The Turkish Journal of Pediatrics 2003; 45: 198-202 *Original*

Analysis of the modifying effects of SAA1, SAA2 and TNF-alpha gene polymorphisms on development of amyloidosis in FMF patients

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SUMMARY: Yılmaz E, Balcı B, Kutlay S, Özen S, Ertürk Ş, Öner A, Beşbaş N, Bakkaloğlu A. Analysis of the modifying effects of SAA1, SAA2 and TNF-alpha gene polymorphisms on development of amyloidosis in FMF patients. Turk J Pediatr 2003; 45: 198-202.

The aim of this study was to examine whether polymorphisms at serum amyloid A (SAA) and tumor necrosis factor-alpha (TNF-α) genes are associated with development of amyloidosis in familial Mediterranean fever (FMF) patients. Seventy-three FMF patients with amyloidosis and 100 other FMF patients without amyloidosis of known genotypes and 100 healthy control subjects were analyzed. There was a significant difference in the frequency of α/α genotype at the SAA1 locus between FMF patients with amyloidosis and controls and FMF patients without amyloidosis. The frequencies of the α/α genotype and α alleles at SAA1 locus were significantly higher in the FMF patients with amyloidosis. The frequencies of the α allele at SAA1 locus in FMF patients with amyloidosis, without amyloidosis and controls were 85.6%, 49.5% and 42.5%, respectively. We demonstrated that α/α genotype at SAA1 gene might have modifying effects on the development of amyloidosis.

Determination of genotypes at SAA1 locus can play a key role in conferring genetic susceptibility and patient's prognosis to renal amyloidosis.

Key words: Serum amyloid A1 (SAA1), SAA2 and tumor necrosis factor-α gene polymorphisms, amyloidosis, familial Mediterranean fever.

Familial Mediterranean fever (FMF) is an autosomal recessive disease affecting mainly non-Ashkenazi Jews, Armenians, Turks and Arabs[1].The disease is characterized by recurrent short episodes of inflammation and serositis including fever, peritonitis pleuritis, synovitis and, rarely, pericarditis. Amyloidosis is the most important complication of the disease that determines the prognosis[2,3]. The identification in 1997 of the gene (MEFV) responsible for FMF has been fundamental in our understanding of the disease[4,5]. Nevertheless, we still do not know the exact pathogenesis of amyloidosis. The first genotype-phenotype correlation studies showed that the milder V726A mutation may have a protective effect against amyloidosis, whereas patients homozygous for M694V would be more prone to amyloidosis[6]. However, recent studies indicate that renal amyloidosis can be accompanied by other mutations. Furthermore, clinical studies have shown that the presence of a family member with amyloidosis increases the risk of this complication significantly[7,8].

The rate of amyloid deposition and thus the clinical expression of amyloid disease may theoretically depend on other genetic factors, such as the genetic predisposition to convert serum amyloid A (SAA) into AA amyloid fibrils, or some cis-acting elements which can increase transcription of SAA, such as proinflammatory cytokines, interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-alpha etc.

Serum amyloid A protein is one of the apolipoproteins of the high-density lipoprotein (HDL) family, and AA which accumulates in amyloidosis derives from SAA by proteolytic

cleavage. The factors associated with both the synthesis of SAA and the activity of macrophages in processing SAA to AA make SAA loci a candidate region for amyloid development[9-11].

A prolonged high plasma level of SAA in chronic inflammation may lead to the deposition of its degraded products, AA proteins, in tissues. However, a high concentration of SAA is not sufficient for the development of amyloidosis[10]. Recent studies have focused on the polymorphisms of SAA as a genetic risk factor for amyloidosis[12]. Some alleles in the SAA1 gene have been proposed as positive risk factors in white and Japanese rheumatoid arthritis patients[13].

In this study, SAA and TNF-α gene loci were chosen for the genetic studies of susceptibility to amyloidosis in FMF patients.

Material and Methods

Patients

Of more than 500 patients with FMF seen in our outpatient clinic, we chose 173 cases. These 173 FMF patients were divided into two groups according to the presence (n=73) or absence (n=100) of renal amyloidosis. One hundred healthy individuals were included in the study as the control population. All the patients were diagnosed as having FMF based on the following criteria: recurrent attacks of fever with peritonitis, pleuritis or synovitis, favorable response to colchicine, and/or family history of FMF in a first-degree relative. The diagnosis of AA-amyloidosis had been confirmed histologi-cally by staining renal biopsies with Congo red, daylon and anti-amyloid A antibody. At presentation, 16.4% of FMF patients with amyloidosis had proteinuria, 77.0% had nephrotic syndrome and 6.7% had already developed chronic renal failure. Mutation analysis was performed in all FMF patients. Informed consent was received from the patients and control group prior to the study.

Molecular Genetic Analysis

Detection of MEFV gene mutations

Genomic DNA was isolated from peripheral leukocytes using standard procedures. The hot spot exon 10, which harbors 18 mutations of the MEFV gene, was first analyzed by denaturing gradient gel electrophoresis (DGGE)[14]. According to the band pattern, subsequent analysis was done by restriction endonuclease enzyme digestion. Furthermore, E148Q muta-tion in exon 2 was analyzed by restriction endonuclease enzyme BstNI digestion.

Analysis of Polymorphisms in SAA1, SAA2 and TNF-alpha Genes

The SAA1α, SAA1β and SAA1γ isoforms are encoded by the V52-A57, A52-V57 and A52-A57 SAA1 alleles, respectively. The SAA2α and SAA2β isoforms are encoded by H71 and R71 SAA2 alleles, respectively[11]. TNF-α 308 G->A polymorphism exists in the promoter region of the gene[15]. The polymerase chain reaction (PCR) products from the SAA1 gene

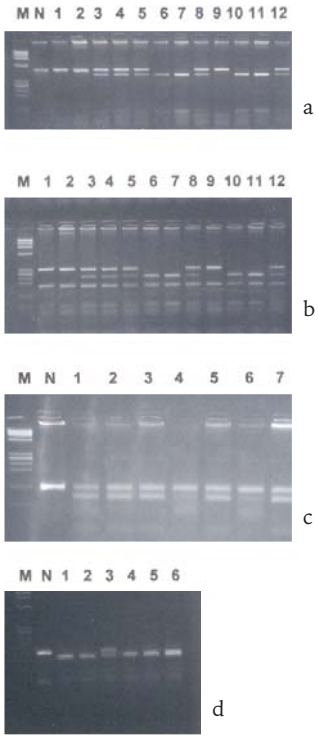

Fig. 1. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of SAA1, SAA2 and TNF-α gene polymorphisms. The PCR products of these genes were digested with different restriction enzymes as follows:

a and b: BcI I and Ban I digestion of SAA1 gene. DNA samples 1,2,9 α/α; 3,4,5,8,12 α/β; 6,7,10,11 β/β genotype.

c: Nco I digestion of SAA2 gene. DNA samples 1,2,3,5,7 α/β; 4,6 α/α genotype.

d: Nco I digestion of TNF-α gene. DNA samples 1,2,4,5,6, G/G; 3, G/A genotype.

N: Non-digested PCR products.

were digested with BcI I and Ban I restriction enzyme to detect α, β, and γ alleles (Fig. 1). The genotypes at the SAA2 and TNF-α gene were determined by Nco I restriction enzyme digestion (Fig. 1).

Statistical Analysis

The allele and genotype frequencies were determined both in the FMF patient groups and controls. The results were compared with the values predicted by the Hardy-Weinberg equilibrium by means of the $\chi 2$ test.

Results

Mutation analysis showed that the most common mutation was M694V (62.2%) in FMF patients with amyloidosis. Other mutations accounted for a further 20.5% of the alleles: M680I (13.7%), V726A (6.2%) and E148Q (0.66%) In 25 alleles (17.1%) no mutation could be detected.

The frequency of the α/α genotype in SAA1 gene was significantly higher in the FMF patients with amyloidosis ($P \leq 0.001$) compared with the FMF patients without amyloidosis and healthy control subjects (Table I).

SAA1 genotypes were compared among different MEFV genotypes and the distribution is given in Table II. SAA1 α/α genotype frequency was observed to be distributed among various MEFV genotypes in FMF patients with amyloidosis with different frequencies. However, other SAA1 genotypes were either low or absent in the various MEFV genotypes. In FMF patients without amyloidosis different SAA1 genotypes were randomly distributed, as seen in Table III.

Table I. Comparison of the Numbers and Frequencies of the Genotype and Alleles at the SAA1 Locus in FMF Patients with and Without Amyloidosis and in Healthy Control Subjects

	FMF patients with amyloidosis (n=73)	FMF patients without amyloidosis (n=100)	Healthy controls (n=100)
Genotype at SAA1 locus			
α/α	54 (73.97)	27 (27.0)	19 (19.00)
α/β	16 (21.91)	42 (42.0)	43 (43.00)
α/γ	1 (1.36)	3 (3.00)	4 (4.00)
β/β	1 (1.36)	22 (22.0)	25 (25.00)
β/γ	1 (1.36)	5 (5.00)	9 (9.00)
γ/γ	0 (0.00)	1 (1.00)	0 (0.00)
Allele at SAA1 locus			
α	125 (85.61)	99 (49.5)	85 (42.5)
β	19 (13.01)	87 (43.5)	92 (46.0)
γ	2 (1.40)	10 (5.00)	13 (6.50)

$\chi^2=65.16$.
p=0.000.
FMF : familial Mediterranean fever.
SAA1: serum amyloid A1.

Table II. Genotype Distribution at SAA1 Locus in FMF Patients with Amyloidosis

	α/α	α/β	α/γ	β/β	β/γ	γ/γ
M694V/M694V	28/37 (75.67)	7/37 (18.91)	1/37 (2.70)		1/37 (2.70)	
M694V/M680I	5/7 (71.42)	2/7 (28.85)				
M694V/V726A	6/6 (100.00)					
M680I/M680I	4/5 (80.00)	1/5 (20.00)				
M680I/V726A	1/1 (100.00)					
M680I/E148Q		1/1 (100.00)				
M694V/other	2/3 (66.66)	1/3 (33.33)				
M680I/other	1/1 (100.00)					
V726A/other	1/1 (100.00)					
Other/other	6/11 (54.54)	4/11 (36.36)		1/11 (9.09)		

FMF : familial Mediterranean fever.
SAA1: serum amyloid A1.

Table III. Genotype Distribution at SAA1 Locus in FMF Patients with Amyloidosis

	α/α	α/β	α/γ	β/β	β/γ	γ/γ
M694V/M694V n=40 (2.50)	9/40 (22.50)	17/40 (42.50)	1/40 (2.50)	10/40 (25.00)	2/40 (5.00)	1 / 4 0
M680IV/M680I n=10	3/10 (30.00)	4/10 (40.00)		3/10 (30.00)		
E148Q/E148Q n=10	4/10 (40.00)	4/10 (40.00)	1/10 (10.00)	1/10 (10.00)		
M694V/M680I n=20	7/20 (35.00)	9/20 (45.00)		3/20 (15.00)	1/20 (5.00)	
M694V/V726A n=20	4/20 (20.00)	8/20 (40.00)	1/20 (5.00)	5/20 (25.00)	2/20 (10.00)	

Table IV. Comparison of the Numbers and Frequencies of the Genotype and Alleles at the SAA2 and TNF-α Locus in FMF Patients with and without Amyloidosis and in Healthy Controls

	FMF patients with amyloidosis (n=73)	FMF patients without amyloidosis (n=100)	Healthy controls (n=100)
Genotype at SAA2 locus			
α/α	60 (82.19)	75 (75.00)	73 (73.00)
α/β	11 (15.06)	23 (23.00)	24 (24.00)
β/β	2 (2.73)	2 (2.00)	3 (3.00)
Genotype at TNF-α locus			
G/G	61 (83.50)	91 (91.00)	89 (89.00)
G/A	12 (16.40)	8 (8.00)	11 (11.00)
A/A			

χ^2=2.52, p=0.642 for genotypes at SAA2 locus.
χ^2=2.93, p=0.231 for genotypes at TNF-α locus.
FMF: familial Mediterranean fever, SAA2: serum amyloid A2, TNF-α: tumor necrosis factor-alpha.

The frequencies of the genotypes at the SAA2 and TNF-α genes in FMF patients with and without amyloidosis and healthy control subjects are given in Table IV.

There were no significant differences in the frequencies of SAA2 and TNF-α alleles in these groups.

Discussion

In most Mendelian disorders, it is now well established that individuals with identical mutations may differ clinically. These observations reflect the existence of additional environmental factors or genes that contribute to the disease phenotypes. In the case of FMF the variable risk for patients carrying the same MEFV gene mutations to develop amyloidosis strongly suggests a role for other genetic and/or environmental factors. These candidate modifiers of genetic origin in FMF-associated renal amyloidosis would include factors that participate in amyloid deposits. We therefore investigated the genes encoding the major acute-phase serum amyloid SAA1 and SAA2 proteins, which are precursors of the amyloid A proteins presently found in AA secondary amyloidosis. In this study we focused on these genetic factors that may have a role in the development of amyloidosis by comparing the genotypes at SAA1, SAA2 and TNF-α genes in a group of 73 FMF patients with amyloidosis, 100 FMF patients without amyloidosis and 100 healthy controls. One conclusion of this study concerned the MEFV gene mutation frequencies in FMF patients with amyloidosis. When we compared the distribution of the MEFV gene mutations in the FMF patients with amyloidosis in this study with FMF patients without amyloidosis of our previous study[16], the frequency of the M694V homozygosity was higher in the FMF patients with amyloidosis (50.7%) than that observed in the group of FMF patients without amyloidosis (36.2%). ($p \leq 0.001$). In contrast, there was no significant difference in the frequency of the other most common MEFV genotypes.

The second observation of this study was that the genotypes at SAA1 gene were related to the development of renal AA-amyloidosis. The frequency of the patients homozygous for SAA1 α/α was much higher in the renal AA group than in the non-renal AA and healthy control groups.

When we compare the α/α genotype at SAA1 locus with the genotypes at MEFV gene of FMF patients with amyloidosis, we can see

that nearly all of the patients with MEFV gene mutations have SAA1 α/α genotype, thus confirming an additive effect of the MEFV gene and SAA gene locus in the pathophysiology of AA type amyloid development in FMF (Table II). Low frequency of SAA1 α/α genotype in M694V homozygous non-amyloid patients and high frequency of SAA1 α/α genotype in patients with rather mild MEFV gene mutations (Table III) also support our additive effect hypothesis for amyloid development. There was no significant correlation between the α/α genotype and the genotypes at the MEFV gene. On the other hand, α/α genotype at SAA1 locus in the same group was much higher in the FMF patients with amyloidosis. One study showed a positive association of the allele α with amyloidosis[17]. The present study similarly showed a positive association of the allele α with amyloidosis in FMF patients with amyloidosis. Despite the structural and functional similarities between SAA1 and SAA2 genes, the SAA2 locus did not significantly affect the risk of occurrence of renal amyloidosis in our study group. In the study done by Cazeneuve et al.[17] with both FMF and rheumatoid arthritis (RA) patients, no association was present between SAA2 locus and amyloid development. Although SAA2 locus is closely linked to the SAA1 locus, our results have excluded its possible responsibility for the development of AA type amyloidosis. This may have been related to the minority representation of AA2 in amyloid deposits.

TNF-α-308 polymorphism was also not found to be associated significantly with the outcome of the AA type amyloidosis phenotype. Thus, this polymorphism was not a defining factor for the development of AA type amyloidosis in FMF. It is rather difficult to draw a firm conclusion on the effect of TNF-α since more specific analysis of multiple genetic markers at this locus are needed if we are to understand whether this polymorphism is functional per se or is in linkage disequilibrium with another polymorphism.

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