, 21]. Again, allele frequency in healthy people in the gnomad database was reported as 0.23. However, we found that there was a significant difference in our comparison with FMF patients. While looking into p.R202Q AF of cases with and without other FMF related variants (Table 5), it shows that p.R202Q alone AF is not that much in FMF population. So, we can conclude that it is common variant in population and its elevated AF in this study is related to co-existence with other pathogenic variants and the statistically significant difference of R202Q frequency between present study and gnomAD (<0.0001)

Table 5 p.R202Q AF of cases with and without other FMF related variants

p.R202Q cases	Allele number	AF
Without any other detected variant	78	0.04
Detected with p.M694V	268	0.13
In combinations with detected other variants	299	0.15
Total	645	0.31

is not sufficient factor for correlation of this variant with FMF as AF of R202Q is greater than 0.05 (Table 4) which is stand-alone criteria in favor of "Benign" classification according to guidelines (ACMG). This difference may be explained with the regional difference of variant. Currently it is not possible to prove this hypothesis as there is not appropriate database but there are many articles describing R202Q frequency in FMF population [22].

While comparing of NGS (sequencing of all exons) and Sanger sequencing (exon 2, 3, 5 and 10) to see if sequencing of all exons is needed for routine at first step or not, as seen in Table 4 there were detected only 3 variants in exon 1 and 9, and intron 6 in 12 patients (1,2%) of all 1028 patients. So, if in future with evolution of technologies there will not be any financial benefit NGS should not be first step, but in case of strong clinical features despite negative exon 2, 3, 5 and 10 sequencing, sequencing of all exons may reveal pathogenic variants.

The frequency of fever was less than expected as it was seen in 68.6% (Table 2) of patients. Despite Tel Hashomer criteria where fever accepted as mandatory complaint were not meet in some patients, while applying Livneh criteria patients were diagnosed with FMF without any doubt. But we must also acknowledge limitations which can slightly affect results such as; not 100% reliable information from patients which is main bias problem of retrospective studies, patients may have incomplete attacks as described by Livneh et al. [9] and despite clinical complaints were noted in first examination of patients it is possible that some patients already started irregular colchicine use which can alter clinical picture.

As a conclusion, it is useful to analyse all exons in the *MEFV* gene with the next generation sequence analysis in the detection of FMF disease. S104C, R116S, G152R, E336K, R461Q, L508Q and c.1611–1 G > C variants are also new variants in literature. c.1611–1 G > C variant is a possible pathogenic variant.

Compliance with ethical standards

Conflict of interest The authors of this paper have no conflicts of interest, including specific financial interests, relationships, and/or affiliations relevant to the subject matter or materials included.

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