Analysis of SAA1 gene polymorphisms in the Greek population: rheumatoid arthritis and FMF patients relative to normal controls. Homogeneous distribution and low incidence of AA amyloidosis

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Abbreviations: R = rheumatoid arthritis; FMF = familial Mediterranean fever; SAA = serum amyloid A protein

Abstract

Objective. To address whether or not the rarity of amyloidosis in Greek patients with rheumatoid arthritis (RA) is related to specific alleles of single nucleotide polymorphisms (SNPs) in the 5'-flanking region and the exon 3 of the SAA1 gene.

Methods. The genotypes of the −13T/C SNP in the 5'-flanking region of the SAA1 gene and the two SNPs within exon 3 of SAA1 (2995C/T and 3010C/T polymorphisms) were determined in 88 Greek patients with RA, 14 patients with familial Mediterranean fever (FMF) and 110 healthy controls. Linkage disequilibrium and haplotype frequencies involving −13T/C, 2995C/T and 3010C/T in these populations were tested and estimated, respectively.

Results. The genotypic distribution and allelic frequencies were similar in all groups tested. SNPs 2995 and 3010 were in linkage disequilibrium for all study populations (p < 0.05), whereas SNP −13 was not in linkage disequilibrium with either 2995 or 3010 (p ≥ 0.05). Two major haplotypes presented in all patients with RA and FMF and controls: −13C; 2995T; 3010C (−13C; z) and −13C; 2995C; 3010T (−13C; f). The −13T allele was linked with the γ haplotype in Greek patients with RA and controls. The frequency of the −13T allele was found to be very rare in all groups tested.

Conclusions. In conclusion, the rarity of the putative amyloidogenic −13T allele in Greek populations may be related to low prevalence of AA amyloidosis development in Greek RA patients.

Introduction

Reactive or AA amyloidosis is considered to be one of the major long-term complications of chronic inflammatory disorders, such as rheumatoid arthritis (RA) and familial Mediterranean fever (FMF), characterized by extracellular deposition of fibrils usually composed of fragments of the acute-phase reactant serum amyloid A (SAA) protein. SAA is synthesized predominantly in the liver increasing 1000-fold in response to various inflammatory insults [1–3].

The incidence of amyloidosis in patients with RA has a considerable geographic variation between ethnic groups, in a range from 1 to 26% [4]. For instance, in the Japanese population, amyloidosis in longstanding RA has been reported to be 13% [5], in contrast to the USA, where the corresponding figure was estimated to be 1% [6]. In our large series of Greek patients with RA, development of AA amyloidosis was very rare (unpublished observations). Given that amyloidosis is more likely to occur in patients with poorly controlled, more severe and longstanding disease [7] and aggressive treatment of the underlying disease reduces the risk for amyloidogenesis later in life [8,9], the differences reported between populations may reflect differences in the population selected, disease duration or the type of therapy established. In addition, increasing evidence suggests that genetic factors such as single nucleotide polymorphisms (SNPs) within exon 3 of the SAA1 gene may play an important role in the development of AA amyloidosis in RA and FMF populations [10–15].

SAA is encoded by a family of 4 SAA genes, the SAA1, SAA2, SAA3 and SAA4, all clustered in the

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short arm of chromosome 11. The presence of two SNPs within exon 3 of the SAA1 gene, 2995C/T and 3010C/T define three haplotypes that correspond to the SAA1x (2995T-3010C), SAA1β (2995C-3010T), and SAA1γ (2995C-3010C) isoforms (SAA1.1, SAA1.5, SAA1.3, respectively) [16–18].

While data on Japanese patients with RA [10,11] suggest that the SAA1.3 isoform is a definite risk factor for amyloidosis development, while SAA1.1 is protective, in Caucasian populations the presence of the SAA1.1 isoform has been shown to be an amyloidogenic risk factor [12]. The above discrepancy was elucidated in a subsequent study demonstrating that the −13T allele in the 5′-flanking region of SAA1 is a major risk factor for amyloidosis and exon 3 haplotypes display different linkage disequilibrium with the above allele in the different ethnic groups studied [13].

In the present study, we sought to investigate whether known polymorphisms of the SAA1 gene associated with AA amyloidosis risk in Japanese population may be associated with the development of amyloidosis in Greek RA and FMF populations.

Patients and methods

Patients

DNA samples from 88 consecutive patients with RA and disease duration >3 years according to the American College of Rheumatology criteria [19], from 12 Greek patients with FMF without amyloidosis and two FMF patients with AA amyloidosis were evaluated for the presence of three SNPs in the SAA1 gene (−13T/C, 2995C/T and 3010C/T) polymorphisms. RA patients were followed in the Outpatient Rheumatology Clinic, Department of Pathophysiology, University of Athens, Athens, Greece. The mean age (±SD) and disease duration (±SD) of patients with RA were 53.22 ± 12.47 and 11.89 ± 8.48 years, respectively. Three out of 88 patients developed proteinuria in the course of their disease. Two had renal involvement due to concomitant presence of diabetes mellitus, while in the third patient proteinuria was reversible attributed to previous D-penicillamine use. No patient in our RA population had experienced manifestations of secondary amyloidosis such as nephrotic syndrome or intractable diarrhea, although histological evidence of amyloidosis was not excluded. All FMF patients fulfilled the Tel Hashomer criteria [20]. They were followed in the Department of Internal Medicine, Medical School, Democritus University of Thrace, Alexandropolis, Greece. Finally, 110 healthy controls of similar age distribution to the RA patients were evaluated. Patients and controls were all of Greek origin.

DNA analysis

Genomic DNA was extracted from peripheral nucleated blood cells by the salting-out method [21]. Genotypes of the −13T/C SNP in the 5′-flanking region of the SAA1 gene and the two SNPs within exon 3 of SAA1 (2995C/T and 3010C/T polymorphisms) were determined after restriction fragment length polymorphism analysis of polymerase chain reaction amplified DNA, as described previously by Moriguchi et al. [11,13].

Statistical analysis

The genotypes were tested for Hardy–Weinberg equilibrium using the exact test according to Weir [22] as implemented by the GDA software [23]. The loci were tested for linkage disequilibrium using the exact test according to Weir [22] as implemented by the GDA software. The haplotype frequencies with the corresponding standard errors were estimated using an iteration algorithm [24] as implemented by PHASE Version 2 (http://www.stat.washington.edu/stephens/). The genotype distribution and the allelic frequencies of the two diseased groups were compared with the control group using log-linear model [25]. The differences in haplotype frequencies between groups were compared using a permutation test (PHASE Version 2). The significance levels were adjusted using the Bonferroni’s correction.

Results

As it is shown in Table I, no differences were detected in the distribution of genotypes of the three SNPs between the RA, FMF and control groups (p > 0.05). In all groups tested, absence or low

Table I. Distribution of genotypes of three single nucleotide polymorphisms (SNPs) at the SAA1 locus in Greek patients with rheumatoid arthritis (RA) and familial Mediterranean fever (FMF), and controls. (The corresponding standard errors are in parentheses).

<table>
<thead>
<tr>
<th>SNPs</th>
<th>RA (n=88)</th>
<th>FMF (n=12)</th>
<th>Controls (n=110)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−13C/T</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>TC</td>
<td>0.07 (0.025)</td>
<td>0 (0)</td>
<td>0.05 (0.021)</td>
</tr>
<tr>
<td>CC</td>
<td>0.93 (0.025)</td>
<td>1 (0)</td>
<td>0.95 (0.021)</td>
</tr>
<tr>
<td>2995C/T</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>0.25 (0.043)</td>
<td>0.17 (0.110)</td>
<td>0.24 (0.041)</td>
</tr>
<tr>
<td>TC</td>
<td>0.52 (0.050)</td>
<td>0.75 (0.125)</td>
<td>0.50 (0.048)</td>
</tr>
<tr>
<td>CC</td>
<td>0.23 (0.042)</td>
<td>0.08 (0.080)</td>
<td>0.25 (0.042)</td>
</tr>
<tr>
<td>3010C/T</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>0.20 (0.040)</td>
<td>0.0 (0)</td>
<td>0.25 (0.041)</td>
</tr>
<tr>
<td>TC</td>
<td>0.48 (0.050)</td>
<td>0.75 (0.125)</td>
<td>0.50 (0.048)</td>
</tr>
<tr>
<td>CC</td>
<td>0.32 (0.050)</td>
<td>0.25 (0.125)</td>
<td>0.25 (0.041)</td>
</tr>
</tbody>
</table>
frequency of TT and TC genotypes, respectively, was noted in the \(-13\) locus of the \textit{SAA1} gene.

No differences \((p > 0.05)\) between patients with RA and FMF, and healthy controls were noted in terms of allele frequencies (Table II). A low frequency of the \(-13T\) allele was found in the RA group and controls \((0.034\) and \(0.027\), respectively), while in FMF patients the \(-13T\) allele was completely absent.

As shown in Table III, genotype distribution of three SNPs \((-13, 2995, 3010)\) for all study groups was in Hardy–Weinberg equilibrium \((p > 0.05)\), indicating that in the population there was no structure [22].

Table IV shows that SNPs 2995 and 3010 were in linkage disequilibrium for all study populations \((p < 0.05)\), whereas SNP \(-13\) was not in linkage disequilibrium with either 2995 or 3010 \((p > 0.05)\).

The distribution of the haplotype frequency of the three SNPs for the RA and FMF groups and controls is presented in Table V. The differences in haplotype frequencies between groups were compared using a permutation test (PHASE Version 2). The significance levels were adjusted using the Bonferroni’s correction. There was no statistical difference between the controls and the RA group \((p = 0.30)\), and between the RA and the FMF groups \((p = 0.87)\).

### Table II. Allelic frequencies in patients with rheumatoid arthritis (RA) and familial Mediterranean fever (FMF), and in healthy controls. (The corresponding standard errors are in parentheses).

<table>
<thead>
<tr>
<th>SNPs</th>
<th>RA</th>
<th>FMF</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-13C/T)</td>
<td>0.034 (0.018)</td>
<td>0 (0)</td>
<td>0.027 (0.016)</td>
</tr>
<tr>
<td>T</td>
<td>0.966 (0.018)</td>
<td>1 (0)</td>
<td>0.973 (0.016)</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2995C/T</td>
<td>0.512 (0.050)</td>
<td>0.542 (0.144)</td>
<td>0.486 (0.048)</td>
</tr>
<tr>
<td>T</td>
<td>0.488 (0.050)</td>
<td>0.458 (0.144)</td>
<td>0.514 (0.048)</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3010C/T</td>
<td>0.443 (0.049)</td>
<td>0.375 (0.140)</td>
<td>0.496 (0.048)</td>
</tr>
<tr>
<td>T</td>
<td>0.557 (0.049)</td>
<td>0.625 (0.140)</td>
<td>0.504 (0.048)</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SNP, single nucleotide polymorphisms.

### Table III. \(p\)-Values for testing Hardy–Weinberg equilibrium for the three single nucleotide polymorphisms (SNPs) in the \textit{SAA1} gene in Greek patients with rheumatoid arthritis (RA) and familial Mediterranean fever (FMF), and in controls.\(^*\)

<table>
<thead>
<tr>
<th>SNPs</th>
<th>RA ((p)-value)</th>
<th>FMF ((p)-value)</th>
<th>Controls ((p)-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-13C/T)</td>
<td>0.999</td>
<td>0.999</td>
<td>0.999</td>
</tr>
<tr>
<td>2995C/T</td>
<td>0.560</td>
<td>0.241</td>
<td>0.999</td>
</tr>
<tr>
<td>3010C/T</td>
<td>0.817</td>
<td>0.093</td>
<td>0.999</td>
</tr>
</tbody>
</table>

\(^*\)The genotypes were tested for Hardy–Weinberg equilibrium using the exact test according to Weir [22] as implemented by the GDA software [23].

There was marginal association between the controls and the FMF group \((p = 0.06)\); however, the sample size in the FMF group is rather small to draw safe conclusions. Interestingly, the same two major haplotypes presented in all RA and FMF patients and controls: \(-13C; 2995T; 3010C\) \((-13C; \alpha)\) and \(-13C; 2995C; 3010T\) \((-13C; \beta)\). The \(-13T\) allele, when present was linked to the \(\gamma\) haplotype in Greek RA patients and controls. The two patients with FMF and amyloidosis had the following same genotype distribution: \(-13\) SNP: C/C, 2995 SNP: T/T, 3010 SNP: C/C, i.e. lack of the \(-13T\) allele.

### Discussion

In the current report, we asked whether or not the low prevalence of previously shown amyloidogenic SNPs of the \textit{SAA1} gene, could account for the rarity of AA amyloidosis in Greek patients with RA.

Several possibilities need to be considered for the rarity of amyloidosis in Greek RA patients. First, it has been previously shown that these patients are characterized by a milder disease course compared to the RA population of North European origin [26]. Second, certain genetic markers such as the presence of the histocompatibility antigen HLA-DR4 – previously shown to increase amyloidogenesis risk in RA patients [27] – was found to be lower in Greek RA patients compared with their British counterparts [28]. In the present report, we offer a potential alternative explanation for the low prevalence of amyloidosis in Greek RA patients; we suggest that this may be due to the rarity of the amyloidogenic \(-13T\) allele, although solid conclusions in the

### Table IV. \(p\)-Values for testing linkage disequilibrium between the three single nucleotide polymorphisms (SNPs) in Greek patients with rheumatoid arthritis (RA) and familial Mediterranean fever (FMF), and in healthy controls.\(^*\)

<table>
<thead>
<tr>
<th>SNPs</th>
<th>RA</th>
<th>FMF</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-13)</td>
<td>0.526</td>
<td>0.152</td>
<td>0.000</td>
</tr>
<tr>
<td>2995</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3010</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^*\)The loci were tested for linkage disequilibrium using the exact test according to Weir [22] as implemented by the GDA software [23].
absence of a well-matched control population with amyloidosis cannot be drawn.

Given the scarcity of AA amyloidosis in our large RA cohort (approximately 1000 patients followed for more than 10 years), in the current study, only patients with RA without amyloidosis were evaluated. In addition, a number of patients with FMF were also included, as a disease control group prone to develop AA amyloidosis. It should, however, be acknowledged that given the rarity of the disease, the number of recruited FMF patients was limited and no definite conclusions could be made.

Four major findings were revealed from the present study: first, the $-13T$ allele was rare in Greek RA and FMF patients and controls; second, the $-13$ and 2995 polymorphic sites were not in linkage disequilibrium in the Greek RA and FMF patients as well as in the controls; third, the same two major haplotypes appear in these three populations ($-13C, 2995T, 3010C$ and $-13C, 2995C, 3010T$); and fourth, the $-13T$ allele was linked to the $\gamma$ haplotype in all three groups.

All of the above findings in the Greek RA and FMF populations constitute characteristics previously observed in healthy Caucasian populations; the $-13$ SNP site is not in linkage disequilibrium with the 2995 SNP and the $-13T$ allele is rare and mainly associated with the $\gamma$ haplotype [13,14]. While in the Japanese study [13], the $-13T/C$ and 2995C/T SNPs were in linkage disequilibrium and both SNPs were associated with amyloidosis, in our study, the two SNPs were not in linkage disequilibrium and the 2995C allele was frequent in the three study groups.

Consistent with our initial hypothesis, a strong association between the $-13T$ allele and AA amyloidosis in different inflammatory diseases has been recently reported in Caucasian populations [29]. However, in the study of Gerchoni-Baruch et al. [14] no correlation was found between the $-13C/T$ polymorphism and the development of renal amyloidosis in Caucasian FMF patients. While both of our FMF patients shared the SAA1.1 isoform (\(\gamma\) haplotype) – previously proposed as an amyloidogenic risk factor in Caucasians – they also lacked the $-13T$ allele; these findings suggest that other factors may promote active inflammation and amyloidosis development in FMF populations.

If we consider that the $-13T$ allele of the SAA1 gene increases susceptibility for amyloidosis in Caucasian as well as in Japanese RA populations, based on our work, we could assert that our RA patients may not develop amyloidosis since this allele is very rare in this population. While a case–control study including RA patients with and without amyloidosis would be more informative, this was not feasible owing to the rarity of the disease.

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**References**
