RESEARCH ARTICLE

Evidence of digenic inheritance in autoinflammation-associated genes

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Abstract

Familial Mediterranean fever (FMF) has traditionally been considered as a monogenic autosomal recessive disorder caused by mutations in the *MEFV* gene with highest incidence among Mediterranean populations. In a considerable number of patients with typical FMF, only one *MEFV* mutation was identified and the possibility that more than one autoinflammatory gene may be responsible for their disease was investigated. In the present study, an extensive search for possible mutations in three hereditary recurrent fever (*HRF*) genes was performed in 128 *MEFV* heterozygous Greek–Cypriots clinically diagnosed based on their phenotype with FMF-like disease from a previous study. Sequence analysis was performed for *MVK*, *TNFRSF1A* and *NLRP3* genes which is also known to cause HRFs. In total, three patients were identified with heterozygous mutations and a second mutation in an autoinflammatory gene. Two patients carried a *MEFV* mutation and a *NLRP3* mutation, and an additional third carried a *MEFV* mutation and a *TNFRSF1A* mutation. Patient 1 carried *MEFV* p.[Val726Ala] (NM_000243.2:c.2177T>C) and *NLRP3* p.[Val198Met] (NM_001243133.1:c.592G>A) variants and patient 2 carried *MEFV* p.[Glu148Gln] (NM_000243.2:c.442G>C) variant which is of uncertain significance and *NLRP3* p.[Arg176Trp] (NM_001243133.1:c.526C>T). Lastly, patient 3 was identified to carry *MEFV* p.[Met694Val] (NM_000243.2:c.2080A>G) and *TNFRSF1A* p.[Arg121Gln] (NM_001065.3:c.362G>A) variants. The results from this study indicate that screening of genes known to cause HRFs in patients already identified with a single *MEFV* mutation, can reveal quite rare but potentially causative mutational combinations at different loci. Such interaction provide further evidence for possible locus–locus interactions and phenotypes resulting from digenic inheritance.

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Introduction

Hereditary recurrent fevers (HRFs) are an emerging group of autoinflammatory disorders and till now several genes have been identified as potentially causal (Horneff 2015). There is difficulty in establishing stringent clinical criteria for the diagnosis of patients affected by HRFs as the symptoms may be nonspecific, generally manifesting as recurrent fever episodes usually accompanied by a number of clinical features such as rash, serositis, lymphadenopathy and arthritis (Federici *et al.* 2012, 2015; Horneff 2015). The best characterized HRFs are the two recessively inherited diseases: familial Mediterranean fever (FMF) (MIM 249100) and hyper-IgD/mevalonate kinase deficiency syndrome (HIDS/MKD) (MIM 610377 and 260920), and two dominantly inherited diseases, the tumour necrosis factor receptor-associated periodic syndrome (TRAPS) (MIM 142680) and the cryopyrin-associated periodic syndrome (CAPS, FCAS 120100, MWS 191900 and CINCA 607115) (Shinar *et al*. 2012).

Mutations in the *MEFV* gene have been reported among individuals of Mediterranean origin and particularly in non-Ashkenazi Jews, Armenians, north Africans, Arabs, Greeks, Cypriots and Turks, and are known to be implicated

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in the development of FMF (Konstantopoulos *et al.* 2003; La Regina *et al.* 2003; Lidar and Livneh 2007; Neocleous *et al.* 2015). Mutations in the *MVK* gene causing MKD are very rare and the disorder is mostly found among individuals in the Netherlands and other northern European countries (Bader-Meunier *et al.* 2011; Vuch *et al.* 2013). Mutations in the *TNFRSF1A* and *NLRP3* genes have been reported in individuals with TRAPS and CAPS syndromes, respectively (De Pieri *et al.* 2015).

More than 800 variants have been identified and reported in the Infevers database (http://fmf.igh.cnrs.fr/ISSAID/infevers/) since the identification of these four HRF causative genes, (Sarrauste de Menthière *et al.* 2003; Touitou *et al.* 2004; Milhavet *et al.* 2008). Several of these variants appear to be causative but most are unconfirmed or seemingly non-pathogenic (Shinar *et al.* 2012). A considerable number of Mediterranean ancestry patients clinically diagnosed with HRF have been found to carry only a single mutation in the *MEFV* gene despite extensive analysis for a second pathogenic mutation in the coding and regulatory region of the gene. Therefore, it is still uncertainty as how patients with a single *MEFV* mutation develop the disease (Booty *et al.* 2009; Marek-Yagel *et al.* 2009; Jeru *et al.* 2013; Soylemezoglu *et al.* 2015).

Additionally, several recent studies have reported the coexistence of *MEFV* haplotype variants along with *NLRP3*, *TNFRSF1A* and *MVK* genes that determined possible synergistic effects with a *MEFV* heterozygote haplotype (Kubota *et al.* 2013; Mereuta *et al.* 2013; Timerman and Frank 2013; Moussa *et al.* 2015). Finally, there is always the possibility that some *MEFV* gene mutations may act in a dominant fashion, thus being solely responsible for the cause of the disease (Booth *et al.* 2000; Aldea *et al.* 2004; Caglar *et al.* 2008; Marek-Yagel *et al.* 2009; Stoffels *et al.* 2014).

Since the genetic basis of heterozygous *MEFV* gene patients is still uncertain, the aim of the present study was to screen a large cohort of FMF-like patients who carried a single *MEFV* mutation for *MVK*, *TNFRSF1A* and *NLRP3* mutations. Since patients with HRF frequently exhibit comparable inflammatory symptoms, the potential synergistic effect of variants in alternative hereditary autoinflammatory genes was exploited.

Materials and methods

Patients

A total of 128 unrelated patients (59 males and 69 females) with FMF or FMF-like symptoms were referred to the Cyprus Institute of Neurology and Genetics (Neocleous *et al.* 2015). The diagnosis of FMF was made after clinical suspicion based on the Tel-Hashomer criteria and the patients were characterized based on the recurrent self-limiting episodes of fever and serositis that appeared for every few weeks to months or years (Pras 1998).

Amplification and direct sequencing of MEFV, MVK, TNFRSF1A and NLRP3 genes

The genetic investigation of the present study using genomic DNA isolated from peripheral blood samples was performed in accordance with the latest guidelines for the genetic diagnosis of HRFs by Shinar *et al.* (2012). DNA sequencing was performed in exons 2, 3, 5 and 10 of *MEFV* (ENSG0000010 3313), whole coding sequence of *MVK* (ENSG00000110921), in exons 2, 3 and 4 of *TNFRSF1A* (ENSG00000067182) and exon 3 of the *NLRP3* (ENSG00000162711) genes. The PCR conditions and primers used for the amplification of *MEFV*, *MVK*, *TNFRSF1A* and *NLRP3* genes are available upon request. PCR amplification was carried out using BigDye Terminator v1.1, cycle sequencing kit (Applied Biosystems, Foster City, USA). Amplification products were run on an automated Applied Biosystems 3130xl Genetic Analyzer. This study has been approved by the Cyprus National Bioethics Committee and informed consent was obtained from all patients who participated in this study.

Results

The spectrum and frequency of *MEFV* gene defects in the cohort of 128 heterozygote Cypriot patients with clinical suspicion of FMF or FMF-like disease is depicted in table 1. The most frequent defect among the 128 Cypriot identified alleles was p.[Val726Ala] (25.8%) followed by p.[Glu148Gln] (25.0%), p.[Met694Val] (13.3%), p.[Met694Ile] (10.9%), the complex allele p.[Phe479Leu(;)Glu167Asp] (10.15%) and p.[Met680Ile] (7.0%). The missense mutations p.[Arg761His] (3.9%) and p.[Ala744Ser] (3.9%) were identified as the rarest (table 1).

In the present study, an extensive screen for possible locus–locus interactions was performed in 128 *MEFV* heterozygous Greek–Cypriots clinically diagnosed with FMF. Sequence analysis of the *MVK*, *TNFRSF1A* and *NLRP3* genes was performed. These genes were selected as they are also known to cause HRF. A total of three patients were identified with heterozygous mutations in two separate HRF loci

Table 1. Types and frequency of molecular *MEFV* defects in the cohort of 128 Cypriot HRF patients.

Heterozygotes in *MEFV* gene	No. of HRF patients with *MEFV* defects	Per cent
p.[Val726Ala];[=]	33	25.8
p.[Met694Val];[=]	17	13.3
p.[Met694Ile];[=]	14	10.9
p.[Arg761His];[=]	5	3.9
p.[Met680Ile];[=]	9	7.0
p.[Glu148Gln];[=]	32	25.0
*p.[Phe479Leu(;)Glu167Asp];[=]	13	10.15
p.[Ala744Ser];[=]	5	3.9
Total	128	100

*p.[Phe479Leu(;)Glu167Asp] is known to be coinherited.

and the possibility of DI explaining their clinical presentation was speculated. Two patients were identified with a *MEFV* and a *NLRP3* mutation and a third patient with a *MEFV* and a *TNFRSF1A* mutation.

Patient 1, a 21-year-old Greek–Cypriot woman, who carried the *MEFV* p.[Val726Ala] and *NLRP3* p.[Val198Met] mutations was initially evaluated due to frequent episodes of abdominal pain with no associated pyrexial episodes (figure 1a; table 2). The first episode was reported at the age of 3.5 years and was characterized by intense abdominal pain, vomiting, adenopathy and urticaria. She had no accompanying fever or any other signs of viral or bacterial infection. She had lower abdominal tenderness and elevated inflammatory markers. She was treated with intravenous fluids and was discharged when symptoms resolved. Between the age 3.5 and 6 years she had cyclical (2–3 monthly) similar episodes of abdominal pain with vomiting, with or without urticaria but again with no fever. At the age of 4.5 years, she underwent a tonsillectomy and at the age of 6 years, an appendectomy for acute abdominal pain and lower diffuse lower abdominal tenderness. In her adolescence and later she had no recurrent episodes apart from lower abdominal pains especially during menstruation. She was never treated with colchicine and by far, she has not developed any systemic complications associated with FMF.

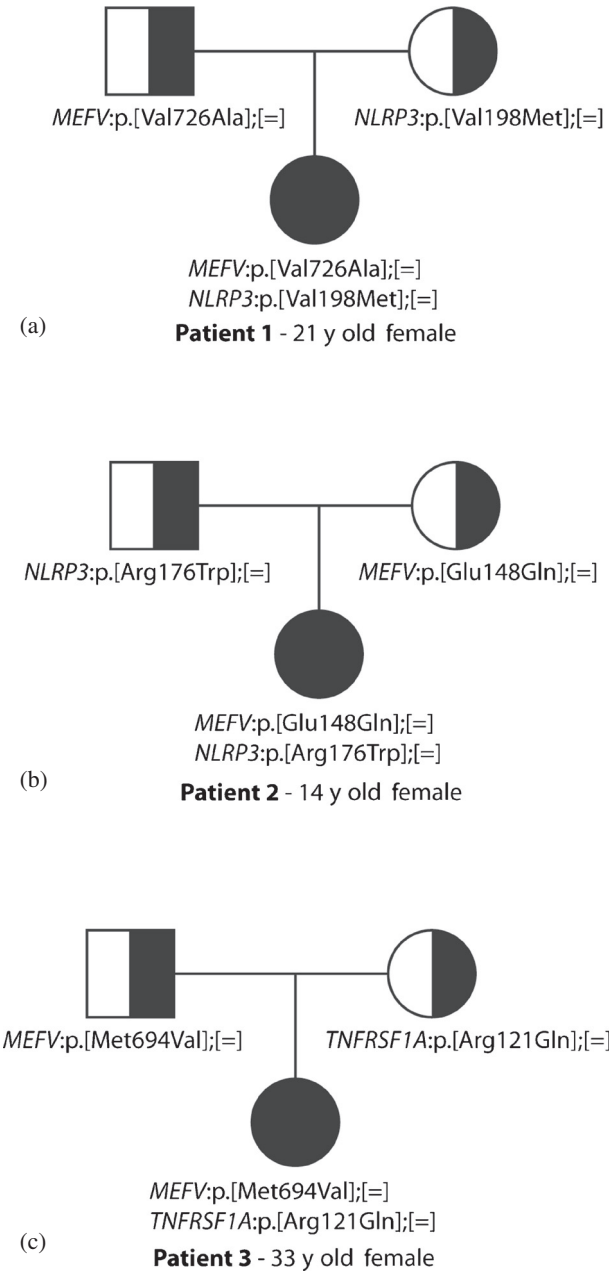

Figure 1. (a, b and c) With three genealogical trees and the mutations inherited by each family member.

Patient 2, a 14-year-old girl with clinical HRF manifestations such as urticarial rash and fever spikes of short duration (<24 h) carried the known missense *MEFV* p.[Glu148Gln] and *NLRP3* p.[Arg176Trp] (NM_001243133.1:c.526C>T) mutations (figure 1b; table 2). This patient did not show a good response for colchicine but to a certain extent showed satisfactory response for IL-1 blockade. In the *NLRP3* amino acid position at 176, the replacement of *Arg* residue with a *Trp*, according to the *in silico* computational algorithms (Polyphen-2 and SIFT) is predicted to interfere with the protein function (http://exac.broadinstitute.org/variant/1-247587277-C-T). As the *MEFV* p[.Glu148Gln] is considered as a variant of uncertain or mild significance, the diagnosis of CAPS was considered based on the clinical features and the response to IL-1 blockade (Shinar *et al.* 2012).

Patient 3, a 33-year-old female carried the severe *MEFV* p.Met694Val and *TNFRSF1A* p.[Arg121Gln] missense mutation in the heterozygous state (figure 1c; table 2). She had an overlapping FMF and TRAPS phenotype associated with mild periorbital swelling, short duration of fever episodes ranging between 24 and 48 h, abdominal and chest pain with a mediocre response to colchicine (table 2).

Parental analysis of patients 1, 2 and 3 revealed heterozygosity for either variant in either parents. Neither of the parents reported any HRF-associated symptoms. The father of patient 2 identified with p.[Arg176Trp];[=] in the *NLRP3* did not display any sign of inflammatory symptoms similar to his affected daughter with CAPS, despite the fact that the disease is reported to be dominantly inherited. The p.[Arg176Trp];[=] variant of the *NLRP3* gene when in heterozygous state seems to be as pathogenic as when it is coinherited with the *MEFV* p.Glu148Gln variant. Therefore, based on the latest 'Standards and Guidelines for the interpretation of sequence variants' it can be characterized as 'likely pathogenic' or of 'uncertain significance' (Richards *et al.* 2015).

Discussion

The present study included an extensive genetic screen of three HRF-associated genes with the aim to identify potential causative epistatic phenomena and locus–locus interactions in a total of 128 Cypriot FMF patients, heterozygous

Table 2. Clinical and genetic features in patients identified with digenic inheritance in autoinflammatory genes.

Gender (age of onset)	Mutations in different autoinflammatory gene	Clinical feature	Response to medication
Female (21 y)	*MEFV*: p.[Val726Ala];[=] *NLRP3*: p.[Val198Met];[=]	Frequent episodes of abdominal pain without accompanied fever	Never treated with colchicine or IL-1 blockade
Female (14 y)	*MEFV*: p.[Glu148Gln];[=] *NLRP3*: p.[Arg176Trp];[=]	Urticarial rash, fever spikes of short duration	Satisfactory response to IL-1 blockade
Female (33 y)	*MEFV*: p.[Met694Val];[=] *TNFRSF1A*: p.[Arg121Gln];[=]	Mild periorbital swelling, short duration of fever episodes from 24–48 h, abdominal and chest pain	Mediocre response to colchicine

for an *MEFV* mutation. The only objective tool that confirms HRFs is *MEFV*, *MVK*, *TNFRSF1A* and *NLRP3* sequencing. Therefore, the testing approach adopted here is similar to the one suggested by the HRF genetic diagnosis guidelines that were prepared in a consensus document disseminated through the European Molecular Genetics Quality Network, and involved direct sequencing of specific exons in *MEFV*, *MVK*, *TNFRSF1A* and *NLRP3* genes, where most of the frequent mutations are located (Shinar *et al.* 2012).

In general, mutations of the *NLRP3* gene are found in almost 70% of patients with CAPS which is inherited in an autosomal dominant manner (Federici *et al.* 2012). The coexistence of the *NLRP3* p.[Val198Met] along with the *MEFV* p.[Val726Ala] variant as seen in patient 1 with the FMF-like phenotype, most likely does not act synergistically due to the absence of associated pyrexial episodes that are typical of a FMF phenotype. In general, the missense *NLRP3* p.[Val198Met] mutation is considered as a low penetrance mutation than a benign polymorphism and which may contribute to inflammatory disease processes, such as familial cold autoinflammatory syndrome, Muckle–Wells syndrome and recurrent fevers (Hoffman *et al.* 2001; Aganna *et al.* 2002).

To our knowledge, variant *MEFV* p.[Glu148Gln] identified in patient 2 is of uncertain clinical significance (Ben-Chetrit *et al.* 2000; Tchernitchko *et al.* 2006). Several opposing studies have, however, established it as a pathologic variant associated with a milder form of FMF (Stoffman *et al*. 2000; Konstantopoulos *et al*. 2005; Solak *et al*. 2008; Tomiyama *et al*. 2008). In the *NLRP3* amino acid position at 176, the replacement of *Arg* residue with a *Trp*, according to the *in silico* computational algorithms (Polyphen and SIFT) is predicted to interfere with the protein function. As the *MEFV* p.[Glu148Gln] is considered a variant of uncertain or mild significance, the diagnosis of CAPS was finally considered based on the clinical features and the response to IL-1 blockade.

Currently, more than 100 mutations have been described in the *TNFRSF1A* with the missense p.[Arg121Gln] as the most frequent in the gene (Hull *et al.* 2002; Pelagatti *et al.* 2011). The missense *TNFRSF1A* p.[Arg121Gln] is a low-penetrance mutation that is associated with mild severity, high rate of spontaneous amelioration and lower prevalence of amyloidosis. Case of patient 3 suggests that the overlapping FMF/TRAPS phenotype is likely to be the result of synergistic behaviour of the two mutations residing in two different genes, thus suggesting another example of DI (Booty *et al.* 2009; Kubota *et al.* 2013; Timerman and Frank 2013; Abdwani *et al.* 2015). Cases with mutations in more than one gene suspected to lead to disease are increasingly identified and reported in the literature. In contrast to well recognized monogenic disorders, DI is the simplest form of inheritance associated with genetically complex diseases (Touitou *et al.* 2006; Schaffer 2013; Touitou 2013). There is no clear definition of DI but a good operational definition was given by Schaffer (2013). This states that DI exists when the variant genotypes at two loci explain the phenotypes of some patients and their unaffected (or more mildly affected) relatives more clearly than the genotypes at one locus alone. The definition excludes evidence of polygenic inheritance, and the presence of 'modifier loci' with a modest effect on the phenotype with only statistical evidence of significance (Schaffer 2013). Based on our results, we feel that the patients presented in this study in combination with their genotypes potentially represent examples of DI. Further studies are needed to confirm this assumption. The era of high-throughput sequencing is exquisitely temporally placed to clarify, delineate and accelerate the identification of potential associations and confirm the existence of DI in HRF disorders.

In the present study, the failure to identify a second mutation in the great majority of the Cypriot HRF heterozygous *MEFV* patients could also raise the possibility, under certain circumstances of codominant inheritance in FMF. This observation is consistent with the hypothesis that clinical symptoms of the disorder may also be present in carriers (Booth *et al*. 2000; Jeru *et al*. 2013). Another potential explanation for this observation may include the presence of rare unidentified mutations at other loci despite the fact that the sequenced mutational hot spot regions of the present study have 95% mutation coverage (Konstantopoulos *et al.* 2003; Shinar *et al.* 2012). Disease-causing mutations may also exist in the noncoding or regulatory regions that affect the splicing or the messenger RNA expression (Booty *et al.* 2009). The presence of dominant negative mutations or mutations with a high frequency could also be another potential cause (Booty *et al.* 2009). Finally, the fact that no mutations were identified in the entire coding sequence of *MVK* gene in any

of the screened patients suggest HIDS as almost nonextant in Cypriot population.

In conclusion, the present study identified the spectrum of *MEFV* mutations in a large cohort of *MEFV* heterozygote Cypriot patients who were presented with FMF-like symptoms reflecting the allelic heterogeneity which characterizes FMF in the island. The observation that FMF-like phenotypes can be seen in patients with combined heterozygous mutations involving more than one hereditary autoinflammatory genes, raises the hypothesis that some cases could be due to locus–locus interactions potentially providing evidence of DI. Insights obtained from studies that identify the genetic basis of detrimental disorders such as HRFs are extremely useful since they can provide a better understanding of disease pathogenesis, used for more effective diagnostic confirmation, assist in genetic counselling and used in the development of newer therapeutic approaches.

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