Investigation of different genomic variants in familial Mediterranean fever cases with monoallelic *MEFV* mutation

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Abstract

Objective

Familial Mediterranean Fever (FMF) is an inflammatory disease characterised by periodic fever and concurrent episodes of serous membrane inflammation. FMF is considered to be inherited in autosomal recessive manner and biallelic mutations in the MEFV gene are associated with the disease. However, approximately 20-25% of patients only have a single mutation in MEFV gene, which creates confusion in differential diagnosis of many patients. This study aimed to reveal rare variants that may act in conjunction with the single pathogenic MEFV variant in the pathogenesis of FMF.

Methods

We performed whole exome sequencing in 17 individuals from 5 different families who were diagnosed according to the clinical criteria, responded positively to colchicine treatment, but had no biallelic MEFV mutation.

Results

A disease-causing variant or a common affected cellular pathway that was shared in all index cases was not detected. When cases were examined individually, two de novo variants were identified in the BIRC2 and BCL10 genes, both of which play a role in inflammatory pathways. Functional studies are needed to confirm the physiopathological relationship of these genes with FMF.

Conclusion

This study is one of the most extensive aetiological researches in FMF cases with monoallelic MEFV mutation. We have shown that genotype-phenotype correlation in these cases may not be established by rare genetic variants and discussed underlying causes. Clinical criteria with emphasis on colchicine response and family history should be the main tool and genetic results should only be used for support in FMF diagnosis.

Key words

BCL10, BIRC2, familial Mediterranean fever, *MEFV*, whole exome sequencing

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Introduction

Familial Mediterranean fever (FMF) is an autoinflammatory disease characterised by self-limiting episodes of fever and serous membrane inflammation. It is believed to be a monogenic disease with an autosomal recessive inheritance but there are several issues requiring an explanation regarding *MEFV*-related phenotype such as low penetrance, difficulties in clinical interpretation of *MEFV* variants, the lack of a reliable genotype-phenotype correlation and patients with monoallelic mutations.

FMF is most common in Eastern Mediterranean populations, but studies report a wide range of disease prevalence. In Turkish population the prevalence, as determined by clinical evaluation, is reported in the range of 0.027%–0.82% (1-3). While the carrier rate as determined by genetic evaluation of *MEFV* is reported in the range of 3.75%-27%. (4, 5). The common point of all these studies is that, contrary to expectations, they have a lower prevalence compared to high carrier rate. Either FMF is largely underdiagnosed or *MEFV* variants alone are insufficient in determining carrier state and other genetic/environmental factors affect disease penetrance.

There are more than 350 variants identified within *MEFV* in the Infevers database (6). Variants can be found across all parts of the gene (5' UTR, 3' UTR, all exons and introns) but only a limited number of well-established mutations (mostly exon 10 variants) are associated with FMF. The rest of the variants are generally classified as variants of uncertain significance (6). This high degree of uncertainty is another aspect of FMF genetics and until date, an internationally recognised genotype-phenotype correlation that enables predicting disease outcome of *MEFV* variants is not established (7). Apart from the difficulties of missing penetrance and uncertain variants, it is shown that around 20%–26% of patients only have a single mutation (8). Many studies searched for a second mutation. Booty *et al*. analysed the entire 15 Kb genomic region of *MEFV* in 10 cases, but they were unable to show a second mutation (9). Marek-Yagel *et al*. performed complementary DNA sequencing with expression analysis in 20 cases and excluded the possibility of a loss of expression due to an unknown mutation or a mosaic mutation specific to leukocytes (10). Deletions and duplications were also evaluated as possible underlying causes but no rearrangements were found (10, 11). Haplotype analyses were conducted in affected siblings, but different alleles were found to be inherited in wild-type MEFV alleles (9, 10). Considering these results, explaining monoallelic FMF patients by autosomal recessive inheritance of *MEFV* variants alone is not possible. This prompted the definition of a subtype of FMF with autosomal dominant inheritance (OMIM#134610), and also another clinical entity named as Pyrin Associated Autoinflammation with Neutrophilic Dermatosis (OMIM#608068) which is associated with dominant inheritance of two specific *MEFV* mutations (p.S242R, p.E244K) (12-15). This also led to the hypothesis that *MEFV* variants have a dose-dependent phenotypic effect in FMF pathogenesis (16). Although these subtypes show that dominant inheritance is possible, they do not answer the main question. What causes the classical FMF disease, in individuals carrying common heterozygous mutations? Contrary to the expectations from dose-dependent phenotypic effects, there are no clinical differences that can be clearly distinguished between biallelic mutant cases and monoallelic mutant cases (10, 17). Additionally, the vast majority of monoallelic mutant individuals carrying known mutations are asymptomatic carriers.

In this study we aimed to reveal rare variants that acts in conjunction with a single *MEFV* mutation in the pathogenesis of FMF. We performed an extensive search with whole-exome sequencing (WES) in 5 unrelated index patients and their first-degree relatives who are clinically diagnosed with FMF and carrying only one *MEFV* mutation.

Material and methods

Patient selection

All patients referred for FMF in Dokuz Eylul University Paediatric Nephrology and Paediatric Genetics outpatient

Table I. Phenotypic characteristics of the cohort.

Cases	Gender	Age (year)	Age at onset	Abdominal pain	Fever	Arthralgia	Arthritis	Frequency of episodes†	Episode duration (Day)
Family I									
I.1 (index)	F	11	5	+	+	+	-	1-2/year	5-7
I.2 (sister)	F	19	-	-	-	-	-	-	-
I.3 (mother)	F	45	6	+	+	+	+	6-7/year	5-10
Family II									
II.1 (index)	M	12	8	+	+	-	-	6/year	3
II.2 (mother)	F	34	-	-	-	-	-	-	-
II.3 (father)	M	35	-	-	-	-	-	-	-
Family III									
III.1 (index)	F	8	2	-	+	+	+	4-5/year	1-2
III.2 (mother)	F	35	18	+	+	+	+	10-12/year	1-2
III.3 (father)	M	37	-	-	-	-	-	-	-
Family IV									
IV.1 (index)	F	7	1	+	+	+	-	4-6/year	2-3
IV.2 (mother)	F	27	-	-	-	-	-	-	-
IV.3 (father)	M	31	-	-	-	-	-	-	-
Family V									
V.1 (index)	M	4	1	-	+	-	-	12/year	2-3
V.2 (dizygotic twin)	M	4	-	-	-	-	-	-	-
V.3 (brother)	M	8	2	-	+	+	+	3-4/year	3-7
V.4 (mother)	F	37	-	-	-	-	-	-	-
V.5 (father)	M	38	-	-	-	-	-	-	-

Table I (continued). Phenotypic characteristics of the cohort.

Cases	ELE	Chest pain	Diarrhoea / cons.	Nausea /vomit	Myalgia	ESR/CRP	Leukocyte	Laparotomy	Colchicine
Family I									
I.1 (index)	-	-	+	+	+	41‡ / 0,3‡	8300‡	-	1 mg/day
I.2 (sister)	-	-	-	-	-	-	-	-	Healthy
I.3 (mother)	-	+	-	-	-	13§ / 3,9§	5400§	+	1,5 mg/ day
Family II									
II.1 (index)	+	-	-	-	-	3§ / 0,7§	9900§	-	1 mg/ day
II.2 (mother)	-	-	-	-	-	-	-	-	Healthy
II.3 (father)	-	-	-	-	-	-	-	-	Healthy
Family III									
III.1 (index)	-	-	-	-	+	40‡ / 2,9‡	5000§	-	1 mg/ day
III.2 (mother)	-	+	+	+	-	30‡ / 11‡	9900‡	-	1,5 mg/ day
III.3 (father)	-	-	-	-	-	-	-	-Healthy	
Family IV									
IV.1 (index)	-	-	+	+	-	32‡ / 6,1‡	3100‡	-	1 mg/ day
IV.2 (mother)	-	-	-	-	-	-	-	-	Healthy
IV.3 (father)	-	-	-	-	-	-	-	-	Healthy
Family V									
V.1 (index)	+	-	-	-	-	81‡ / 110,6‡	12100‡	-	0,5 mg/ day
V.2 (dizygotic twin)	-	-	-	-	-	-	-	-	Healthy
V.3 (brother)	-	-	-	-	-	17‡ / 20,3‡	15300‡	-	0,5 mg/ day
V.4 (mother)	-	-	-	-	-	-	-	-	Healthy
V.5 (father)	-	-	-	-	-	-	-	-	Healthy

M: male; F: female; ELE: erysipelas-like erythema; Cons: constipation; ESR: erythrocyte sedimentation rate (normal range: 0-15 mm/h); CRP: C-reactive protein (normal range: 0-5 mg/L).
Leukocyte normal range: 4000-10000/μl.
†The frequency of episodes reflects the frequency before treatment, and it is decreased in all cases after colchicine initiation.
‡Laboratory values (ESR, CRP, leukocyte) taken during crises.
§Basal values of patients in asymptomatic intervals when episodic data were not available.

clinics in 2020 were retrospectively reviewed. *MEFV* mutation status was determined after a two-step genetic testing procedure as recommended in the literature (18, 19). Patients with a confirmatory genotype for any autoinflammatory disease as explained in Eurofever criteria were excluded (20). Among the rest, priority was given to those who benefited from colchicine treatment, and to those with a family history of FMF. A total of 17 individuals including 5 index cases, who were clinically diagnosed with FMF but only have a single *MEFV* mutation, and their first-degree relatives were selected. All individuals (or parents when the patient was a minor) provided written informed consent for molecular analysis and for the publication of clinical findings. The study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Dokuz Eylul University (date: 01.11.2018/No. 2018/28-25).

MEFV mutation analysis

Peripheral blood samples were collected from all individuals. Total genomic DNA were isolated using QIAamp® DNA Blood Mini kit (Qiagen, Germany) via Qiacube® automated DNA isolation device (Qiagen, Germany). In 5 index patients, PCR amplification with melting curve analysis for hotspot mutations (p.E148Q, p.R202Q, p.P369S, p.M680I, p.M694V, p.M694I, p.V726A, p.A744S) was performed as a first step analysis. LightSNiP® FMF kit (TIB Molbiol, Germany) were used according to the manufacturer's protocols in Cobas® z480 Analyzer (Roche Diagnostics, Switzerland). As a second step testing in index patients, target capture and enrichment based next generation sequencing was performed to analyse the entire coding region and splice junctions (± 10 bp) of *MEFV* and 11 other autoinflammatory disease related genes [*TNFRSF1A*, *MEFV*, *MVK*, *IL-1RN*, *LPIN2*, *NOD2*, *NLRP3*, *NLRP12*, *ADA2*, *PSTPIP1*, *TNFRSF11A*, *ELANE*]. Library preparation was carried out using autoinflammatory diseases panel (Celemics, South Korea) and massively parallel sequencing was conducted on Illumina NextSeq550 system (Illumina, San Diego, CA, USA). Bioinformatic steps and data analysis were performed as stated below.

Whole-exome sequencing and data analysis

To generate exome capture libraries, Human Core Exome Kit (Twist Bioscience, USA) that enriches 33 Mb of the human consensus coding sequence was used according to the manufacturer's protocols. The enriched DNA was paired-end sequenced on HiSeq2000 Sequencer (Illumina, San Diego, CA, USA). Sequence alignment and variant calling were performed against the reference human genome (hg19) using SEQ programme v. 16.7 (https://seq.genomize.com/) (Genomize, Turkey). The SEQ algorithm works according to the GATK recommendations and incorporates Burrows-Wheeler Aligner for read mapping, FreeBayes for variant calling, PCR dedup for removing duplicates and Indel Realignment to remove alignment artifacts (21). SEQ also annotates variants using several online resources such as Ensembl Variant Effect Predictor v. 78, Clinvar, dbSNP v. 151 and dbNSFP v. 3.5 (22-25).

Variant filtering and interpretation

Every patient's data were analysed individually and then cross examined for joint variants. For every individual, a two-step variant filtering strategy was applied. At the first step, we excluded variants with an allele frequency greater than 0.1% or 1% in Gnomad version 2.1.1 and in SEQ's in-house database. SEQ contains around 2500 individual's exome sequencing data from Turkey (26). Two minor allele frequency thresholds were applied between the different variant types. A less restrictive 1% threshold was used for null type variants since these mutations are relatively few and have serious phenotypic consequences. For missense and in-frame variants 0.1% frequency threshold is chosen because, the disease prevalence in our region is 0.1% and monoallelic FMF patients are a smaller cluster within all patients. Also, since this study was conducted with five index cases, it would not be possible to associate more frequent variants that could act through oligogenic/polygenic inheritance. Then, benign/likely benign variants in ClinVar were omitted from the remaining rare exonic non-synonymous variants (missense, nonsense, frameshift, canonical splice site, stop loss, initiator codon variants, in-frame deletions, and in-frame insertions). Therefore, we revealed rare variants with likely phenotypic consequences. No candidate gene-based filtering was applied. Instead in the second step, all remaining variants were searched for functional relevance with the *MEFV* gene.

Functional enrichment analyses was performed on the frequency filtered exonic non-synonymous variants by DAVID (27). In this analysis, we searched whether any of our filtered variant is present in a gene involved in the 'Nod-like receptor signalling pathway' or not. We also searched for other cellular pathways where the variant burden is increased. To distinguish this, the list of genes to which the filtered variants belong was entered as 'Official Gene-Symbols'. Homo sapiens was used as the reference genome. $p<0.05$ was considered statistically significant.

GeneMANIA and STRING web applications were also used for functional filtering (28, 29). 13 genes that play an active role in pyrin inflammasome activation [*MEFV*, *CASP1*, *IL18*, *IL1B*, *MVK*, *PKN1*, *PKN2*, *PSTPIP1*, *PYCARD*, *RHOA*, *RHOB*, *RHOC*, *YWHAB*] were searched along with filtered rare variant genes. The idea was that any physiopathologically important gene must play a biological part (co-localisation, physical interaction, pathway interaction, etc.) with aforementioned genes inside the cell. While determining the functional interactions in the STRING program, high-confidence interactions with a minimum interaction score of 0.7 and above were taken as the basis. Known physical interactions and same pathways were also taken as basis on the GeneMANIA and other interactions were not included.

In silico data of the REVEL algorithm were used for missense variants and thresholds were decided in line with that of Ioannidis *et al*. (30). REVEL scores

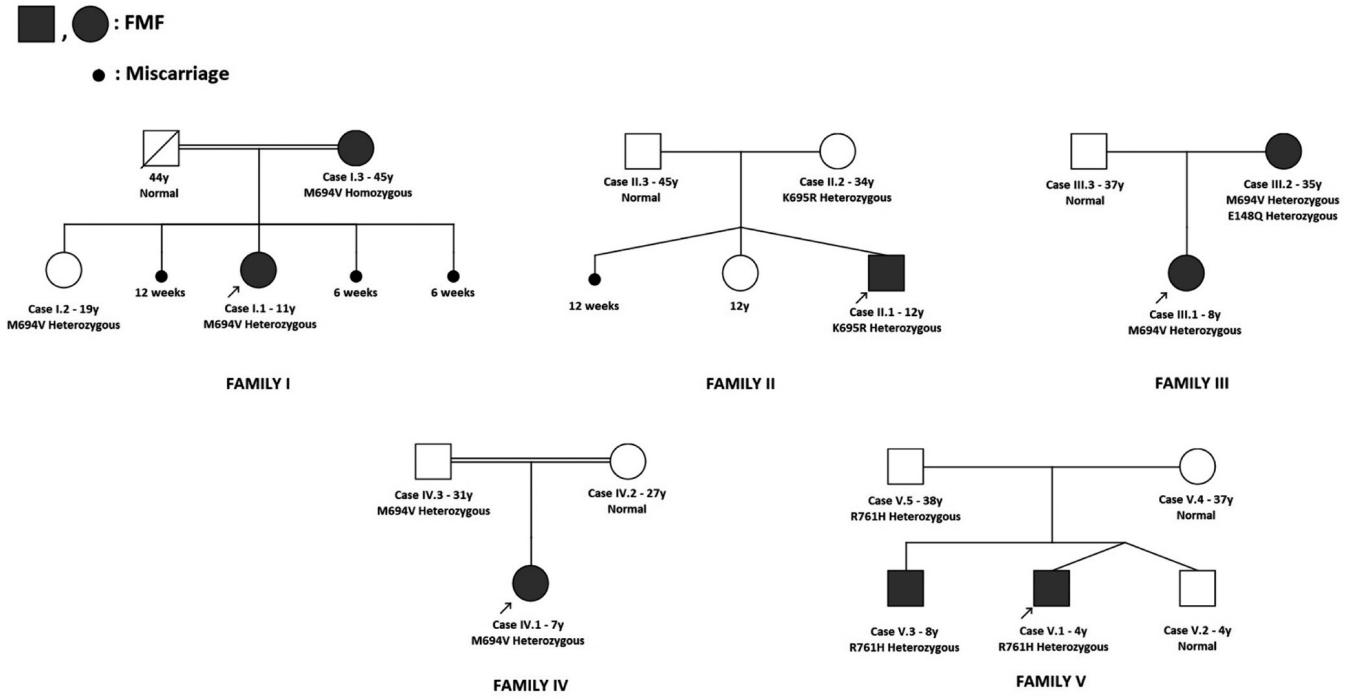

Fig. 1. Pedigrees showing index cases and their first-degree relatives. Age, MEFV mutation status and case numbers of participating family members are shown below each individual. Benign and likely benign variants are not included.

between 0.5 and 0.75 were considered as uncertain [variants of uncertain significance (VUS)] and scores greater than 0.75 were considered as supporting evidence of pathogenicity. Also, Gnomad database were used to obtain observed/expected (o/e) ratios of different gene-variant type combinations. Since o/e scores reflect the ratio of the actual number of specific type variants encountered in the general population to the evolutionarily expected number of that type of variant, a score lower than 0.35 threshold is used to support pathogenicity.

After evaluation of functional predictive information in the second step and filtering, pathogenicity classification were determined according to the American College of Medical Genetics and Genomics (ACMG) standards (31). Cross examination of joint variants between different families was performed and possible causal relationship in FMF is discussed in light of current literature.

Results

Patient information

A total of 17 people, including 5 index cases and their first-degree relatives, were included in the study. The age of index cases was between 4 and 12 (Mean: 8.4). The mean age of the entire study group was 23.6. The total number of individuals diagnosed with FMF was 8. One sibling and two parents from different families were receiving colchicine treatment. All the clinical data are summarised in Table I. *MEFV* testing results of the members are shown alongside pedigrees in Figure 1.

Whole-exome sequencing

The mean depth was 176.4 reads and the mean target coverage in at least 50x depth was 91.73%. 50x coverage was below 85% in only case V.5 (father of the index patient in family V), but the coverage rose to 93.38% at 20x. Therefore, it was deemed sufficient and evaluated accordingly. A mean of 33,474 variants per index case was detected (Fig. 2). Two-step filtering was performed on these variants. In the first step, variants were filtered separately according to variant types for their minor allele frequencies (MAF) and ClinVar reports which left an average of 252 variants per index case (Fig. 2).

In the second step, variants without any functional relevance to pyrin inflammasome pathway in DAVID, GeneMANIA and STRING *in silico* analysis were filtered out and *de novo* variants were uncovered by parental and sibling analyses (Fig. 3). One heterozygous *de novo* variant was detected in *BIRC2* gene in Case V.1, *PRR21* gene in Case IV.1, *PELP1* gene in Case III.1, *BCL10* and *IMP4* genes in Case II.1. Since the father of Case I.1 was not available, *de novo* variants were not evaluated in that case.

The results of DAVID's pathway enrichment analysis were as follows,

- 'Tight junction' pathway (p=0.045) in Case I.1,
- No statistically significant pathway in Case II.1,
- 'Autoimmune thyroid disease' pathway (p=0.031) in Case III.1,
- 'Gap junction' (p=0.014), 'Inflammatory mediator regulation of TRP channels' (p=0.02), 'Neuroactive ligand-receptor interaction' (p=0.027), 'Oocyte meiosis' (p=0.03) and 'Calcium signalling' (p=0.04) pathways in Case IV.1,
- 'Retinol metabolism' (p=0.036) and 'Adherens junction' (p=0.046) pathways in Case V.1 were found to be enriched in variant load.

All variants after second-step filtering [*MEFV* variants, pathogenic (P) or likely pathogenic (LP) variants, *de novo* variants, Nod-like receptor sig-

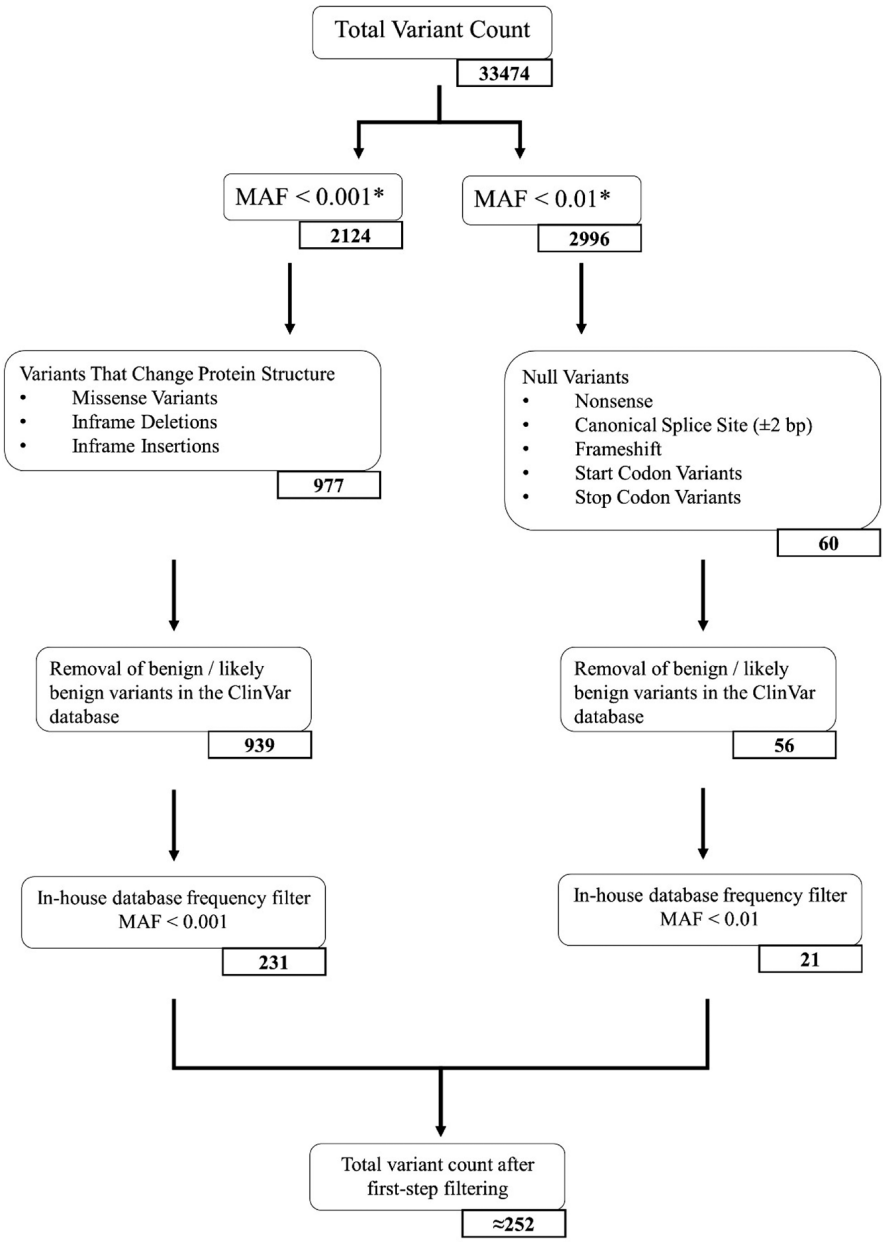

Fig. 2. First-step variant filtering strategy.
Numbers in the lower right boxes are the average number of variants detected in an index case after every stage. The numbers for each individual case are reported in the Supplementary Figure S1.
MAF: minor allele frequency. *Population frequencies from Gnomad database.

nalling pathway variants and pyrin inflammasome pathway related variants] are shown case by case in Supplementary Table S1. Variant name in accordance with the international nomenclature, position, zygosity, parental origin, *in silico* prediction score, o/e score, rs number, in-house MAF and pathogenicity criteria are included in Supplementary Table S1.

In cross-comparison, a variant that is overlapping in all five families were not found. In separate comparisons, it was seen that there were two shared variants in the *CACNA1H* and *HRNR* genes in cases I.1 and V.1, while there were two other shared variants in the *MYH13* and *TTN* genes in cases II.1 and IV.1.

Pathogenicity classification was made to the rest of the filtered variants according to the ACMG criteria. Eight variants were interpreted as P/LP (apart from *MEFV* mutations). P/LP variants were detected in *ABCA13* and *XPA* genes in case I.1, *TACR3* gene in case II.1, *SLC19A3* gene in case III.1, and *BIRC2* (also *de novo*), *KRIT1*, *LZTR1*, and *RPE65* genes in case V.1.

In total, 115 variants in 5 index cases were manually curated. 7 of them were previously known *MEFV* variants, 8 of them were rare variants in the same gene across index cases (*CACNA1H, HRNR, MYH13, TTN*), 5 of them were *de novo* variants (*BCL10, IMP4, PELP1, PRR21, BIRC2*) and 7 of them were P/LP variants (*ABCA13, XPA, TACR3, SLC19A3, KRIT1, LZTR1, RPE65*). 88 different gene variants were left which are identified as possibly related to the pyrin inflammasome pathway. Among them, *CHUK* p.Leu354Ile found in case II.1 is discussed separately with the closely related *de novo BCL10* variant. The remaining 87 variants were classified as VUS (Supplementary Table S1).

Discussion

The strongest evidence to associate a genetic variant with FMF would have been the detection of the same variant in many unrelated cases. Genes of the overlapping variants detected in this study (*CACNA1H, HRNR, MYH13, TTN*) were not involved in inflammatory pathways. No information regarding possible FMF association was found in the literature for these genes. All variants were classified as VUS. Therefore, an association with FMF was not considered and the fact that these rare variants overlapping in two out of five cases were considered to be coincidental. The absence of any shared pathway in gene enrichment analysis was also interpreted as the lack of other cellular pathways involved in the FMF phenotype.

De novo variants play an important role in many diseases, but *de novo* inheritance is a strong indicator of pathogenicity, only if it can be associated functionally with disease (31). The function of *PRR21* is unknown, *PELP1* encodes an oestrogen receptor co-activator and the *IMP4* gene encodes a protein involved in ribosome biogenesis localised in the nucleolus. No functional relationship to the pyrin inflammasome pathway or a known association with any disease was found for these genes. Therefore, *de novo* variants other than *BCL10* and

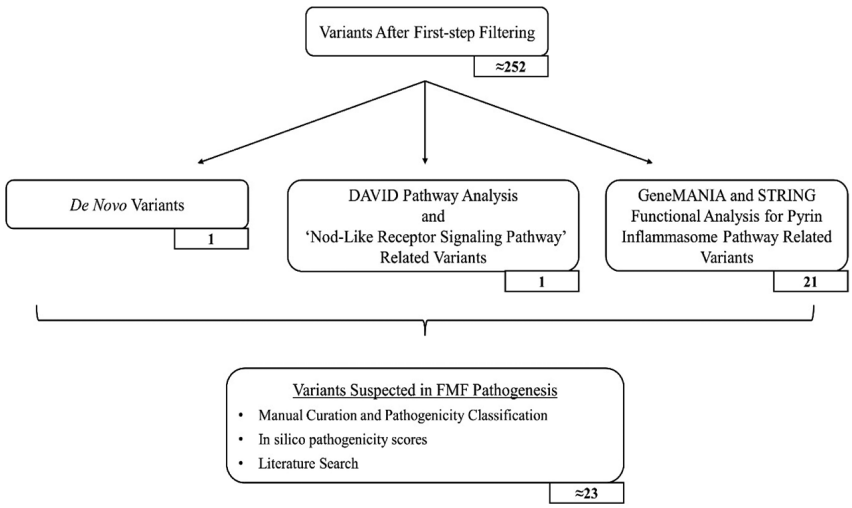

Fig. 3. Second-step variant filtering strategy.
Numbers in the lower right boxes are the average number of variants detected in an index case after every stage. The numbers for each individual case are reported in Supplementary Figure S1.

BIRC2 were not considered to be associated with FMF.

BIRC2 gene (formerly CIAP1) is a member of the inhibitor of apoptosis protein family. It functions as an E3 ubiquitin ligase and regulates many substrates, including itself, by ubiquitination (32). It's most relevant function in terms of FMF is the inhibition of IL-1 beta formation with other inhibitors of apoptosis proteins (XIAP and CIAP2). This function occurs via Caspase-8 along with NLRP3 inflammasome (33, 34). However, contrary to this information, there are also publications reporting that CIAP1 and CIAP2 are essential for effective caspase-1 activation and therefore IL-1 beta formation (35). In Case V.1, a *de novo* heterozygous p.Glu443GlyfsTer7 frameshift variant was detected in the 6th exon of the *BIRC2* gene. BIRC2 protein has 6 different domains. From the N to C terminus; three BIR domains, that are responsible for protein-protein interactions, UBA domain that is responsible for ubiquitin binding, CARD and RING domains that are responsible for E2 protein interactions, respectively (Fig. 4) (36). The frameshift variant coincides just before the CARD domain and leads to a stop codon after 7 amino acids (Fig. 4). The *BIRC2* gene has an o/e score of 0.22, and therefore haploinsufficiency has a high risk of clinical disease. Mutations associated with inhibitors of apoptosis proteins have generally been reported in somatic form, so there is no clear information about germline mutations (37). In family V, index case (V.1), his older brother (V.3) and his father (V.5) has *MEFV* p.R761H variant in heterozygous state while *BIRC2* variant is only found in the index case. While this is not compatible with segregation, their symptoms are also not the same. V.1 has erythematous eruptions around ankles and frequent shorter episodes of fever, whereas V.3 has arthralgia, arthritis, and less frequent longer episodes of fever. V.5 is healthy. We interpreted the frameshift *BIRC2* variant as likely pathogenic, but functional studies are required to determine the physiopathological relationship with FMF.

BCL10 gene encodes a signal protein involved in adaptive immune system activation and regulation. When B or T cell receptors are stimulated; BCL10 forms a complex called 'CBM' with various CARD proteins and MALT1 protein. The main result of this complex is NFκB activation. This happens by ubiquitin-regulating protein recruitment and NEMO ('NFκB essential modulator') activation along with IKK alpha ('inhibitor of NFκB kinase', also known as CHUK) and beta proteins. When IKK proteins are activated, they deactivate NFκB kinase and therefore, active NFκB is released in the cytosol (38). In 2012, Gringhuis *et al*. reported that Dectin-1, a pattern recognition receptor protein such as pyrin, causes the formation of the CARD9-BCL10-MALT1 complex in response to extracellular fungal pathogens, which in turn increases IL-1 beta transcription and maturation (39). Therefore, it has been shown that although the physiological processes in which BCL10 is involved are not directly related to the Pyrin inflammasome pathway, it can increase IL1-beta synthesis. In case II.1, we detected a *de novo* heterozygous missense variant (p.Arg25Cys) in the *BCL10* gene and a paternally inherited heterozygous missense variant (p.Leu354Ile) in the *CHUK* gene together. Lymphoma occurs in somatic mutations of the CBM complex, particularly in translocations involving *BCL10* (40). Whereas in biallelic germline mutations, immunodeficiency syndromes called CBM-opathies occur

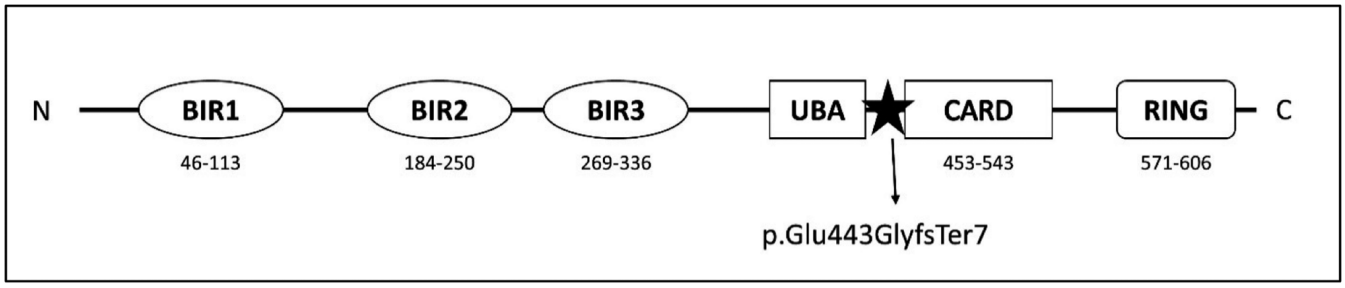

Fig. 4. BIRC2 protein structure. Amino acid positions are given under each domain. The mutation detected in Case V.1 is marked with a star.
BIR: baculoviral IAP repeat domain; UBA: ubiquitin associated domain; CARD: caspase recruitment domain; RING: really interesting new gene domain.

Table II. In silico predictions and allele frequencies*.

Prediction tool	BCL10:c.73C>T:p.R25C	CHUK:c.1060C>A:p.L354I
PROVEAN	Damaging (-7,35)	Tolerable (-0,95)
SIFT	Damaging (0)	Tolerable (0,145)
Polyphen2_HDIV	Probably_damaging (1)	Probably_damaging (0.983)
Polyphen2_HVAR	Probably_damaging (0.998)	Probably_damaging (0.656)
CADD	Damaging (24.3)	Damaging (24.7)
LRT	Deleterious (0)	Deleterious (0)
MetaSVM	Tolerable (-0.79)	Tolerable (-0.773)
MetaLR	Tolerable (0.15)	Tolerable (0.205)
MutationAssessor	Medium (2.175)	Medium (2.58)
VEST3	Damaging (0.977)	Damaging (0.383)
MutationTaster	Disease_causing (1)	Disease_causing (1)
REVEL	Damaging (0.68)	Tolerable (0.119)
GERP++	Conserved (3.7)	Conserved (5.93)
PhastCons	Conserved (1)	Conserved (1)
PhyloP	Conserved (3.827)	Conserved (4.957)
FATHMM	Tolerable (1.31)	Tolerable (0.37)
FATHMM_MKL	Damaging (0.987)	Damaging (0.981)
DANN	Damaging (0.999)	Damaging (0.994)
Eigen	Damaging (0.351)	Damaging (0.664)
Population databases	**Allele frequency**	**Allele frequency**
gnomAD_exome_ALL	-	-
gnomAD_genome_ALL	-	-
ExAC_ALL	-	-
1000genomes_ALL	-	-
ESP6500_ALL	-	-

*All information was obtained from VarCards database (42).

(41). Biallelic mutations of the *CHUK* have been associated with Cocoon syndrome (OMIM#613630), which is fatal in the prenatal period. No clinical report of heterozygous mutations for both gene (*BCL10*/*CHUK*) has been found in the literature. Also, o/e scores for both genes (*CHUK*:0.608-*BCL10*:0.648) reflect that haploinsufficiency may be tolerant. *In silico* predictions were concordant as both variants were not predicted as pathogenic (Table II) (42). We classified both variants as VUS, but reserved judgement as only gain-of-function type variants in these genes can be associated with autoinflammatory symptoms. Also, case II.1 has no other family member diagnosed with FMF for comparison. Healthy mother (II.2) carrying the heterozygous *MEFV* p.K695R mutation did not have *BCL10* or *CHUK* variant. Therefore, functional research is needed to conclude whether these variants are pathogenic or not.

At the end of second step filtering; besides *MEFV* variants, overlapping variants and *de novo* variants, 95 variants remained (Supplementary Table S1). All these variants were classified and searched for potential relationship with Pyrin inflammasome pathway. No such evidence was found (except for CHUK p.Leu354Ile). Genetic counselling was provided where required, but potential clinical consequences of these mutations are irrelevant to the scope of this study. An important point is that 57 of the 95 (60%) variants were filtered because of their association with RhoGTPases and 14-3-3 chaperone proteins. This is a reflection of the large number of interactions these proteins make within the cell. Therefore, it can be predicted that a multitude of targets can alter the Pyrin inflammasome pathway via Rho proteins and 14-3-3 proteins.

There can be several hypotheses explaining why a causal genetic variant in every family was not detected. It is possible that FMF is caused by several different genetic aetiologies in conjunction with *MEFV* mutations. Similarly, these cases might be phenocopies of more than one autoinflammatory disease, independent of *MEFV*. For example, the *de novo BCL10* and *BIRC2* variants might be the sole aetiology of a distinct autoinflammatory phenotype in their respective patients but in order to prove this, identification of new patients without *MEFV* mutations are needed. Our cases do not follow a clear Mendelian inheritance pattern and periodic fever is commonly seen in autoinflammation process. Deep phenotyping is required because to date, studies have not shown any distinguishing features between monoallelic mutant FMF cases and other FMF cases (10, 17). The small number of cases is the most important limitation of this study. If monoallelic FMF cases follow a polygenic/multifactorial inheritance, this study is not suitable to uncover any related variant. Finally, Pyrin inflammasome is in direct interaction with environmental factors, especially infectious agents, as a part of the innate immune system. Considering the geographical distribution of the disease, an environmental agent specific to the Eastern Mediterranean may prompt the symptoms in cases with monoallelic mutations independent from other genetic variants. Various infectious agents such as Clostridium difficile, Vibrio parahaemolyticus, Histophilus somni, and Clostridium botulinum are known to trigger pyrin inflammasome activation (43). Studies of epigenetic factors and environmental/microbiota related

processes that can trigger attacks in monoallelic and biallelic cases will have interesting results.

This study is one the most extensive etiological search conducted in monoallelic mutant FMF cases to date. In the past, only few genes are searched as candidate genes (9, 44). Mainly other autoinflammatory disease genes or pyrin inflammasome pathway genes were investigated. One of the strengths of our study is that no gene filter was put in during bioinformatic analysis. The diagnostic potential of WES is higher than analysis based on a limited number of selected genes, but this approach has limitations. As the target region expands, coverage and depth may decrease. Even though an average of 91.73% coverage at a minimum 50x reading depth was achieved, an important limitation of this study is the possibility of missing overlapping variants due to unread exonic regions. Also, causal variants may reside in genomic regions outside the scope of the research since epigenetic changes, intronic regions and intergenic DNA regions are not covered. Recently, Umar *et al*. performed whole genome sequencing in 50 patients who has zero, single and double mutations in *MEFV* gene (45). With a gene filtering approach (MAF<1%, autoinflammatory disease related genes), they found a heterozygous deletion in exon 11 of *IL1RL1* gene which was present in 9 monoallelic mutant FMF cases. They then validated their results in 184 FMF patients and 218 controls. Same deletion was found in 12%, 21%, 23% of zero, single and double MEFV mutation carriers, respectively. Notwithstanding this important finding, they also interpreted that a single rare variant would not be sufficient to explain the pathophysiology of FMF and a multifactorial model should be investigated (45).

Conclusion

In conclusion, we have shown that genotype-phenotype correlation in these cases may not be established by rare genetic variants and discussed underlying causes. Due to the incomplete understanding of FMF genetics, we recommend that genetic testing should only be used as support for diagnosis. Clinical criteria with emphasis on colchicine response and family history should be the main tool in FMF diagnosis. Clarifying the genetics of FMF, is critical for developing accurate diagnosis, treatment, and follow-up algorithms in the future. It is necessary to collect a large amount of clinical and genetic data and to interpret the information by combining it with modelling systems. We hope that the results of this study will contribute to the existing knowledge in examining genotype-phenotype relationships.

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