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Diagnostic utility of a targeted next-generation sequencing gene panel in the clinical suspicion of systemic autoinflammatory diseases: a multi-center study

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Abstract

Systemic autoinflammatory diseases (sAIDs) are a heterogeneous group of disorders, having monogenic inherited forms with overlapping clinical manifestations. More than half of patients do not carry any pathogenic variant in formerly associated disease genes. Here, we report a cross-sectional study on targeted Next-Generation Sequencing (NGS) screening in patients with suspected sAIDs to determine the diagnostic utility of genetic screening. Fifteen autoinflammation/immune-related genes (*ADA2-CARD14-IL10RA-LPIN2-MEFV-MVK-NLRC4-NLRP12-NLRP3-NOD2-PLCG2-PSTPIP1-SLC29A3-TMEM173-TNFRSF1A*) were used to screen 196 subjects from adult/pediatric clinics, each with an initial clinical suspicion of one or more sAID diagnosis with the exclusion of typical familial Mediterranean fever (FMF) patients. Following the genetic screening, 140 patients (71.4%) were clinically followed-up and re-evaluated. Fifty rare variants in 41 patients (20.9%) were classified as pathogenic or likely pathogenic and 32 of those variants were located on the *MEFV* gene. We detected pathogenic or likely pathogenic variants compatible with the final diagnoses and inheritance patterns in 14/140 (10%) of patients for the following sAIDs: familial Mediterranean fever ($n=7$), deficiency of adenosine deaminase 2 ($n=2$), mevalonate kinase deficiency ($n=2$), Muckle–Wells syndrome ($n=1$), Majeed syndrome ($n=1$), and STING-associated vasculopathy with onset in infancy ($n=1$). Targeted NGS panels have impact on diagnosing rare monogenic sAIDs for a group of patients. We suggest that *MEFV* gene screening should be first-tier genetic testing especially in regions with high carrier rates. Clinical utility of multi-gene testing in sAIDs was as low as expected, but extensive genome-wide familial analyses in combination with exome screening would enlighten additional genetic factors causing disease.

Keywords Hereditary autoinflammatory diseases · *MEFV* gene · Genetic testing · Sequence analysis

Introduction

Systemic autoinflammatory diseases (sAIDs) are characterized by recurrent episodes of inflammation and fever that are driven by impairment of inflammasome in the absence of autoantibody response and microbial infection. The clinical manifestations are variable and include relapsing fever with rash, serositis, lymphadenopathy, arthritis, as well as involvement of muscular and the central nervous systems [1, 2].

The concept of periodic fever and autoinflammatory diseases was first described by McDermott et al. in 1999 [3]. To date, 31 inflammatory-related genes have been highlighted in the Infevers database [4]. The most commonly referred sAIDs are monogenic; familial Mediterranean fever (FMF), TNF receptor-associated periodic fever syndrome (TRAPS), *NLRP3*-associated autoinflammatory disease (*NLRP3*-AID, formerly known as CAPS: cryopyrin-associated periodic

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fever syndrome), mevalonate kinase deficiency (MKD), Blau syndrome, deficiency of adenosine deaminase 2 (DADA2), *PSTPIP1*-associated arthritis, pyoderma gangrenosum and acne (PAPA), STING-associated vasculopathy with onset in infancy (SAVI). Further, frequency of these diseases exhibit geographical differences most likely due to genetic stratification and require exhaustive analysis for the penetrance of variants and clinical significance of the responsible genes. It is known that approximately half of sAID patients do not carry any known pathogenic variants either due to misdiagnosis and/or inadequate gene screening [5–8]. In some cases, nevertheless definitive diagnosis can be more reliable with the aid of genetic data, which also provides a better understanding of the molecular mechanisms and evolving therapeutic interventions.

In this study, we aimed to screen 15 genes using a targeted next-generation sequencing (NGS) panel in suspected sAID patients, further re-evaluating the patients for a definitive diagnosis considering the genetic data to determine the diagnostic utility of the panel.

Patients and methods

Patients

The study investigated 196 patients who had a history of periodic fever. These patients originated from Turkey and were enrolled in the study to identify underlying genetic defects. Patients with symptoms suggestive of a sAID were included in the study and sent for genetic analysis from tertiary pediatric or adult rheumatology clinics based on expert opinion. Patients were referred to the genetic analysis with a clinical pre-diagnosis which included either one or more specific sAIDs, or who were referred as 'unknown sAID' if no specific sAID was suspected. A subgroup of patients was referred for genetic analysis with the pre-diagnosis of 'atypical FMF' which included patients who did not fulfill Tel-Hashomer criteria for adults or Yalçınkaya criteria for pediatric cases [9, 10]. Patients who fulfilled the above diagnostic criteria and had biallelic pathogenic *MEFV* variants were diagnosed as having FMF; therefore, not included in the study. No definite *MEFV* gene-screening method was predefined for the referral centers. After genetic analysis, all clinicians were requested to re-evaluate the patients for a definitive diagnosis.

Methods

DNA extraction

Genomic DNA isolation from blood samples was performed using the DNA Isolation Kit for Mammalian Blood (Roche Diagnostics, Mannheim, Germany) according to manufacturer's protocol in Istanbul Technical University, MOBGAM Laboratories. Concentration and purity of the isolated DNA samples were measured by Nanodrop and the samples were stored at − 20 °C until further use.

Next-generation sequencing

Gene amplicons of the targeted panel were designed through the Ion AmpliSeqTM designer software. Our panel covers coding and UTR regions of 15 genes (*ADA2, CARD14, IL10RA, LPIN2, MEFV, MVK, NLRC4, NLRP12, NLRP3, NOD2, PLCG2, PSTPIP1, SLC29A3, TMEM173, TNFRSF1A*) which are autoinflammation/immune-related. The panel is composed of two multiplexed primer pools (total of 283 amplicons) and requires 20 ng of DNA template for each sample (10 ng per pool). DNA samples were quantified with Qubit dsDNA HS Assay Kit (Life Technologies) to 10 ng in maximum of 6 µl volume. According to manufacturer's protocols, 10 ng of DNA for each sample was used for library preparation of per amplicon pool with the custom Ion AmpliSeq Panel and the Ion AmpliSeq Library Kit 2.0-96LV. The amplicons were ligated to adapters with the barcodes of the Ion Xpress Barcode Adapters Kit. Barcoded libraries were purified using Agencourt AMPure XP reagent (Beckman Coulter, CA) and combined to a final concentration of 8 pM. Template preparation by emulsion PCR (emPCR) was performed on the Ion OneTouch 2 system. Template-positive Ion Sphere Particles (ISP) were enriched using Ion OneTouch ES system. Sequencing primer and polymerase were added to the final enriched ISPs prior to loading onto Ion 520 and 530 chips. Sequencing was carried out using 400 bp kit on the Ion S5 system.

Bioinformatic analysis

Bioinformatic processing of sequenced samples was performed by Torrent Suite Software 5.4.0 (Life Technologies). After alignment to the hg19 human reference genome, variant calling was performed by the Ion Torrent Variant Caller Plugin v5.4.0.46 using default germline variant detection parameters. Coverage of each amplicon was determined using Coverage Analysis Plugin v5.4.0.5. The output of Torrent Suite pipeline, which is a raw VCF file, was annotated using Annovar [11]. After variant annotation, filtering and statistical analysis were accomplished using in-house analysis scripts written in Python for this project. We first filtered out called variants which have read depths < 50 and variant allele frequencies < 0.25. Then all detected variants were manually reviewed from processed BAM files for sequencing errors such as false-positive calls near homopolymer or repeat regions and those variants were excluded from the analysis. Transcripts used for variant annotation and nomenclature were as follows: *ADA2* (NM_001282225), *CARD14* (NM_024110.3),

IL10RA (NM_001558.3), *LPIN2* (NM_014646.2), *MEFV* (NM_000243.2), *MVK* (NM_000431.2), *NLRC4* (NM_021209), *NLRP12* (NM_144687.2), *NLRP3* (NM_004895.4), *NOD2* (NM_022162.1), *PLCG2* (NM_002661.2), *PSTPIP1* (NM_003978.3), *SLC29A3* (NM_018344.5), *TMEM173* (NM_198282.2), *TNFRSF1A* (NM_001065.3).

Variant classification

Variant pathogenicity assessment was performed mainly according to variant's population frequency and classifications in public databases such as ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), HGMD (http://www.hgmd.cf.ac.uk), and InFevers (https://infevers.umai-montpellier.fr/). First, synonymous and intronic variants were filtered out. Secondly, we excluded common variants since most of rare monogenic diseases are caused by rare variants. Variants with 1% or higher population frequency among all 1000G samples were considered to be common. Identified rare variants were individually evaluated and classified in pathogenicity groups: pathogenic, likely pathogenic, variant of unknown significance (VUS), likely benign, and benign. If classifications in the databases were in consensus for benign or pathogenic, we classified as benign or pathogenic, respectively. When the databases were in conflict including uncertainty alongside with benign or pathogenic entries, we classified them as likely benign or likely pathogenic, respectively. If there is a conflict on classifications among the databases, we classified as VUS. Additionally, protein truncating variants which were not classified as benign in databases before, were classified as likely pathogenic in this study. We further assessed genotype–phenotype association using pathogenic, likely pathogenic, and VUS variants, but not likely benign or benign variants. All variants located in UTR regions were classified as VUS if they were not reported oppositely in public databases, due to lack of information on disease pathogenesis.

Statistical analysis

Descriptive statistics were used to analyze the data. Proportions and percentages were used for the categorical data and mean values and standard deviations were used for the continuous data. Statistical significance of pathogenic or likely pathogenic variant carrier rate difference among the patient groups was calculated by Fisher's exact test. A value of $p < 0.05$ was considered statistically significant.

Results

A total of 196 unrelated patients (112 female and 84 male) were enrolled from eight tertiary adult or pediatric rheumatology clinics across Turkey. The study included 72 adult and 124 pediatric patients. Mean ages of the pediatric and adult patients were 8.1 ± 3.8 and 36.7 ± 12.4, respectively. Blood samples were obtained for genetic analysis which included 156 patients with a probable clinical diagnosis of one or more specific sAID (79.6%), 31 patients with 'unknown sAID' (15.8%), and nine with 'atypical FMF' (4.6%). In 44 (35.5%) of the pediatric and 12 (16.7%) of the adult patients, more than one sAID was suspected ($p = 0.005$). The distribution and frequency of the suspected diagnoses of patients sent for genetic screening were as follows: CAPS (62/196, 31%), MKD (59/196, 30%), TRAPS (46/196, 23%), DADA2 (31/196, 16%), and unknown sAID (31/196, 16%) (Fig. 1). The remaining included probable diagnoses with a referral frequency below 10%.

Among the 196 patients, 58 did not to carry any rare variants (29.6%), 41 had at least one pathogenic or likely pathogenic variants (20.9%), and the remaining 97 had at least one rare variant classified as benign, likely benign or VUS (49.5%). Rare variants excluding benign or likely benign variants of those 41 patients were given in Table 1. Forty-one patients with 50 rare variants were classified as

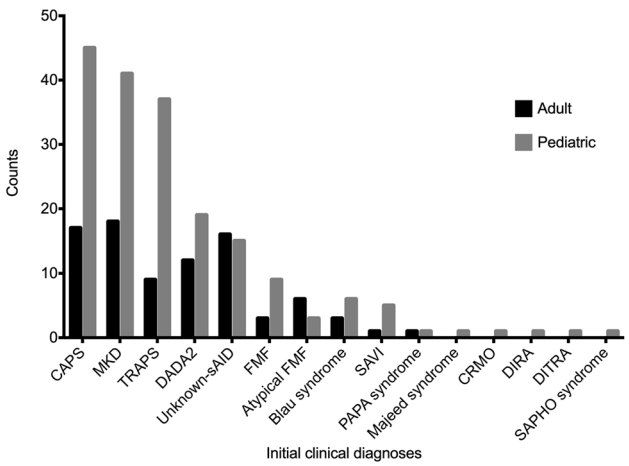

Fig. 1 Distribution of suspected clinical diagnoses in initial forms of patients with systemic autoinflammatory diseases (*CAPS* cryopyrin-associated periodic fever syndrome, *CRMO* chronic recurrent multifocal osteomyelitis, *DADA2* deficiency of adenosine deaminase 2, *DIRA* deficiency of the interleukin-1-receptor antagonist, *DITRA* deficiency of interleukin 36–receptor antagonist, *FMF* familial Mediterranean fever, *MKD* mevalonate kinase deficiency, *PAPA PSTPIP1*-associated arthritis, pyoderma gangrenosum and acne, *sAID* systemic autoinflammatory disease, *SAPHO* synovitis–acne–pustulosis–hyperostosis–osteitis, *SAVI* STING-associated vasculopathy with onset in infancy, *TRAPS* TNF receptor-associated periodic fever syndrome)

pathogenic or likely pathogenic according to their population frequency and classification of public databases such as ClinVar, HGMD, and InFevers. Those variants, all exonic, belong to ten genes: *ADA2* ($n=2$), *CARD14* ($n=1$), *LPIN2* ($n=1$), *MEFV* ($n=32$), *MVK* ($n=5$), *NLRC4* ($n=2$), *NLRP3* ($n=1$), *PSTPIP1* ($n=2$), *SLC29A3* ($n=3$), and *TMEM173* ($n=1$). The distribution of these 41 patients according to their referral categories was as follows: 33 belonged to category one (specific one or more sAID), 5 to category two (unknown sAID), and 3 to category three (atypical FMF). Thirteen of the 41 cases were enrolled from adult and the remaining 28 patients from pediatric rheumatology clinics. Even though the pathogenic variant carrier rate in the adult group (26.4%) was higher than in the pediatric group (17.7%), the difference was not statistically significant.

After the genetic screening, 140 patients (71.4%) were reassessed by clinicians for a definitive diagnosis. Among these 140 patients, 64 carried either no mutation or had rare, benign/likely benign variants (45.7%). Consistency among the genetic findings, inheritance patterns and final diagnosis was achieved in 14 patients (10%). The distribution of the responsible genes in this patient group was as follows: *ADA2* ($n=2$), *LPIN2* ($n=1$), *MEFV* ($n=7$), *MVK* ($n=2$), *NLRP3* ($n=1$), and *TMEM173* ($n=1$). There were seven patients who carried biallelic pathogenic variants in *MEFV* gene suggesting recessive inheritance and final diagnosis of FMF. All of these seven patients were from the first category with a clinical suspicion of one or more specific sAID. At referral for genetic analysis, probable diagnoses of these patients were FMF/MKD ($n=2$), CAPS ($n=2$), CAPS/MKD ($n=1$), DADA2/MKD/TRAPS ($n=1$), CRMO/PAPA ($n=1$). In this group, sAIDs other than FMF were considered initially, mainly because of the phenotypes that were compatible with well described sAIDs.

Regarding performance characteristics of the NGS panel, the final AmpliSeq panel design consists of 283 amplicons (125–375 bp) to cover 99.24% of 15 target genes. Total panel size was 74.35 kb. An average of 295,627 mapped reads per sample were produced of which 91% were on target. Average of Q20 bases per sequenced sample was 65.4 million bp which yielded mean depth of 886x. More than 95% of all amplicons were sequenced at least 50x depth. Missing regions in the panel design were given in Supplementary table 1 and low coverage regions in Supplementary table 2.

Discussion

Systemic autoinflammatory diseases are a heterogeneous group of disorders with both clinical and genetic heterogeneity. Besides rare monogenic diseases, vast majority of patients' clinical findings suggest multifactorial involvement. Recent improvements in sAID diagnosis have been partially achieved through genetic testing. In this study, 15 genes in 196 patients with sAID manifestations were sequenced. After genetic screening, 140 patients (71.4%) were reassessed by clinicians for a definitive diagnosis. Concordance was obtained among genetic results, inheritance patterns, and final definitive diagnosis only in 14 patients (10%).

The availability of high throughput sequencing allows to screen more than one gene in a timely manner. In a heterogeneous group of disorders, especially when diagnostic challenges are obvious, multigene screening assays are expected to be quite helpful. Recently, a few NGS-based panel screenings in patients with autoinflammatory syndromes have been published, but differed with regards to patient selection criteria, targeted genes, and study design. Omoyinmi et al. studied 50 prospective autoinflammatory and vasculitis patients using two tier of sequencing panels targeting 113 and 166 genes. They detected pathogenic or likely pathogenic variants in 32% of the patients [6], while this ratio was 20.9% in our cohort. Although there is no remarkable difference between the two ratios, Omoyinmi et al. focused on a wider disease and gene spectra. In addition, we further re-evaluated the patients for a definitive diagnosis considering the genetic data which yielded a final of 10% of consistency between clinical and genetic data. This finding appears to contradict with the generally accepted genotype-phenotype correlation of 50% in sAID patients [5]. However, it is important to note that the previous targeted gene panel screenings are quite different in terms of study design here which includes many centers, excludes common and/or low penetrance disease associated variants, and evaluates patients before and after the genetic analysis.

We detected pathogenic variants in 41 patients independently from clinical concordance. Thirty patients carried at least one *MEFV* pathogenic or likely pathogenic allele. Eight of them were biallelic and helped to conclude an *MEFV* gene contribution in a recessive manner in seven of the patients with a definitive diagnosis of FMF. All of these eight patients belonged to category one, which included referrals for genetic analysis with the suspicion of one or more well described sAIDs other than FMF. None of the patients referred under other two categories of unknown sAID and atypical FMF were biallelic for *MEFV*. Except one patient (patient 80, definitive diagnosis was SAVI), the definitive diagnosis of seven patients were finalized as FMF. This result indicates that biallelic pathogenic variants in *MEFV* gene have an important role in FMF symptoms, but patients' manifestations may evoke to misdiagnose with other syndromes during initial examinations without genetic analysis.

Two patients among the biallelic pathogenic *MEFV* variant carriers, were initially reported as *MEFV*-negative and suspected of FMF or MKD. These patients had atypical clinical presentations such as presence of rashes, prolonged

Table 1 Systemic autoinflammatory disease patients with at least one pathogenic or likely pathogenic variants

Patient	Sex	Age	Group	Initial diagnosis	Definitive diagnosis	Variants			
						Gene	Variant	Classification	Status
66	F	6	Pediatric	CAPS, TRAPS	JIA	*ADA2*	c.-31A > G	VUS (UTR)	Het
						MEFV	c.C704T (p.S235L)	VUS	Het
						MEFV	c.T2177C (p.V726A)	Pathogenic	Het
80	F	6	Pediatric	DADA2, SAVI	SAVI	*MEFV*	c.T2177C (p.V726A)	Pathogenic	Hom
						MVK	c.*571G > A	VUS (UTR)	Het
121[b]	M	2	Pediatric	CAPS, MKD	MKD	*MVK*	c.G1129A (p.V377I)	Pathogenic	Het
						MVK	c.G431T (p.G144V)	Pathogenic	Het
						PLCG2	c.*35T > C	VUS (UTR)	Het
133[b]	M	15	Pediatric	Blau syndrome, DADA2	DADA2	*ADA2*	c.G139A (p.G47R)	Pathogenic	Hom
136	M	53	Adult	CAPS	NA[a]	*MEFV*	c.A443T (p.E148V)	Likely pathogenic	Het
						NLRP3	c.A617G (p.E206G)	VUS	Het
145[b]	F	12	Pediatric	DADA2	SAVI	*TMEM173*	c.G842A (p.R281Q)	Pathogenic	Het
151	F	18	Adult	SAVI	Lupus	*MEFV*	c.T2177C (p.V726A)	Pathogenic	Het
185	M	5	Pediatric	DADA2	FMF, BD	*IL10RA*	c.T1209G (p.I403M)	VUS	Het
						MEFV	c.A2080G (p.M694V)	Pathogenic	Het
186[b]	M	25	Adult	DADA2	MKD	*MEFV*	c.G2230T (p.A744S)	Pathogenic	Het
						MVK	c.G52A (p.G18R)	Likely pathogenic	Het
						MVK	c.G1129A (p.V377I)	Pathogenic	Het
						NLRP3	c.G598A (p.V200M)	VUS	Het
187	F	16	Pediatric	DADA2	PAN	*MEFV*	c.A2080G (p.M694V)	Pathogenic	Het
189	F	4	Pediatric	Unknown sAID	PFAPA syndrome	*CARD14*	c.G646A (p.A216T)	VUS	Het
						MVK	c.G1129A (p.V377I)	Pathogenic	Het
						NLRP3	c.A2182G (p.S728G)	VUS	Het
191	M	12	Pediatric	CAPS	JIA	*MEFV*	c.A2084G (p.K695R)	Pathogenic	Het
197[b]	F	2	Pediatric	CAPS, MKD	FMF, JIA	*CARD14*	c.1530dupG (p.P510fs)	Likely pathogenic	Het
						MEFV	c.A2080G (p.M694V)	Pathogenic	Hom
						NLRP12	c.A2191C (p.K731Q)	VUS	Het
205[b]	F	12	Pediatric	FMF, MKD	FMF	*CARD14*	c.C1091T (p.A364V)	VUS	Het
						IL10RA	c.G706A (p.V236I)	VUS	Het
						MEFV	c.A2080G (p.M694V)	Pathogenic	Hom
206[b]	F	3	Pediatric	CAPS	FMF	*ADA2*	c.*159G > A	VUS (UTR)	Het
						MEFV	c.A2080G (p.M694V)	Pathogenic	Hom
208	F	2	Pediatric	CAPS, MKD	FMF	*MEFV*	c.G2040C (p.M680I)	Pathogenic	Het
209	F	24	Adult	Unknown sAID	CAPS	*MEFV*	c.A2080G (p.M694V)	Pathogenic	Het
218	M	37	Adult	Unknown sAID	MKD	*MEFV*	c.A2084G (p.K695R)	Pathogenic	Het
236[b]	M	10	Pediatric	DADA2	DADA2	*ADA2*	c.G139A (p.G47R)	Pathogenic	Hom
						PLCG2	c.A2393G (p.N798S)	VUS	Het
251	M	8	Pediatric	CAPS, TRAPS	NA[a]	*NLRC4*	c.C928T p.R310X	Likely pathogenic	Het
						SLC29A3	c.C38A p.S13X	Likely pathogenic	Het
						MVK	c.G538A p.E180K	VUS	Het
263	M	47	Adult	DADA2	FMF	*MEFV*	c.A2080G (p.M694V)	Pathogenic	Het
269	M	31	Adult	Atypical FMF	NA[a]	*MEFV*	c.A2084G (p.K695R)	Pathogenic	Het
272[b]	M	7	Pediatric	CAPS, TRAPS	MWS	*NLRP3*	c.G2425A (p.A809T)	Likely pathogenic	Het
						SLC29A3	c.G1339A (p.E447K)	Pathogenic	Hom
275	M	12	Pediatric	ADA2, SAVI	Vasculitis	*MEFV*	c.G2040C (p.M680I)	Pathogenic	Het
291	M	9	Pediatric	CAPS	NA[a]	*MEFV*	c.G2282A (p.R761H)	Pathogenic	Het
297	M	34	Adult	MKD, TRAPS	NA[a]	*MEFV*	c.G2282A (p.R761H)	Pathogenic	Het

Table 1 (continued)

Patient	Sex	Age	Group	Initial diagnosis	Definitive diagnosis	Variants			
						Gene	Variant	Classification	Status
300[b]	F	3	Pediatric	FMF, MKD	FMF	*MEFV*	c.A2080G (p.M694V)	Pathogenic	Hom
						NOD2	c.A2909G (p.E970G)	VUS	Het
307[b]	F	9	Pediatric	DADA2, MKD, TRAPS	FMF, JIA	*MEFV*	c.A2080G (p.M694V)	Pathogenic	Hom
316	M	5	Pediatric	CAPS	CAPS	*ADA2*	c.A1213T (p.I405L)	VUS	Het
						MEFV	c.T2177C (p.V726A)	Pathogenic	Het
331[b]	M	12	Pediatric	CAPS	FMF	*CARD14*	c.G452A (p.R151Q)	VUS	Het
						MEFV	c.T2177C (p.V726A)	Pathogenic	Het
						MEFV	c.A2080G (p.M694V)	Pathogenic	Het
334	M	27	Adult	Atypical FMF	FMF	*NLRC4*	c,G283T p.E95X	Likely pathogenic	Het
						NLRC4	c.T847G p.C283G	VUS	Het
339[b]	M	9	Pediatric	Unknown sAID	Majeed syndrome	*LPIN2*	c.1456_1456del p.E486fs	Likely pathogenic	Hom
						NOD2	c.C2031G p.F677L	VUS	Het
344	F	59	Adult	Blau syndrome, FMF	FMF	*PSTPIP1*	c.C682T (p.R228C)	Pathogenic	Het
345	F	10	Pediatric	MKD	FMF	*PSTPIP1*	c.C682T (p.R228C)	Pathogenic	Het
						SLC29A3	c.G688A (p.A230T)	VUS	Het
347	M	11	Pediatric	MKD	FMF	*MEFV*	c.G2282A (p.R761H)	Pathogenic	Het
						NOD2	c.G1277A (p.R426H)	VUS	Het
						PLCG2	c.*35T > C	VUS (UTR)	Het
349	M	31	Adult	CAPS, MKD	FMF	*MEFV*	c.A2084G (p.K695R)	Pathogenic	Het
363[b]	M	8	Pediatric	CRMO, PAPA syndrome	FMF	*MEFV*	c.A2080G (p.M694V)	Pathogenic	Het
						MEFV	c.G2040C (p.M680I)	Pathogenic	Het
365	M	39	Adult	DADA2	Vasculitis	*MEFV*	c.A2080G (p.M694V)	Pathogenic	Het
						NLRP12	c.G154A (p.G52S)	VUS	Het
380	F	8	Pediatric	MKD	FMF	*MEFV*	c.A2080G (p.M694V)	Pathogenic	Het
384	F	52	Adult	Atypical FMF	NA[a]	*MEFV*	c.A2080G (p.M694V)	Pathogenic	Het
						SLC29A3	c.118delC (p.P40fs)	Likely pathogenic	Het
389	F	9	Pediatric	Unknown sAID	NA[a]	*MEFV*	c.G2082A (p.M694I)	Pathogenic	Het

BD Behcet's disease, *CAPS* cryopyrin-associated periodic fever syndrome, *CRMO* chronic recurrent multifocal osteomyelitis, *DADA2* deficiency of adenosine deaminase 2, *F* female, *FMF* familial Mediterranean fever, *Het* heterozygous, *Hom* homozygous, *JIA* juvenile idiopathic arthritis, *M* male, *MKD* mevalonate kinase deficiency, *MWS* Muckle–Wells syndrome, *NA* not available, *PAN* polyarteritis nodosa, *PAPA PSTPIP1*-associated arthritis, pyoderma gangrenosum and acne, PFAPA periodic fever with aphthous stomatitis pharyngitis, cervical adenitis, *sAID* systemic autoinflammatory disease, *SAVI* STING-associated vasculopathy with onset in infancy, *TRAPS* TNF receptor-associated periodic fever syndrome, *UTR* untranslated region, *VUS* variant of unknown significance

[a]Clinical follow-up and definitive diagnosis could not be achieved for those patients

[b]Patients with pathogenic/likely pathogenic variants compatible with final diagnosis

fever, and unresponsiveness to colchicine. Although those patients and other biallelic pathogenic *MEFV* variant carriers were reported as *MEFV*-negative, we relied on the genetic test results by the referring clinician. Some of these test results were later found to be false negative and this may be due to the methodology of mutation analysis with relatively low sensitivity and specificity, such as strip assays. It has been well documented that FMF carrier rate is very high in the Mediterranean and Middle East regions including Turkey, Armenia, Israel, etc. Total *MEFV* pathogenic allele frequency was 9.7% (38/392 alleles) in the studied cohort. Excluding patients with biallelic *MEFV* pathogenic variants, mostly presenting clinical FMF manifestations, the cohort includes 22 heterozygous *MEFV* pathogenic variant carriers with a pathogenic allele frequency of 5.8% (22/376 alleles). To this end, when the patients with biallelic pathogenic *MEFV* variants were excluded, the carrier rate was found to be as high as expected ($p > 0.05$) in a healthy control population originated from Turkey; around 5–9% [12–16]. Dominant or digenic inheritance had been rarely reported in recessive diseases such as FMF with atypical presentations [17–21]. *MEFV* gene contribution in sAID manifestations could not be elucidated in the study, since *MEFV* pathogenic variant carrier rate was around the healthy

population frequency in Turkey. Although possible *MEFV* gene contribution to other sAIDs findings still remained unknown, it is wise to use *MEFV* gene screening as a first-tier genetic diagnostic test not for only FMF cases, but also in other sAID patients manifesting atypical FMF symptoms.

NGS panel screening in sAID patients yielded higher diagnostic rate compared to Sanger sequencing of selected exons in related genes in our laboratory (data not shown) possibly due to pathogenic variants mostly located outside of suspected genes. Previous gene screening studies using both Sanger and NGS methods, revealed that detection rate of pathogenic variants explaining clinical manifestations was very low [6, 22]. Regardless of sequencing technique and coverage, diagnostic rates of genetic screening in sAIDs are not sufficient and this indicates that involvement of additional genes may play a role on pathogenesis of sAIDs. Extensive phenotyping in those heterogeneous group of disorders may help to recognize different syndromes, caused by defects of known or novel genes. XXXX et al. have previously reported two families with atypical FMF findings without *MEFV* gene involvement. Detailed genome-wide familial analyses let us reach a true definitive diagnosis [23]. Genome-wide screening of families with unusual clinical findings not only help to improve diagnosis or orient the treatment but also expands the clinical spectrum of diseases.

Russo and Brogan reported that approximately 50% of patients with sAID do not have a known genetic cause of the disease [5]. sAIDs are clinically heterogeneous diseases and phenotypic manifestations can be the result of each patient's genomic architecture differences, as can be caused by ancestral differences. Most of the time, known defects in causative genes manifest similar phenotypes and Ben-Chetrit et al. proposed a new nomenclature for AIDs based on primarily their known genetic cause where appropriate [24]. This approach is very useful for most of the monogenic sAIDs but there are still some sAID patients/families whose diagnosis could not be established. Diagnostic characteristics are usually derived from patients who were first identified in these heterogeneous syndromes which sometimes manifest differently based on geography or other environmental parameters. Detailed clinical and genetic evaluation for each disease may unveil the complete spectrum and provide more effective and rapid evaluation of diagnosis and treatment options.

Advanced genetic testing is widely used for diagnostic purposes in autoinflammatory diseases and has substantial contributions for diagnostic purposes. In consistence with previous findings, we report pathogenic variants only in 20.9% of the patients, the half of which did not correspond with the expected monogenic inheritance pattern. We concluded that patients with sAID manifestations and originating from a region with high carrier rate for FMF, should first be screened for *MEFV* gene, preferably using a low-cost but gold standard technique such as Sanger sequencing. Any patients or families without known genetic defects would benefit from extensive genome-wide analysis methods such as whole exome or genome sequencing.

Limitations of the study

One of the major limitations of this study is the need for better standardization of the study population. Another limitation is that all variants are not verified by Sanger sequencing and familial segregation is not tested which could have led to better classification of such "unclassified" VUS alleles. Novel missense variants are not classified as pathogenic or likely pathogenic unless any pathogenicity information was present in related databases. Another limitation is the possibility that the variants located in uncovered genes such as *TNFAIP3* gene, mutations of which cause A20 haploinsufficiency, regions with low coverage, or deep intronic regions > 20 bp away from exon junctions may have been missed. Also, heterozygous exonic deletions could not be detected reliably using amplicon sequencing approach. The allele frequency of 0.25 that we used to call heterozygous variants, may have caused to miss any mosaicism under this threshold.

Author contributions İK: analysis and interpretation of the genomic data, drafting the work and critically revising the final version of the study. AB: laboratory genome analysis of the sample, drafting the work, checked the accuracy of the genotyping in line with phenotypic data. SU: contributed samples to the study, analysed the genotype–phenotype correlations and revised the study. AKA: laboratory genome analysis of the sample, drafting the work, checked the accuracy of the genotyping in line with phenotypic data. EE: analysis and interpretation of the genomic data, drafting the work. SZ: analysis and interpretation of the genomic data, drafting the work. MÖÖ: laboratory genome analysis of the sample, drafting the work, checked the accuracy of the genotyping in line with phenotypic data. SD: contributed genome analysis and drafting the work. OÖ: contributed samples to the study, analysed the genotype–phenotype correlations and revise the study. BS: contribute samples to the study, analysed the genotype–phenotype correlations and revised the study. AT: contributed samples to the study, analysed the genotype–phenotype correlations and critically revised the study. DGY: contributed samples to the study, analysed the genotype–phenotype correlations and revised the study. SY: contributed samples to the study, analysed the genotype–phenotype correlations and revised the study. NAA: contributed samples to the study, analysed the genotype–phenotype correlations and revised the study. RE: contributes samples to the study, analysed the genotype–phenotype correlations and revised the study. KÖ: contributed samples to the study, analysed the genotype–phenotype correlations and revised the study. MÇ: contribute samples to the study, analysed the genotype–phenotype correlations and revised the study. OS: contribute samples to the study, analysed the genotype–phenotype correlations and revised the study. SŞ: contributed samples to the study, analysed the genotype–phenotype correlations and revised the study. KB: contribute samples to the study, analysed the genotype–phenotype correlations and revised the study. AA: contributed samples to the study, analysed the genotype–phenotype correlations

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This study was approved by the Ethics Review Committee of XXXXXXX (approval number and dates: B.30.2.İST.0.30.90.00/19756, 3 July 2012 and 83045809-604.01.02, 6 December 2016). This article does not contain any studies with animals performed by any of the authors.

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