Serum Amyloid A Type 1 Gene Polymorphism in Egyptian Children with Familial Mediterranean Fever

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Key Words

Familial Mediterranean fever · Gene polymorphism · Serum amyloid A type 1

Abstract

Background: Since spontaneous inflammation is an important contributor to familial Mediterranean fever (FMF), genetic variants mediating inflammation are of interest. We investigated gene variants in the acute-phase serum amyloid A type 1 (*SAA1*), a sensitive marker of inflammatory activity, and their association with susceptibility and severity of FMF. **Methods:** The genotypes of 2 single-nucleotide polymorphisms within exon 3 of *SAA1* (2995C/T and 3010C/T) were determined in 105 Egyptian children with FMF and in 125 controls by polymerase chain reaction-restriction fragment length polymorphism. Genotyping of the causative *MEFV* mutations was performed by reverse hybridization. **Results:** The *M694I* mutation was the most frequent allele (42.8%), followed by *V726A* (18.6%), *M680I* (17.1%), *E148Q* (11.9%) and *M694V* (9.0%). The frequency of the *SAA1* α , β and γ alleles was not significantly different between FMF patients and controls. The genotype frequency of *SAA1* α/α was higher in patients than in healthy subjects (21.0 vs. 14.4%) although it did not reach statistical significance. The clinical manifestations including age at disease onset, number of FMF attacks, colchicine dose and severity score were not re-

lated to genotypes of *SAA1*. However, *M694V* mutation and female gender were significantly associated with severity. **Conclusion:** The genetic polymorphism of *SAA1* is not associated with susceptibility and severity of FMF in Egyptian children.

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Introduction

Familial Mediterranean fever (FMF) is a systemic autoinflammatory disease characterized by self-limited recurrent episodes of fever accompanied by peritoneal, pleural or synovial inflammation. FMF was originally described as a disease of autosomal recessive inheritance and early onset leading to significant morbidity. Missense mutations in the Mediterranean fever (*MEFV*) gene located on chromosome 16p13.3 have been observed to be causative of FMF [1]. Pyrin, the 781-amino acid protein product of the *MEFV* gene, is expressed in the cytoplasm of monocytes, and the nucleus of dendritic cells, neutrophils, and synovial fibroblasts [2–6]. Five mutations, *M694I*, *M680I*, *M694V* and *V726A* in exon 10 and *E148Q* in exon 2, account for almost 90% of FMF mutations [7]. A major role of pyrin is the regulation of inflammation. FMF-associated mutations in pyrin activate interleukin (IL)-1 β and induce the acute phase response.

Serum amyloid A (SAA) is markedly expressed in the acute inflammatory state during attacks as well as in between attacks of FMF [8]. In general, the plasma concentrations of SAA are biomarkers for host response to trauma, stress or infection. SAA synthesis, which occurs in the hepatocytes and epithelial cells, is induced by the inflammation-associated cytokines IL-6 and IL-1 and tumor necrosis factor- α (TNF- α) [9]. Because cytokine regulation of SAA and cytokine inducing functions of SAA determine the magnitude and duration of the immune response, genetic variation in the acute-phase reactant may be related to the development of inflammatory diseases [10, 11] such as FMF.

The genes on chromosome 11 encode for the acute-phase SAA type 1 (SAA1) protein, which is the predominant form of the SAA gene family [12]. Three SAA1 allelic variants have been defined on the basis of 2 single-nucleotide polymorphisms (SNPs) located in exon 3, i.e. 2995 C/T and 3010 C/T, that correspond to the isoforms SAA1 α (2995T–3010C), SAA1 β (2995C–3010T) and SAA1 γ (2995C–3010C) or SAA1.1, SAA1.5 (originally considered as SAA1.2) and SAA1.3, respectively [10, 13–14]. The 2 polymorphisms of the SAA1 gene result in amino acid changes at positions 52 and 57, Val52–Ala57, Ala52–Val57 and Ala52–Ala57, respectively.

There is increasing evidence that genotypes at the SAA1 locus are associated with raised susceptibility to AA amyloidosis [15, 16]. For example, the genotype α/α of the SAA1 gene has been associated with a 7-fold increase in the incidence of renal amyloidosis, [17] while a protective effect of the SAA1 β and γ alleles on the development of amyloidosis was suggested. However, the contribution of these genotypes to the occurrence of nonamyloid, inflammatory disease has not been fully elucidated, especially in Egyptian patients. FMF is presumed to be a monogenic disease, although the role of potential modifying genetic factors other than *MEFV* in the development of FMF has been suggested [11].

In view of the recent genetic studies on FMF, SAA1 allelic variants may contribute to the susceptibility of FMF in addition to *MEFV* mutations. Therefore, we attempted to determine the effect of gene polymorphisms on the susceptibility and severity of FMF in the pediatric Egyptian population.

Methods

Patients and Controls

Patients with FMF were diagnosed at the Rheumatology Department of the Pediatric Hospital of Cairo University. The medi-

cal records of the children with FMF were evaluated retrospectively for gender, age at the onset of symptoms and time of diagnosis, clinical signs and symptoms and *MEFV* genotype. The study included 105 children with FMF diagnosed according to established FMF criteria [18] and genetically confirmed with 2 *MEFV* gene mutations. Age- and gender-matched apparently healthy children ($n = 125$), with no family history or clinical manifestations suggestive of FMF and negative for the *MEFV* gene mutation, were assigned to the control group. Severity score was assessed according to Mor et al. [19] modified for children by Ozen et al. [20] and Pras et al. [8]. The study was approved by the ethical committee of Cairo University.

MEFV Gene Mutation Analysis

Molecular genetic mutation analysis of the *MEFV* gene was performed for patients and controls using the reverse hybridization assay (FMF StripAssay; ViennaLab, Vienna, Austria). In brief, exons 2, 3, 5 and 10 were amplified in a single multiplex polymerase chain reaction (PCR). The amplification program was 35 cycles including 94°C for 15 s, 58°C for 45 s, 72°C for 45 s and a final extension at 72°C for 7 min. Biotinylated PCR products were hybridized to allele-specific oligonucleotide probes and the 12 common mutations, E148Q in exon 2, P369S in exon 3, F479L in exon 5 and M680I (G/C) and M680I (G/A), I692del, M694V, M694I, K695R, V726A, A744S and R761H in exon 10 were determined. In individuals with symptoms of FMF, mutation analysis of the common *MEFV* mutations identified double (homozygous or compound heterozygous) mutations, confirming the diagnosis.

SAA1 Genotyping

The genotyping of exon 3 of SAA1 SNP was performed using PCR-restriction fragment length polymorphism (RFLP) assay.

DNA Extraction and Purification

Total genomic DNA from FMF patients and healthy controls was extracted from EDTA-anticoagulated whole blood using DNA extraction and a purification kit according to the manufacturer's instructions (Thermo Scientific).

PCR Amplification

DNA was amplified by PCR using 5'-GCC AAT TAC ATC GGC TCA G-3' (sense) and 5'-TGG CCA AAG AAT CTC TGG AT-3' (antisense), primers spanning exon 3 of SAA1 [10]. PCR reactions were carried out in 2 $\times$ DreamTaq Green PCR master mix (Thermo Scientific) containing DreamTaqTM DNA polymerase, 0.4 mM of dATP, dCTP, dGTP and dTTP and 4 mM MgCl₂ with 0.4 μ M of each primer. PCR amplification was performed as follows: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 60 s, annealing at 62°C for 60 s and extension at 72°C for 7 min [21].

RFLP Assay

The 530-bp PCR products were then digested by *BanI* and *BclI* (Fermentas, Germany) and DNA fragments were then separated by electrophoresis in 2.5% agarose gel stained with ethidium bromide. With the enzyme *BanI*, the amplified α allele was digested into 3 fragments (317, 188 and 25 bp), while the amplified β and γ alleles were digested into 4 fragments (244, 188, 73 and 25 bp). With *BclI*, the DNA amplified from the β allele was digested into 2 fragments (438 and 92 bp), while that from the α and γ alleles was not digested [13].

Table 1. Demographic and clinical features of 105 FMF patients

Males/females	50/55
Age at disease onset, years	5.4±3.7
Age at diagnosis, years	7.2±4.0
Disease duration, years	3.9±2.8
Number of attacks per month	3.0±2.2
Duration of attacks, days	1.7±1.4
Severity (mild/moderate/severe)	24/36/45
Colchicine, mg/day	1.0±0.4
Duration of colchicine treatment, years	2.0±2.0

Values are presented as mean ± SD or n.

Statistical Analysis

Data were statistically described in terms of mean ± standard deviation (SD), number of cases and percentages. For comparing categorical data, the χ^2 test was performed. All statistical tests were 2-sided. Linear regression analysis was used to study the contribution of independent variables to the severity of FMF. $p < 0.05$ was considered statistically significant. All statistical calculations were done using SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, Ill., USA) v20 for Microsoft Windows.

A power calculation by G*power software was estimated where the χ^2 test with an α value of 0.05 and effect size of 0.3 was employed. For total sample size i.e. 230 (105 FMF patients + 125 controls) a power equal to 0.96 for differences between the genotype frequencies in patients and controls and equal to 0.99 for allele differences was observed.

Results

Clinical Features

The clinical and demographic data of the FMF patients are summarized in table 1. The ratio of males to females in patients with FMF was 0.9 (50:55). Consanguinity was recognized in 40/105 (38.1%) patients. Both family cases and sporadic cases were observed; 20/105 (19.0%) patients had a family history of FMF. The most common clinical features during the attacks were abdominal pain in 96.2%, a high-grade fever ($\geq 38^\circ\text{C}$) in 91.4%, arthralgia in 61.9% and chest pain in 56.2%. Only 33.3% had myalgia and 21.0% had arthritis, while 6.7% of the patients had erysipelas-like lesions.

All the patients were treated with colchicine. The average dose was 1.0 ± 0.4 mg/day and 30.5% of patients received <1.0 mg/day. The disease was mild in 24 (22.9%) patients, moderate in 36 (34.3%) patients and severe in 45 patients (42.9%), according to the modified scoring system of Mor et al. [19] and Ozen et al. [20]. The median

Table 2. Distribution of *MEFV* mutations in FMF patients

Homozygotes		Compound heterozygotes	
Mutation	n (%)	Mutation	n (%)
<i>M694I/M694I</i>	22 (20.9)	<i>M694I/E148Q</i>	16 (15.2)
<i>M680I/M680I</i>	10 (9.5)	<i>M694I/V726A</i>	12 (11.4)
<i>V726A/V726A</i>	5 (4.8)	<i>M694I/M694V</i>	8 (7.6)
<i>M694V/M694V</i>	2 (1.9)	<i>M694I/M680I</i>	6 (5.7)
<i>E148Q/E148Q</i>	1 (0.9)	<i>M680I/V726A</i>	5 (4.8)
		<i>M680I/E148Q</i>	1 (0.9)
		<i>M680I/M694V</i>	1 (0.9)
		<i>M694V/E148Q</i>	3 (2.8)
		<i>M694V/V726A</i>	2 (1.9)
		<i>M694V/A744S</i>	1 (0.9)
		<i>V726A/P369S</i>	1 (0.9)
		<i>V726A/E148Q</i>	1 (0.9)
		<i>V726A/1692del</i>	2 (1.9)
		<i>M694I/V726A/M680I</i>	2 (1.9)
		<i>M694I/V726A/E148Q</i>	1 (0.9)
		<i>M694I/V726A/1692del</i>	1 (0.9)
		<i>M680I/V726A/A744S</i>	1 (0.9)
		<i>V726A/E148Q/1692del</i>	1 (0.9)
Total	40 (38.1)		65 (61.9)

severity score was 6.0 (range 3–11) according to Pras et al. [8].

MEFV Gene Mutations

Among the 105 patients, 40 (38.1%) were homozygous and 65 (61.9%) were compound heterozygous for *MEFV* mutations. Table 2 shows the distribution of *MEFV* mutations in the study. *M694I* and *M694I/E148Q* were the most frequent genotypes in the homozygote and heterozygote mutations, respectively. The *M694I* mutation was the most frequent allele (42.8%), followed by *V726A* (18.6%), *M680I* (17.1%), *E148Q* (11.9%) and *M694V* (9.0%).

Association between *SAA1* Gene Polymorphism and FMF

The polymorphic sites of the *SAA1* gene were subjected to PCR-RFLP analysis. Table 3 shows the frequencies of individuals with various genotypes and alleles at the *SAA1* locus in pediatric FMF patients ($n = 105$) and Egyptian control subjects ($n = 125$). The *SAA1* α , β and γ alleles were encountered in both FMF patients and controls. The genotype frequency of *SAA1* α/α was higher in FMF patients than in healthy subjects (21.0 vs. 14.4%) although it did not reach statistical significance ($p = 0.291$). Conversely,

Table 3. SAA1 genotype and allele frequency in FMF patients and normal controls

	FMF patients (n = 105)	Controls (n = 125)	
SAA1 genotypes			
α/α	22 (21.0)	18 (14.4)	$\chi^2 = 6.200$ $p = 0.291$
α/β	37 (35.2)	43 (34.4)	
α/γ	2 (1.9)	10 (8.0)	
β/β	30 (28.6)	33 (26.4)	
β/γ	7 (6.7)	12 (9.6)	
γ/γ	7 (6.7)	9 (7.2)	
SAA1 alleles			
α	83 (39.5)	89 (35.6)	$\chi^2 = 2.623$ $p = 0.269$
β	104 (49.5)	121 (48.4)	
γ	23 (11.0)	40 (16.0)	

Values are expressed as n (%). The χ^2 test was used to examine differences in allele and genotype frequencies between FMF patients and control subjects.

the allele frequency of SAA1 γ was lower in FMF patients than in healthy subjects (11.0 vs. 16.0%, $p = 0.269$).

FMF Clinical Characteristics Related to Genotypes at the SAA1 Loci

The clinical manifestations, age at disease onset, number of FMF attacks, dose of colchicine required and severity score were not related to genotypes at the SAA1 locus ($p = 0.884, 0.838, 0.778$ and 0.729 , respectively). Furthermore, patients bearing the SAA1 α/α genotype ($n = 22$) did not differ from those bearing the SAA1 α/β , β/β , β/γ , α/γ and γ/γ allelic combinations ($n = 83$) in terms of age at disease onset, number of attacks, dose of colchicine and severity score ($p = 0.697, 0.550, 0.489$ and 0.419 , respectively). The severity score was not significantly different among those carrying the SAA1 α/α or SAA1 β/β or SAA1 γ/γ genotype compared with those bearing other SAA1 allelic combinations ($p = 0.419, 0.970$ and 0.753 , respectively).

Based on the severity scoring system by Pras et al. [8], multivariate linear regression analyses showed that the MEFV mutation M694V [$\beta = 1.226$; 95% confidence interval (CI) 0.290–2.162; $p = 0.011$] as well as female gender ($\beta = 0.648$; 95% CI 0.044–1.252; $p = 0.036$) were significantly associated with increasing severity score. SAA1 alleles α , β and γ did not significantly affect severity ($\beta = 0.024$ and $p = 0.947$, $\beta = -0.180$ and $p = 0.645$ and $\beta = -0.709$ and $p = 0.166$, respectively).

Discussion

Variant alleles are present in SAA1, which is the principal form of the SAA gene family. SAA is a major acute-phase protein, and emerging evidence has shown correlations between SAA1 alleles and diseases including FMF [11]. However, our data indicate that SAA1 gene polymorphism consisting of SNPs within exon 3 (i.e. 2995C/T and 3010C/T) that result in amino acid changes at positions 52 and 57, respectively, are not associated with susceptibility in Egyptian children with FMF. This is the first study reporting on the SAA1 polymorphism in the Egyptian population.

It has recently been reported that in the Japanese population, in whom the SAA1.1 (α) allele occurs with a frequency of 34%, possession of and homozygosity for this allele constitute a significant protective factor for FMF [11]. In contrast, it has been observed that, in healthy Turkish individuals and FMF patients without amyloidosis, the SAA1 α allele did not significantly differ, at a frequency of 42.5 and 49.5%, respectively, while among FMF patients with associated AA amyloidosis, there was a marked increased frequency of the SAA1 α allele (85.6%) [22]. Similarly, no differences were detected in the distribution of genotype and allele frequencies of the 2 SNPs, 2995C/T and 3010C/T, between FMF patients without amyloidosis and the control group in a Greek population [23].

It remains unclear how the proteins encoded by the SAA1 alleles function differently. It was proposed that the differences of the SAA1 isoforms in their selectivity for SAA receptors may influence their roles in modulating inflammation [24]. For example, SAA1.1 (α) was more efficient than SAA1.3 (γ) and SAA1.5 (β) in the activation of the SAA receptor, formyl peptide receptor 2, that is present on phagocytes. In addition, the SAA1.3 (γ) isoform was found to be potent in the induction of pro-inflammatory TNF α in macrophages whereas SAA1.5 (β) stimulated anti-inflammatory IL-10 expression. Gouwy et al. [25] demonstrated that the SAA1 α isoform is able to chemoattract monocyte-derived immature dendritic cells. The chemotactic activity of SAA1 α was mediated by rapid chemokine stimulation, suggesting regulation of leukocyte recruitment to inflammatory sites.

In healthy individuals, SAA concentrations were found to be significantly higher in those possessing a SAA 1.5 (β) allele, and highest in homozygotes for the allele [26]. It was reported that the protein of the allelic variant of SAA 1.5 (β) is cleared from the circulation more slow-

ly than other isoforms [27]. Furthermore, SAA1 induces the production of MMPs by monocytic cells [28] and MMPs degrade SAA1 preferentially in the site of the polymorphism at position 57. The SAA1.1 (α) isoform is more susceptible to cleavage by MMP-1 than SAA1.5 (β), resulting in a higher production of the 1–57 fragments from SAA1.1 (α) [29].

The majority of FMF patients in this study had some combination of the *M694I*, *V726A*, *M680I*, *E148Q* and *M694V* mutations. The *M694I* mutation was the most frequent allele (42.8%), followed by *V726A* (18.6%), *M680I* (17.1%), *E148Q* (11.9%) and *M694V* (9.0%). The presence of *M694I* in exon 10 is important in FMF. Patients with the *M694I* mutation show an early onset, a high frequency and a short duration of attacks, in addition to high percentages of fever and serositis. However, their therapeutic response to colchicine is very good [30].

Our data suggest that the *SAA1* polymorphism does not influence the severity of FMF. Similarly, Gershoni-Baruch et al. [31] showed that disease severity was not associated with genotypes at the *SAA1* locus, but was mainly influenced by *MEFV* mutations. In this study, the *M694V* allele significantly influenced severity ($\beta = 1.226$; 95% CI 0.290–2.162; $p = 0.011$). Several studies have emphasized the observation that the severe phenotype of FMF is associated with the *M694V* mutation [17, 31]. Female gender also contributed to severity in the multivariate analysis ($\beta = 0.648$; 95% CI 0.044–1.252; $p = 0.036$).

Type AA amyloidosis is a serious complication of FMF associated with persistent inflammation in inadequately treated disease. It is caused by the deposition of insoluble amyloid proteins in the extracellular spaces of different tissues [32, 33]. The AA protein that forms the amyloid fibril is primarily derived from the degradation products of SAA1. Homozygosity for *SAA1* α and *SAA1* γ in different populations is a significant risk factor for AA amyloidosis [34]. In FMF, homozygosity for the *M694V* allele, arthritis attacks, male sex and the *SAA1* α homozygous genotype were found to be independently associated with renal amyloidosis [31]. However, the development of amyloidosis differs in various ethnic populations. One consideration in this study is the association between the results of the amyloidogenic *SAA1* genotype frequency in FMF patients and controls with the reported rare incidence of amyloidosis in Arabs (1.7%) [35]. The prevalence of amyloidosis among patients attending renal clinics in North Africa varies between 4 and 9%, and FMF is responsible for 11.6–30% of cases [36, 37].

In conclusion, *SAA1* allelic variants are not modifying genetic factors in the susceptibility or severity of FMF in the pediatric Egyptian population. However, the *MEFV* mutation *M694V* and female gender may be associated with more severe disease.

Disclosure Statement

The authors declare no conflicts of interest.

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