Multiple cytokine analysis in familial Mediterranean fever Egyptian cases using multiplex assay (Luminex technology) as a new inflammatory biomarker for disease activity

Naglaa Kholoussi[a], Shams Kholoussi[a], Hala T. El-Bassyouni[b], Haiam A. Raouf[a], Iman Helwa[a], Mohamed I. Gadelhak[b], Ghada N. Eldeen[c], Hala Elnady[d]

Departments of [a]Immunogenetics, [b]Clinical Genetics, [c]Molecular Genetics and Enzymology and [d]Child Health, National Research Centre, Cairo, Egypt

Correspondence to Naglaa Kholoussi, Professor, MD, Human Genetics and Genome Research Division, National Research Centre, El-Bohous Street, El-Dokki, Cairo 12611, Egypt
Tel: 01006077356;
e-mail: nkholoussi@gmail.com

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Objective
The aim of this study was to investigate the serum cytokines levels in the two most common mutations in Egyptian patients with familial Mediterranean fever (FMF).

Patients and methods
A total of 40 patients with FMF and 27 normal controls were included in this study. FMF mutations were analyzed using PCR-restriction fragment length polymorphism technique. CD4% was estimated using flow cytometry. Serum levels of Intercellular Adhesion Molecule 1 (ICAM-1), interleukin-2 (IL-2), E-selectin, IL-6, tumor necrosis factor-α, interferon-γ, and IL-1α were estimated using luminex multiplex technology.

Results
E-selectin was significantly decreased in patients with FMF having *M680I* mutation. On the contrary, interferon-γ and IL-6 were significantly increased in patients with *M694I* mutation. According to statistical analysis, T helper cells significantly increased in both mutations of FMF groups. T helper cells, IL-1α, IL6, tumor necrosis factor, and IFN-γ serum levels were increased in patients with FMF compared with controls.

Conclusion
T helper cells are increased in patients with FMF compared with controls, and a high percentage of *M694I* and *M680I* mutations was noticed in the study.

Keywords:
cytokines, familial Mediterranean fever, Luminex, M680I, M694I, T helper cells

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Introduction

Familial Mediterranean fever (FMF) is an autoinflammatory disease characterized by intermittent attacks of fever, which is self-limiting, arthritis, abdominal pain, and serositis. It clinically manifests by acute episodes of inflammation, which resolves without residual symptoms (Savran *et al.*, 2013).

Genetic causes for FMF were first proposed in 1990s by several studies (Russo and Brogan, 2014). Monogenic autoinflammatory diseases result from dysregulation of the immune system in response to foreign or endogenous harmful signals, which lead to inflammatory response. These inflammatory signals are composed of neutrophils, monocytes, and cells of the innate immune system (Samuels *et al.*, 1998).

FMF is the first monogenic auto-inflammatory disorder to be known and identified of its genetic cause (Russo and Brogan, 2014). Cases with FMF experience repeated attacks of fever, arthritis, pleuritic chest pain, abdominal pain, skin rash, and splenomegaly. These attacks are self-limiting, which may be mild or severe (Ozdogan and Ugurlu, 2017).

FMF results from mutation in *MEFV* gene situated on chromosome 16 coding for pyrin protein, which modifies neutrophil abundance and is important for the regulation of apoptosis and inflammation (Stehlik and Reed, 2004). The protein pyrin also causes dysfunction in the production of IL-1β and IL-18, which activate nuclear factor κß signaling pathways with the result of increase in tumor necrosis factor-α (TNF-α) and IL-6 (Hoffman, 2009).

FMF is considered as an autosomal recessive disorder. Nevertheless, some patients may experience significant classic picture, with only one allele of the *MEFV* gene (Booty *et al.*, 2009).

Colchicine is used in the prophylaxis of FMF attacks and works by abolishing the E-selectin-mediated adherence of neutrophils to cytokine-stimulated vascular endothelium, which leads to the decrease in the effect on neutrophil; consequently, the inflammatory reaction subsides (Kallinich *et al.*, 2007).

Specific biomarkers are not yet used in the diagnosis or disease monitoring of the patients with FMF during attacks. The focus of the present article is to highlight the autoinflammatory condition in patients with FMF and correlating it with their cytokine profile.

Patients and methods

In this case–control study, 76 patients were included, comprising 28 males and 39 females. There were 40 patients with FMF and 27 apparently normal controls. Their age ranged from 2 to 17 years old. All patients had acute attacks of FMF and were receiving colchicine as treatment. The study was approved by the Local Medical Research Ethics Committee (approval no 13/146) of the National Research Centre. A written consent was taken from each guardian and laboratories. All cases were subjected to full clinical examination in the specialized Genetics Clinics at the NRC. However, all laboratory tests, immunogenetics, and molecular studies were performed at the specialized laboratories of the Human Genetics Division at the NRC.

Complete blood picture was done for each case, and CD4% was estimated using BD monoclonal antibodies (BD Biosciences, San Jose, California, USA) by Flow cytometer and then CD4 absolute count was calculated (Rahman 2006).

Molecular methods

Genomic DNA was extracted from two milliliters of whole blood collected in EDTA-containing tubes, according to the standard procedures using QIAamp Spin Columns by QIAamp DNA Blood Kits (catalog no. 51104). Genotyping was performed by PCR-restriction fragment length polymorphism technique to detect the most common reported five-point mutations in both exons 10 and 2. The oligonucleotide primers were selected from an earlier study (Brik, *et al.*, 2001) and optimized for appropriate annealing temperatures in our laboratory. PCRs were performed in a final volume of 25 μl under the following conditions: 94°C for 3 min followed by 35 cycles of 94°C, for 30 s, 55–60°C, for 30 s and 72°C, for 30 s, and final extension at 72°C, for 3 min. Restriction enzyme digestion of amplicons was performed for the four common mutations, that is, M680I, M694V, M694I, and V726A, of exon 10 and E148Q of exon 2 of the MEFV gene by using restriction enzymes HinfI, HphI, PagI, AluI, and Ava1, respectively. Digested fragments were monitored in 3% agarose gel.

Luminex immunoassay technology

Cytokines, IL-2, ICAM-1, E-selectin, IFN-γ, TNF-α, IL-6, and IL-1α, levels were estimated using fluorescent bead-based Luminex multiplex immunoassay kit (R&D Systems Inc., Minneapolis, CA, USA). Luminex is a flow analyzer, and the LABScan 100 identifies the fluorescent intensity of phycoerythrin on each microsphere. Antigen–antibody reaction between the serum of patients and antibodies attached on the microspheres is the principle of this technology. The assignment of cytokines and its concentration is based on the reaction pattern, which applies Luminex technology recommended by Luminex Corporation R&D.

Statistical analysis

Data were analyzed using suitable statistical tests.

Results

Demographic as well as clinical characteristics of 40 patients with FMF and 27 apparently normal controls are presented in Table 1. There were no statistically significant differences between the two groups with respect to demographic data.

The distribution of the *MEFV* genotype is shown in Table 2, with high percentage of *M694I* and *M680I* mutations in the study patients with FMF (37.5 and 32.5%, respectively).

Table 3 summarizes laboratory findings of IL-2, ICAM-1, E-Selectin, IFN-γ, TNF-α, IL-6, and IL-1α levels in addition to TLC and CD4 absolute count in the FMF group and control group. The serum levels of IFN-γ, IL-6, and CD4 count in the patients with M694I mutation were considerably higher compared with the healthy controls (P = 0.018, 0.021, and 0.000003, respectively). However, only CD4 was significantly higher in M680I mutation patients compared with controls (P = 0.0112).

Discussion

FMF is considered the most common monogenic autoinflammatory disease, principally affecting ethnic groups living in the Mediterranean region Zarouk *et al.* (2018). It is characterized by recurrent,

self-limited episodes of fever and serositis. It is difficult to diagnose when signs are atypical, and this may lead to considerable delay in starting treatment (Berkun and Eisenstein, 2014). An increasing number of monogenic inflammatory diseases have been studied, and their respective genes responsible have been recognized. Moreover, proteins encoded by these genes are included in the regulatory pathways of inflammation and are expressed in the cells of the innate immune system. However, clinical diagnosis comes first, with genetic verification where possible (Khalil *et al.*, 2018).

Even though a group of patients show intermittent systemic inflammation (periodic fevers), these disorders are mediated by constant overproduction and release of proinflammatory mediators, such as IL-1 and IL-6, and TNF. Hence, they are considered as autoinflammatory diseases rather than periodic fevers. Blocking these cytokines with biologic agents has been proved to be significantly helpful in some patients. Nevertheless, considerable autoinflammation cases show no genetic abnormalities, and treatment continues to be suboptimal, bringing up the issue of novel pathogenic mutations in unknown genes and pathways (Russo and Brogan 2014).

In this study, we investigated cytokine levels in patients with FMF. Forty patients with FMF and 27 healthy normal controls were included in this study.

In this study, the CD4 was increased in the FMF group in comparison with the apparently healthy controls. This was in agreement with Musabak *et al.* (2004) and Kholoussi *et al.* (2018).

The Mediterranean Fever (*MEFV*) gene linked with the disease is present on chromosome 16. It encodes a 781-amino acid protein called pyrin. Pyrin seems to have a key role in the regulation of both inflammation and apoptosis. Moreover, the mutant pyrin causes autoinflammation characterized by excessive secretion of cytokines in FMF (Berkun and Ben-Chetrit, 2007). The *MEFV* genotype done in this study is listed in Table 2, showing high percentages of *M694I* and *M680I* mutations in patients with FMF. However, on comparing patient groups with M694I and M680I mutations, there was a statistically significant difference in E-selectin serum level (Tables 4 and 5).

Interleukin (IL) 6 and IFN-γ serum levels were increased in patients with FMF with *M694I* mutation as compared with the control group; this coincides with the findings of Baykal *et al.* (2003). They stated that the balance between the cytokines may help to understand the pathophysiology of FMF and to develop immune therapies. However, the cost of cytokine measurement analyses seems disadvantageous. IL-6 is an inflammatory cytokine that has a central role in autoimmune and chronic inflammatory diseases. High levels of IL-6 in the serum throughout FMF attacks were stated by Koga *et al.* (2016). They showed that

Table 1 Clinical and demographic features of patients with familial Mediterranean fever and apparently healthy controls

	FMF (*n*=40)	Control (*n*=27)
Age (range) (years)	2-17	2-12
Sex		
Male	19	20
Female	21	7

FMF, familial Mediterranean fever.

Table 2 Analysis of *MEFV* gene mutation

Mutation type	*n* (%)
Hetero for M694I	15 (37.5)
Hetero for M694V	1 (2.5)
Hetero for M680I	13 (32.5)
Compound Hetero for M680I Exon 10 and Hetero for 148Q Exon 2	1 (2.5)
V726A	2 (5)
Hetero for E148Q	2 (5)
Hetero for E148	1 (2.5)
Wild type no mutation in Exon 10,2,3,5	1 (2.5)
Negative for 5 mutations: M694I, M694V, M 6801, V726A, E148Q	1 (2.5)
No mutation	3 (7.5)

MEFV, Mediterranean fever.

Table 3 The laboratory features of the patients with familial Mediterranean fever and healthy controls

	Control (*n*=27)	FMF (*n*=40)	M694I (*n*=15)	M680I (*n*=13)
IL-2 (pg/ml)	92.9 (81.3-100.6)	73.5 (38.7-108.4)	73.5 (46.4-100.6)	71.6 (42.6-108.4)
ICAM-1 (ng/ml)	1168.2 (1024.8-1450.4)	1052.6 (208.3-1996.2)	1226.6 (477.3-1586.3)	748.8 (293.4-1996.2)
E-Selectin (ng/ml)	43.5 (38.0-52.9)	40.5 (19.7-57.9)	42.8 (34.5-57.9)	*36.6 (19.7-41.3)*
IFN-γ (pg/ml)	4.7 (1.2-7.1)	4.7 (2.4-28.3)	*4.7 (4.7-28.3)*	4.1 (2.4-7.1)
TNF-α (pg/ml)	1.1 (0.4-4.9)	1.5 (0.7-3.4)	1.1 (0.7-3.7)	1.1 (0.9-2.2)
IL-6 (pg/ml)	0.8 (0.62.8)	1.1 (0.5-50.7).	*1.7 (1.1-50.7)*	1.1 (1.1-1.2)
IL-1α (pg/ml)	0.8 (0.4-1.2)	0.8 (0.7-2.0)	0.8 (0.7-2.0)	0.8 (0.7-0.8)
TLC (cell/mm^6)	7.9 (3.5-9.8)	6.8 (3.3-13.2)	7.2 (3.3-11.9)	6.1 (3.5-10.9)
CD4 (cell/mm^3)	389 (48.9-972.4)	*1090.4 (633-2268)*	*1208.7 (1011.9-2193.1)*	*772.9 (399.4-1102.3)*

Results were expressed as median (minimum-maximum). Italic numbers indicate significant difference compared with control. FMF, familial Mediterranean fever; IFN-γ, interferon-γ; IL, interlukin; TLC, total leukocytic count; TNF-α, tumor necrosis factor-α.

Table 4 Biomarker comparison between M694I and M680I groups

	M694I (*n*=15)	M680I (*n*=13)	*P*
IL-2 (pg/ml)	73.5 (46.4-100.6)	71.6 (42.6-108.4)	0.87
ICAM-1 (ng/ml)	1226.6 (477.3-1586.3)	748.8 (293.4-1996.2)	0.53
E-Selectin (ng/ml)	42.8 (34.5-57.9)	36.6 (19.7-41.3)	0.013*
IFN-γ (pg/ml)	4.7 (4.7-28.3)	4.1 (2.4-7.1)	0.17
TNF-α (pg/ml)	1.1 (0.7-3.7)	1.1 (0.9-2.2)	0.49
IL-6 (pg/ml)	1.7 (1.1-50.7)	1.1 (1.1-1.2)	0.27
IL-1α (pg/ml)	0.8 (0.7-2.0)	0.8 (0.7-0.8)	0.16
TLC (cell/mm^6)	7.2 (3.3-11.9)	6.1 (3.5-10.9)	0.29
CD4 (cell/mm^3)	1208.7 (1011.9-2193.1)	772.9 (399.4-1102.3)	0.085

As comparing the two groups there were statistical significant differences in E-selectin serum levels. Results were expressed as median (minimum-maximum). IFN-γ, interferon-γ; IL, interlukin; TLC, total leukocytic count; TNF-α, tumor necrosis factor-α. *P<0.05.

Table 5 Pearson correlation of CD4 with tested cytokines (results were expressed as *r*)

	CD4 (*r*)	*P*
E-Selectin	0.637517	*0.000460**
ICAM-1	0.712008	*0.000045**
IL-2	0.010	0.961331
IFN-γ	−0.34229	0.086962
TNF-α	0.097328	0.636216
IL-6	−0.25943	0.200606
IL-1α	−0.25979	0.199960

Statistically significant positive correlation was found between CD4 an both E-selectin and ICAM-1 serum levels. IFN-γ, interferon-γ; IL, interlukin; *r*, correlation coefficient; TLC, total leukocytic count; TNF-α, tumor necrosis factor-α. *P<0.05.

IL-6 had the top performance for differentiating patients with FMF during the attack from apparently healthy participants or patients found in remission. Other new case reports have revealed the value of an IL-6 inhibitor in clinical practice for the so called 'colchicine-resistant FMF' or secondary amyloidosis in patients with FMF. This supports the concept of IL-6 as a major inflammatory cytokine in FMF and hence shows potential as a biomarker of this disease (Koga *et al.*, 2016).

There was a statistically significant correlation between CD4 and both E-selectin and ICAM-1, indicating that the T-helper cells had increased activity in the patient group.

Conclusion

T helper cells, IL-1, IL6, TNF, and IFN-γ serum levels were increased in patients with FMF compared with controls. This study elucidates the importance of ILs in the FMF disease, which would highlight the importance of treatment with anti-ILs in FMF disease to ameliorate its inflammatory pathology.

Conflicts of interest

There are no conflicts of interest.

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