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Toll-like receptor-4 gene variations in Egyptian children with familial Mediterranean fever

Yomna Farag^{1*} , Samia Salah¹, Hanan Tawfik¹, Mai Hamed² and Huda Marzouk¹

Abstract

Background: Familial Mediterranean fever (FMF) is an autosomal recessive disorder affecting people in the region of the Mediterranean Sea. It is usually associated with mutation in Mediterranean fever (*MEFV*) gene that encodes the pyrin protein, which affects the innate inflammatory response. Toll-like receptors (TLR) are a family of pattern recognition receptors that recognize pathogenic microbes and activate antimicrobial defense mechanisms. Toll-like receptor 4 (TLR-4) is concerned with recognition of gram-negative organisms. There is growing clinical evidence suggesting a role for expression of TLRs in the immune pathogenesis of FMF. Thus, the aim of the current study was to evaluate the presence of *TLR-4* (p.Asp299Gly) and *TLR-4* (p.Thr399Ile) gene variants in association with Egyptian children having FMF, furthermore, its effect on disease course and severity.

Results: Seventy Egyptian children diagnosed as having FMF, together with 50 age and gender-matched controls were enrolled in the study. The *TLR-4* (p.Asp299Gly) and (Thr399Ile) gene variants were determined by PCR-RFLP analysis for all studied patients and controls. *TLR-4* p.Asp299Gly gene variant was detected in 1 (1.4%) of the patients and p.Thr399Ile gene variant was detected in 2 (2%). None of the controls had any of the two tested gene variants. All found variations were heterozygous. We could not find a statistically significant association with disease severity in cases with or without *TLR-4* gene variants ($P = 0.568$). Patients with M694V gene mutation showed a higher disease severity ($P = 0.035$).

Conclusion: TLR-4 (p.Asp299Gly) and (p.Thr399Ile) gene variants were not found to have a link with the occurrence, the clinical picture of FMF, its severity, and response to colchicine treatment in Egyptian children. M694V gene mutation seems to be associated with higher disease severity. Further larger studies are needed to verify these results.

Keywords: Familial Mediterranean fever, Toll-like receptor 4, Gene variants

Background

Familial Mediterranean fever (FMF) is an auto-inflammatory autosomal recessive disease associated with mutation in *MEFV* (Mediterranean fever) gene, which is located on the short arm of chromosome 16. This gene encodes the expression of pyrin protein, which has a role in the innate immune response. It

suppresses the production of inflammatory cytokines specially interleukin (IL-1 β) [1, 2].

Familial Mediterranean fever (*MEFV*) gene mutations interrupt the expression of pyrin, leading to intermittent episodes of uncontrolled inflammation [3].

Toll-like receptors (TLRs) constitute a family of trans-membrane proteins expressed by various cell types, including immune cells. They identify pathogens and initiate inflammatory signaling pathways [4]. Discovery of TLRs increased our understanding of how the innate immune response senses various structures of

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microorganisms and trigger the signaling pathway, causing release of cytokines [1].

About 15 types of TLRs have been identified; among them is TLR-4. It mediates inflammatory signaling by recognizing the lipopolysaccharide (LPS) on gram-negative bacterial cell wall. *TLR-4* (p.Asp299Gly) and *TLR-4* (p.Thr399Ile) polymorphisms downregulate inflammation [5].

Innate immunity is a common factor between FMF and toll-like receptors [6]. Stimulation of the TLRs causes the activation of protein signal cascades, including Interleukin-1 receptor (IL-1 R) associated kinase pathway. This activates nuclear transcription factor-kappa B. Furthermore, mutant pyrin proteins in FMF are unable to inhibit the production of IL-1 β , a cytokine involved in promoting apoptosis, thus allowing persistence of macrophages and inflammation [7, 8].

Genetic variations of TLRs have been found to increase the susceptibility to autoimmune and allergic diseases [9]. Furthermore, expression of FMF disease might be affected by some unknown modifying genes, besides the well-established *MEFV* gene mutations [10].

Single nucleotide variations of the *TLR-4* genes have been associated with receptor hypo-responsiveness and susceptibility to bacterial, fungal, and viral infections [1]. An important question had been raised; whether *TLRs* gene variations would affect the immune responses against infections, thus, affect the course of FMF [11].

There is some clinical evidence suggesting a role for expression of TLRs in FMF [12]. Furthermore, some studies worked on finding an association between the low prevalence of single nucleotide polymorphisms (SNPs) in *TLRs-4* genes and FMF disease [11, 13, 14].

Kawamoto et al. suggested that FMF patients, whose attacks are triggered by the menstrual cycle, might have fewer *TLR4* promoter genotypes that allow gram-negative bacteria to colonize the urinary tract without symptoms [1]. Thus, we hypothesized that *TLR-4* (p.Asp299Gly) and *TLR-4* (p.Thr399Ile) gene variations might affect FMF in Egyptian children.

The aim of this study was to evaluate the presence of *TLR-4* (p.Asp299Gly) and (p.Thr399Ile) gene variants in association with Egyptian children having FMF, including its effect, if any, on disease course and severity.

Methods

This study was conducted on 120 children, including 70 diagnosed as having FMF. They were recruited from our Pediatric Rheumatology Clinic. The control group included 50 age and gender-matched children who came for follow-up at the outpatient surgery clinic of the same hospital for elective pre-operative assessment. The control group does not have any symptoms suggestive of autoimmune disease. The study was conducted during

the period from January 2017 to December 2018. Laboratory investigations were done in Clinical and Chemical Pathology Department Laboratories, Kasr Al Ainy Hospital.

All patients were diagnosed according to FMF pediatric criteria [15]. Their age at time of study ranged from 2-16 years. Patients diagnosed as having an associated autoimmune disorder were excluded from the study.

Full history was taken from patients including history of parents' consanguinity, family history of FMF and other autoimmune diseases, age at disease onset, age at disease diagnosis, and duration of illness. Number of attacks per year, durations of attacks/hour, and clinical pattern of disease attacks (fever, abdominal pain, chest pain, arthritis, erysipelas-like rash, testicular affection, and history of vasculitis) were also included. History of colchicine dosing was taken in full details including duration of therapy/year, starting dose and current dose in mg/day, frequency of attacks/year, and duration of attacks/hour before and after colchicine therapy and compliance. Finally, response to colchicine therapy was assessed by FMF-50 score [16].

Disease severity was assessed by FMF severity score [17]. Familial Mediterranean fever (*MEFV*) gene variation status of the patients was recruited from their files. Complete blood picture (CBC), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and urine analysis were done to all participants at the time of study.

The study was approved by the Local Ethical Committee of Faculty of Medicine, Cairo University. All participants in this study were informed and written consents were taken from their parents or legal guardian of the child.

Specimen collection and storage

Four milliliters of venous blood was withdrawn from all subjects and divided in to two parts: 2 ml were collected in a sterile vacutainer containing ethylene diamine tetraacetate "EDTA" which were used for DNA extraction. Samples were kept frozen at -20°C till time of DNA extraction and analysis. Two milliliters were collected on plain tubes, left for 10 min to clot, and then centrifuged at 3000 rpm for 5 min, the separated serum was used for routine laboratory investigations including CBC, CRP, ESR, serum creatinine, and urine analysis to check for proteinuria.

Genomic DNA extraction

Extraction of genomic DNA was done from peripheral blood using a (TIAN amp Genomic DNA Kit Intro Biotechnology) according to the manufacturer's instructions.

Table 1 Identified bands of Asp299Gly and Thr399Ile genotypes after trans-illumination by ultraviolet trans-illuminator

Genotype	Size of fragment	Number of bands detected
Asp299Gly		
Wild genotype	249 bp*	One band
Homozygous mutant genotype	223 and 26 bp	Two bands
Heterozygous genotype	249, 223, and 26 bp	Three bands
Thr399Ile		
Wild genotype	406 bp	One band
Homozygous mutant genotype	377 and 29 bp	Two bands
Heterozygous genotype	406, 377, and 29 bp	Three bands

*bp base pair

Analysis of toll-like receptor 4 *TLR-4* (p.Asp299Gly) and *TLR-4* (p.Thr399Ile) gene variants was done using polymerase chain restriction-restriction fragment length polymorphism (PCR-RFLP) procedure.

Amplification of extracted genomic DNA (PCR) was done using the following primers: for *TLR-4* (p.Asp299Gly), sense: 5'-GAT TAG CAT ACT TAG ACTACT ACC TCC ATG-3', and anti-sense: 5'-GAT CAA CTT CTG AAA AAG CAT TCC CAC-3'. As for *TLR-4* (p.Thr399Ile), sense: 5'-GGT TGC TGT TCT CCA AAG TGA TTT TGG GAG AA-3', and anti-sense: 5'-ACC TGA AGA CTG GAG AGT GAG TTA AAT GCT-3'. The thermal cycler was programmed for initial denaturation at 95 °C for 10 min, 35 cycles of amplification consisting of denaturation at 94 °C for 30 s, annealing for 30 s at 61 °C for Asp299Gly (NOCI) enzyme, and 62 °C for Thr399Ile (HINF) enzyme and extension at 72 °C for 1 min. The amplified samples were then run in parallel on 2% agarose gel using gel electrophoresis and visualized on Ultra Violet transilluminator to detect presence of amplified material.

Each PCR product was digested to completion with FastDigest (NOCI-HF) restriction endonuclease for *TLR-4* (p.Asp299Gly) and FastDigest (HinfI) restriction endonuclease for *TLR-4* (p.Thr399Ile).

The digested products were separated by electrophoresis on agarose 3% gel and visualized using ethidium bromide (Table 1), (Figs. 1 and 2).

Statistical methods

Data were analyzed using SPSS© Statistics version 24 (SPSS© Corp., Armonk, NY, USA). Normally distributed numerical data were presented as mean \pm SD and inter-group differences were compared using the unpaired *t* test. Skewed numerical data were presented as median and interquartile range and between-group differences were compared using the Mann-Whitney test. Categorical variables were presented as number and percentage. Fisher's exact test was used to compare nominal data and the chi-squared test for trend to compare ordinal data. Association between FMF genes and disease severity were tested using rank biserial correlation (rrb). *P* values \leq 0.05 were considered statistically significant.

Results

Demographic, clinical, and laboratory characteristics of the studied groups are represented in Table 2. Mean of age at disease onset was 4.9 ± 2.9 (0.5-12) years, while the time between first presentation and disease diagnosis showed a mean of 2.0 ± 2.5 (0.0-12.0) years.

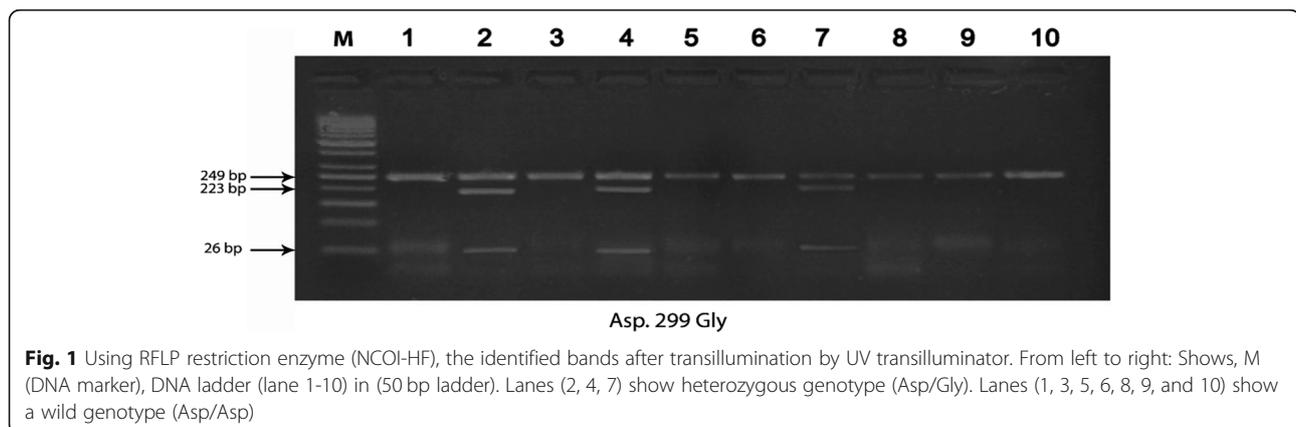


Fig. 1 Using RFLP restriction enzyme (NOCI-HF), the identified bands after transillumination by UV transilluminator. From left to right: Shows, M (DNA marker), DNA ladder (lane 1-10) in (50 bp ladder). Lanes (2, 4, 7) show heterozygous genotype (Asp/Gly). Lanes (1, 3, 5, 6, 8, 9, and 10) show a wild genotype (Asp/Asp)

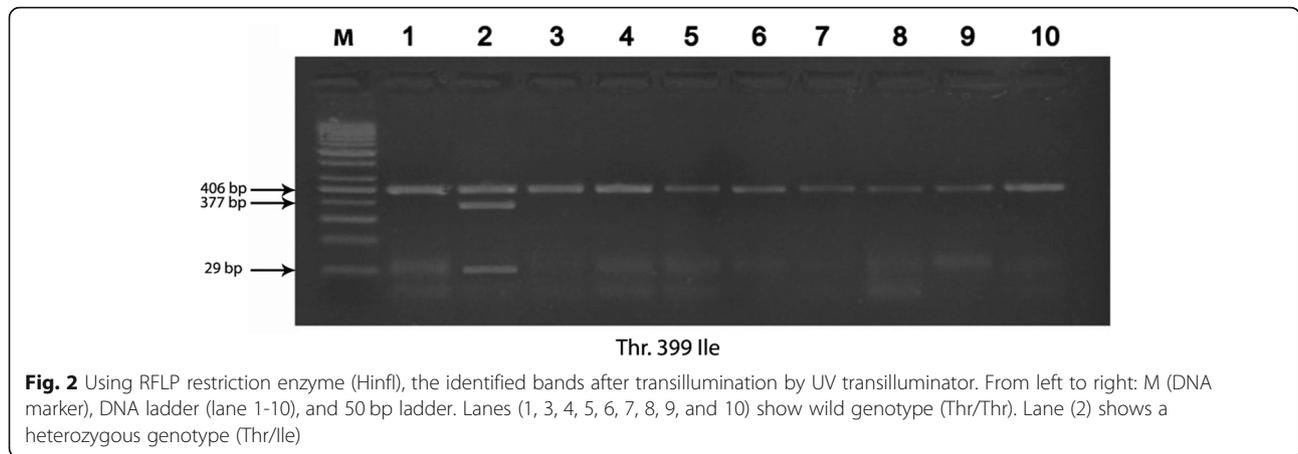


Fig. 2 Using RFLP restriction enzyme (HinfI), the identified bands after transillumination by UV transilluminator. From left to right: M (DNA marker), DNA ladder (lane 1-10), and 50 bp ladder. Lanes (1, 3, 4, 5, 6, 7, 8, 9, and 10) show wild genotype (Thr/Thr). Lane (2) shows a heterozygous genotype (Thr/Ile)

Table 2 Demographic, clinical, and laboratory characteristics of the studied groups regarding age, gender, clinical manifestations, disease severity, and presence of TLR4 gene mutations

Variables	Cases (n = 70)	Controls (n = 50)	P value
Age (years), mean ± SD	9.5 ± 3.5	9.4 ± 2.6	0.840
Gender			
Male n (%)	30 (42.9%)	28 (56.0%)	0.195
Female n (%)	40 (57.1%)	22 (44.0%)	
Clinical manifestations of FMF n (%)			
Abdominal pain	62 (88.6%)		
Recurrent fever	55 (78.6%)		
Arthritis	55 (78.6%)		
Chest pain	30 (42.9%)		
Myalgia	29 (41.4%)		
Skin rash	4 (5.7%)		
Degree of disease severity n (%)			
Mild	14 (20%)		
Moderate	20 (28.6%)		
Severe	26 (51.4%)		
Laboratory findings			
Positive CRP	6 (8.6%)		
Proteinuria	1 (1.4%)		
Anemia	1 (1.4%)		
Leukocytosis	4 (5.7%)		
TLR-4 Asp299Gly genetic variation			
Normal	69 (98.6%)	48 (96.0%)	0.570
Heterozygous	1 (1.4%)	2 (4.0%)	
TLR-4 Thr399Ile genetic variation			
Normal	69 (98.6%)	50 (100.0%)	1.000
Heterozygous	1 (1.4%)	0 (0.0%)	

Furthermore, disease duration had a mean of 4.4 ± 2.7 (1.0-12.0) years. Hemoglobin level had a mean value of 12.2 ± 1.0 (9.0-14.0) g/dl. Total leucocyte count had a mean of 7 ± 2 (4-14) $\times 1000/\text{mm}^3$. One patient (1.4%) had normocytic normochromic anemia and 4 patients (5.7%) had leukocytosis.

The mean duration of colchicine therapy was 2.7 ± 1.9 (0.5-9.0) years. The starting dose in cases showed a mean of 0.89 ± 0.44 (0.25-2.0) mg/day, while the current dose of colchicine at time of study had a mean of 1.13 ± 0.48 (0.25-2.0) mg/day. Median and IQ range seen in Table 3.

Our result showed that the frequency of attacks decreases after colchicine therapy from a mean of 6 ± 4 (1-36) attacks/year to a mean of 2 ± 2 (0.0-12) attacks/year. Similarly, duration of the attacks decreased from a mean of 35 ± 20 (6-72) hours to a mean of 10 ± 11 (0-48) hours. Response to colchicine per FMF50 scores [16] showed non-responsiveness in 10 patients (14.3%) compared to 60 (85.7%) being responsive. Three patients (4.3%) were non-compliant to therapy.

We found that 20 (28.6%) patients carried a heterozygous MEFV gene mutation, 35 (50.0%) were homozygous, and 15 (21.4%) had compound heterozygous mutation. The most prevalent FMF gene among patients was the E148Q, in 22 (25.2%) patients, M694I in 20 (22.9%), V726A in 18 (20.6%), M680I in 16 (18.3%), and M694V was present in 11 (12.6%) patients.

The MEFV gene M694V had a weak association with disease severity with a rank biserial correlation coefficient (r_{rb}) of 0.253 (P value = 0.035). Otherwise, there was no statistically significant association between any of the other FMF genes and severity of disease (all P values > 0.05). Similarly, there was no statistically significant association between disease severity and presence of FMF gene mutation (r_{rb} = 0.069, P value = 0.572), as seen in Table 4.

Table 3 Duration, starting dose, and current dose of colchicine therapy among cases

Variable	Min	Max	Median	IQ range	Percentiles		
					25 th	50 th	75 th
Duration of colchicine therapy (years)	0.5	9.0	2.5	(1.0-3.5)	1.0	2.5	3.5
Starting dose of colchicine (mg/day)	0.25	2.00	1.00	(0.50-1.00)	0.50	1.00	1.00
Current dose of colchicine (mg/day)	0.25	2.00	1.00	(1.00-1.50)	1.00	1.00	1.50

The *TLR-4* (p.Asp299Gly) gene variants were found normal in 69 (98.6%) patients compared to 48 (96.0%) of the controls. One (1.4%) of the cases had heterozygous variation compared to 2 (4.0%) of the controls, with $P = 0.570$. *TLR-4* (p.Thr399Ile) genetic variation was normal in 69 (98.6%) cases compared to 50 (100.0%) of the controls, while 1 (1.4%) of the cases carried a heterozygous variation compared to none of the controls ($P = 1.000$).

Odds ratio of exposure among cases versus among control showed a result of 0.71 with no statistical significance ($P = 0.732$).

We did not find a significant correlation between frequency and duration of attacks and colchicine dosage among cases with and without TLR gene variations (Table 5).

As for disease severity in cases with *TLR4* gene variations, one (7.1%) patient showed mild form of the disease severity, and another patient (2.8%) showed severe form (P value 0.568). Regarding response to treatment, the two (3.3%) patients with *TLR4* gene variations showed good response to colchicine treatment.

Discussion

To the best of our knowledge, this is the first study about TLR-4 gene variants in Egyptian children with FMF. We could not find a significant correlation

between TLR-4 (p.Asp299Gly) and (p.Thr399Ile) gene variants and clinical measures of FMF disease, its severity, and response to colchicine treatment. Furthermore, M694V gene mutation statistically correlated with higher disease severity (P value = 0.035).

The low prevalence of *TLR-4* gene variations that were found in this study was also reported in Egyptian patients with other autoimmune diseases. The Egyptian study by Taha et al. [4] did not find any of the *TLR-4* (p.Asp299Gly) or *TLR-4* (p.Thr399Ile) gene variations in patients with rheumatoid arthritis or systemic lupus erythematosus or in the control group. They stated that the *TLR-4* gene variations display ethnic differences. Africans have the highest frequency (16%) [18], followed by Europeans (4-10%) [19], whereas Asians do not display this type of gene variations [20]. It seems that Egyptians have a low prevalence for these types of gene variations.

This study comes in agreement with the results reported by the study done in Greece [11] who found no association between *TLR-4* gene variations and the frequency of attacks or disease severity. Speletas et al. [11] found the allele frequency of *TLR-4* (p.Asp299Gly) to be lower in FMF patients compared with controls, while two other Turkish studies [13, 21] found a lower prevalence among healthy controls. The latter two studies

Table 4 Association between FMF genes present in study subjects and FMF disease severity

Variable		Severity of FMF						Rank biserial correlation	
		Mild		Moderate		Severe		Coefficient (r_{rb})	P value
		N	Row %	N	Row %	N	Row %		
FMF gene V627A	Absent	10	19.2%	14	26.9%	28	53.8%	-0.069	0.570
	Present	4	22.2%	6	33.3%	8	44.4%		
FMF gene E148Q	Absent	11	22.9%	12	25.0%	25	52.1%	0.043	0.726
	Present	3	13.6%	8	36.4%	11	50.0%		
FMF gene M680I	Absent	9	17.0%	16	30.2%	28	52.8%	-0.099	0.413
	Present	5	29.4%	4	23.5%	8	47.1%		
FMF gene M694V	Absent	13	21.7%	20	33.3%	27	45.0%	0.253*	0.035
	Present	1	10.0%	0	0.0%	9	90.0%		
FMF gene M694I	Absent	11	22.0%	13	26.0%	26	52.0%	0.029	0.813
	Present	3	15.0%	7	35.0%	10	50.0%		
FMF gene mutation	Absent	1	50.0%	0	0.0%	1	50.0%	0.069	0.572
	Present	13	19.1%	20	29.4%	35	51.5%		

Data are counts (N) and row percentage (%).

*There is a weak association between FMF gene M694V and severity of FMF (r_{rb} 0.253; P value, 0.035)

Table 5 Relation between *TLR-4* gene variations and age at disease onset, frequency, and duration of attacks before and after colchicine therapy and colchicine dosage

Variable	Negative <i>TLR-4</i> gene variation (n = 68)	Positive <i>TLR-4</i> gene variation (n = 2)	P value
Age at onset (years)	4.75 (0.5-12)	5 (5-5)	0.750
Frequency of attacks per year before colchicine	4 (1-36)	6 (4-8)	0.545
Frequency of attacks per year after colchicine	1 (0-12)	1 (1-1)	0.794
Duration of attacks before colchicine (h)	24 (6-72)	42 (36-48)	0.459
Duration of attacks after colchicine (h)	6 (0-48)	15 (6-24)	0.388
Starting dose of colchicine (mg/day)	1 (0.25-2)	1.25 (1-1.5)	0.152
Dose of colchicine at time of study (mg/day)	1 (0.25-2)	1.75 (1.5-2)	0.077

found a higher prevalence of *TLR-4* (p.Thr399Ile) gene variations among control. All genetic variations found were heterozygous, a finding similar to our study.

Soylu et al. agreed with our finding that the frequency of these gene variations did not differ between FMF patients and control [13].

Another study looking for the same *TLR4* gene variants in Henoch-Schonlein purpura (HSP) observed a similar finding. They found a low prevalence of these variants in HSP with no effect on disease occurrence and presentation [22].

On the other hand, a meta-analysis study found a weak association between inflammatory bowel disease and the studied gene variants in Caucasians, but not Asians [23], highlighting the fact that the prevalence and effect of *TLR* gene variants are different among ethnicities.

Our study found an association between FMF disease severity and M694V gene mutation. This agreed with several Egyptian studies [23–25]. An Egyptian study by Alhaggar et al. suggested a link between this gene mutation and amyloidosis [26]. Researchers studying other ethnicities found a similar link [27–30].

In our study, the mean age of disease onset was 4.9 ± 2.9 years. This was similar to Talaat et al. [31]. However, Salah et al. [32] and El-Garf et al. [33] found slightly older age of onset. Mean age of onset was also higher in a Syrian study [34] and a Turkish study [35]. Meanwhile, the age of onset in three other Turkish studies was almost similar [36–38]. These differences may be related to different ethnicities and different genetic backgrounds.

The most common disease manifestation in this study was abdominal pain, followed by fever. Abdominal pain came to be the first symptomatic presentation in several studies [32, 34, 36, 39]. However, other studies [33, 40–42] reported more occurrence of fever than abdominal pain. This difference may be due to different sample sizes and different presentations in various regions.

Limitations of our study are the relatively small number of patients and control.

Conclusion

TLR-4 (p.Asp299Gly) and (p.Thr399Ile) gene variants were not found to have a link with the occurrence, the clinical picture of FMF, its severity, and response to colchicine treatment in Egyptian children. M694V gene mutation seems to be associated with higher disease severity. Further larger studies are needed to verify these results.

Abbreviations

FMF: Familial Mediterranean fever; MEV: Mediterranean fever gene; CBC: Complete blood count; CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate; PCR-RFLP: Polymerase chain restriction-restriction fragment length polymorphism; IL-1: Interleukin-1; HSP: Henoch-Schonlein purpura; IBD: Inflammatory bowel disease

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Authors' contributions

All authors have contributed significantly and all authors are in agreement with the content of the manuscript. YM: contributed in searching the literature, writing and editing the manuscript, and being the corresponding author. SS: contributed with the idea of the research, following the results, and writing the manuscript. HT: contributed in searching literature, collecting the data, and performing the statistical part of the research. MH: contributed in the laboratory part of the research and writing the methodology of the manuscript. HM: contributed in following the results and writing the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was approved by the ethical scientific committee of the Faculty of Medicine, Cairo University. Informed written consents were taken from the parents or the patient's guardians according to guidelines of the ethical committee of the Faculty of Medicine, Cairo University.

Consent for publication

Written informed consents to publish the data contained within this study were taken from the parents or the patient's legal guardians.

Competing interests

The authors declare that they have no competing interests.

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