**TLR2 and TLR4 polymorphisms in familial Mediterranean fever**

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It has been suggested that MEFV mutations offer advantage against infections, including tuberculosis. Bearing in mind the central role of TLR-2 and TLR-4 in the recognition of pathogens, we conducted this study to examine whether the TLR2-R753Q, TLR4-D299G, TLR4-T399I common polymorphisms are associated with susceptibility to familial Mediterranean fever (FMF) or affect the course of the disease. A cohort of 169 FMF patients and 245 healthy bone marrow donors were enrolled in the study. FMF patients appeared with a significantly lower frequency of the TLR4-D299G mutated allele (3.2% vs 6.9%, \( p = 0.032 \)). No association was observed with the other analyzed polymorphisms. Moreover, we found no association between polymorphisms and the frequency of attacks or the development of amyloidosis. Our results may reinforce the hypothesis that FMF patients display a better defense against pathogens, providing an additional mechanism and suggesting a positive selection advantage in the area of the Mediterranean basin.

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1. Introduction

Familial Mediterranean fever (FMF) is the most frequent hereditary autoinflammatory disease, characterized by self-limited recurrent attacks of fever and serositis [1]. FMF is caused by mutations in MEFV gene, which encodes pyrin [1–3]. Pyrin is implicated in a complex interplay with proteins involved in Toll-like receptor (TLR) signaling, PYD superfamily and procaspase-1 activation, suggesting a controlling role in inflammatory response through leukocyte apoptosis, interleukin-1β production and NF-κB pathway activation [4–6]. Mutations in the MEFV gene result in a pro-inflammatory state and are highly prevalent in the Middle East and Mediterranean basin, with carrier rates of up to 1.3% in some populations [1–3]. Although, no obvious benefit of MEFV mutations has been identified to date [7], the high prevalence, multiple mutations, and geographic localization suggest a positive selection advantage for the abnormal gene operating in the Mediterranean basin over the last 1000 years. Consequently, it has been proposed that MEFV mutations were possibly protective against frequent and lethal infections before the antibiotic era [2,3], operating as balanced polymorphisms, analogous to the protective effect against malaria that maintains high levels of thalassemia and sickle cell trait in Middle East and sub-Saharan Africa.

Until recent years, infectious diseases with a high prevalence rate in the Mediterranean basin were those deriving from intracellular pathogens, as Mycobacterium tuberculosis and Brucella species [2,3]. Moreover, a Th1 profile has been found to be predominant in such infections, including tuberculosis [8], and carriers of MEFV mutations have a Th1 polarization with high levels of interferon-γ [9,10]. Until now, there have been only indirect arguments concerning the protective role of MEFV mutations against intracellular bacteria, including tuberculosis. For example, Cattan reported a low mortality rate from tuberculosis in Jews living in Tunis, compared with the other ethnic populations, during the first half of the twentieth century, and this finding has attributed to the high prevalence of MEFV mutations in individuals of Jewish origin [3]. However, Ozen et al., when analyzing a Turkish population, did not find any significant difference in the frequency of MEFV mutations between patients with tuberculosis and normal controls [11]. Nevertheless, resistance to tuberculosis, the lower severity of tubercular disease, as well as the lower mortality from tuberculosis remain a good hypothesis for MEFV mutation carriers [3].

On the other hand, population variations in susceptibility to infectious diseases have been associated with single nucleotide polymorphisms (SNPs) in a number of genes, especially of innate immunity receptors [12,13]. Among them, SNPs of the TLR2 and TLR4 genes have been associated with receptor hypo-responsiveness and susceptibility to bacterial, fungal and viral infections (reviewed by Schroder and Schumann) [13]. Moreover, TLR-2 and TLR-4 mediate recognition, among other pathogens, of M. tuberculosis and Brucella species, initiating the immune response to infection [14,15], and carriers of the TLR2-R753Q SNP are more susceptible to tuberculosis [16], whereas the presence of TLR4-D299G and TLR4-T399I SNPs have been associated with an increased risk of developing active tuberculosis in HIV-infected patients [17].
Considering the central role of TLR-2 and TLR-4 in the recognition of pathogens, we postulated that the presence of common TLR SNPs, affecting the immune responses against infections, might modify the course of FMF, namely the frequency of attacks and the development of secondary amyloidosis. At present, only one study has been published addressing the influence of the TLR2-R753Q SNP on the disease course of FMF patients, indicating that carriers display a higher incidence of secondary amyloidosis [18]. Thus, the purpose of our study was to determine whether polymorphisms in genes of major importance for innate immunity, in particular TLR2-R753Q, TLR4-D299G and TLR4-T399I, are associated with FMF course and phenotype.

2. Subjects and methods

2.1. Study population

Blood samples were collected from 169 patients with FMF (86 male and 83 female, mean age, 25.8 years; range, 2–60 years), as previously described [19]. The diagnosis of FMF was based on previously established and internationally accepted clinical criteria, including family history, the exclusion of other hereditary periodic fever syndromes and the patient’s response to colchicine treatment [20]. The genetic analysis of MEFV mutations, for the great majority of the patients (152 of 169), has already been performed by us in a previous report [19], and a mean of 1.69 alleles per individual was detected to carry MEFV mutations. The M694V was the most frequent mutation (55.6%), followed by M680I (26.0%), V725A (17.2%), E148Q (13.6%), and E230K (5.9%). Moreover, R202Q homozgyosity was detected in only half of the FMF patients lacking any other classical MEFV mutation (16%); thus, it is considered to be disease-related [19]. In addition, 32.1% of the patients exhibited more than 15, 24.6% exhibited 10–15, 32.1% exhibited six to 10, and 11.2% exhibited three–five attacks during their life. Only five patients (3%) developed secondary amyloidosis, and 39 patients (23%) experienced an inappropriate surgery at least once during their life.

A cohort of 245 healthy bone marrow donors (BMDs) (110 male and 135 female; mean age, 35.5 years; range, 19–65 years) was recruited to serve as normal control group, for the estimation of the prevalence of the analyzed SNPs in the general Greek population.

All samples came from unrelated individuals who were ethnic Greeks, as assessed by questionnaire. Written informed consent was obtained from each individual or an accompanying relative, in case of FMF patients where consent was not legally applicable (e.g., with children). The study was approved by the institutional genetic review board of the University Hospitals of Larissa and Alexandroupolis.

2.2. Molecular techniques

Genomic DNA was extracted from peripheral blood using the QiAamp DNA Blood Mini Kit (Qiagen, Crawley, UK), according to manufacturer’s instructions. The detection of TLR4-D299G and TLR4-T399I polymorphisms was performed by allele-specific polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP) analysis, as described previously [21,22]. In brief, the forward primers, in both reactions, were modified at the 5’ end, creating restriction enzyme recognition sites (NcoI for the TLR4-D299G polymorphism and HinfI for the TLR4-T399I), so that if a polymorphism is present, PCR-RFLP analysis will create digestion fragments, visible on agarose gels [21,22]. The detection of TLR2-R753Q polymorphism was also performed by PCR-RFLP. The protocol was designed based on the fact that the polymorphism results in the creation of a DNA sequence recognized by the restriction enzyme SfI. Primers used for the PCR amplification of TLR2 gene were previously described by Ogus et al. [16]. All PCR and digestion procedures were carried out in the PCR-engine apparatus PTC-200 (MJ Research, Watertown, MA), and the PCR and digestion products were analyzed in 2% TBE agarose gels.

For the confirmation of PCR-RFLP results, randomly chosen PCR products, positive and negative for the TLR polymorphisms, were purified by Qiagen PCR Purification System (Qiagen, UK) and directed sequenced using an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) and a Big Dye Terminator DNA sequencing kit (Applied Biosystems).

2.3. Statistical analysis

Linear correlation studies were performed using the parametric Pearson’s correlation coefficient (r) when appropriate. To exclude false positive results, correction of type II errors, a Bonferroni correction was applied to compromise the level of statistical significance. A χ² test was used for the comparison between discrete parameters, along with Yates’ correction when appropriate (especially when expected absolute values <5). A χ² of goodness of fit was used for the estimation of poten deviation from Hardy-Weinberg principle. During this procedure, comparison between observed (p²obs, 2pqobs, and q²obs) and expected (p²exp, 2pqexp, and q²exp) relative genotype frequencies based on relative allelic frequencies p and q, were performed for each studied polymorphism.

The Student’s t-test for unpaired samples was used for comparison between quantitative parameters when allowed by the number of cases (>10) and the normality tests (Kolmogorov-Smirnov test, Lilliefors test); otherwise, the non-parametric Mann-Whitney U test or the median test were preferred. All means and odds ratios (OR) are accompanied by their 95% confidence intervals (95% CI). A variable was considered significant at p < 0.05. All tests were carried out using the free online statistical tools found at http://statpages.org and the STATISTICA 6.0 software.

2.4. Population genetics analysis

The ARLEQUIN 2.0 software was used for computing non-differentiation exact p value (after 10,000 Markov steps done), Reynolds’ distance (genetic distance based on F statistics) from haplotype frequencies, gene diversity (H) (the probability that two randomly chosen haplotypes are different in the population), θ (estimation of nucleotide polymorphism, equivalent to the expected heterozygosity from H (θ), and Ewens-Watterson neutrality test (based on Ewens sampling theory in a population at equilibrium) [23]. Finally, the DnaSP 4.1 software was used for computing linkage disequilibrium between TLR4-D299G and TLR4-T399I SNPs [24].

3. Results

3.1. Population demographics

As presented in Table 1, gender was comparable between the two groups (p = 0.230), but FMF patients were much younger than BMDs, considering that FMF group included children. However, this was intentional, to exclude late-onset FMF cases.

3.2. TLR polymorphisms analysis in FMF patients and BMDs

TLR allelic and genotypic data are provided in Table 1. None of the FMF patients showed homozgyosity for any SNP. The allele frequency of TLR4-D299G SNP was lower in FMF patients compared with BMDs (3.2% vs 6.9%), and the difference was statistically significant (p = 0.032). The allele frequencies of the other analyzed SNPs did not differ between the groups (4.1% vs 6.7%, p = 0.227 for the TLR4-T399I and 0.9% vs 0.6%, p = 0.966 for the TLR2-R753Q).

Moreover, no association of TLR2-R753Q, TLR4-D299G and TLR4-T399I SNPs was observed with the frequency of attacks (11.0 ± 3.0 for the carriers of TLR2-R753Q vs 13.6 ± 1.1 for wild-type patients, p = 0.598; 14.9 ± 4.4 for the carriers of TLR4-D299G vs 13.5 ± 1.2 for wild-type patients, p = 0.490; 13.7 ± 4.0 for the carriers of TLR4-T399I vs 13.6 ± 1.2 for wild-type patients, p = 0.908; the
analysis was performed by Mann–Whitney U test), the presence of skin involvement (no one carrier of TLR2-R753Q vs one wild-type patient, \( p = 1.000 \); no one carrier of TLR4-D299G vs one wild-type patient, \( p = 1.000 \); no one carrier of TLR4-T399I vs one wild-type patient, \( p = 1.000 \); the analysis was performed by \( \chi^2 \) test along with Yates’ correction), or the development of secondary amyloidosis (no one carrier of TLR2-R753Q vs five wild-type patients, \( p = 1.000 \); one carrier of TLR4-D299G vs four wild-type patients, \( p = 0.748 \); one carrier of TLR4-T399I vs four wild-type patients, \( p = 0.888 \); the analysis was performed by \( \chi^2 \) test along with Yates’ correction).

Finally, a strong correlation between both TLR4-D299G and TLR4-T399I polymorphisms and the rare MEFV-Q167D mutation was detected (\( p < 0.001 \)). This correlation was still significant after Bonferroni correction, which lowers the level of statistical significance to \( \alpha = 0.00104 \) (derived from the initial level of statistical significance 0.05 divided by the sum of three polymorphisms studied by 16 different MEFV mutations checked).

3.3. Linkage disequilibrium between TLR4-D299G and TLR4-T399I SNPs

We confirmed that the G allele of TLR4-D299G SNP has a high level of linkage disequilibrium with the allele C of TLR4-T399I SNP (\( p < 0.001 \)), for both patients and normal individuals, as reported previously [25]. In detail, D’ and R was 1.000 and 0.786 for FMF patients and 0.938 and 0.924 for BMDs, respectively.

3.4. Hardy–Weinberg principle among TLR SNPs

The analysis suggests that both the TLR4 gene polymorphisms obey Hardy–Weinberg principle in the group of FMF patients (\( p = 0.509 \) and \( p = 0.854 \), for the D299C and T399I SNPs, respectively). In contrast, there is a strong deviation in the group of BMDs (\( p < 0.001 \) for both SNPs). On the other hand, TLR2-R753Q SNP shares the same deviant behavior in both populations (\( p = 0.993 \) and \( p = 0.470 \) for FMF patients and BMDs, respectively).

3.5. Population genetics analysis

FMF patients and BMDs present differences concerning the gene diversity (0.1552 ± 0.0366 and 0.2371 ± 0.0329, respectively), observed (0.84573 and 0.76383, respectively) and expected homozygosity (0.59141 and 0.62204, respectively) and Ewens–Watterson \( p \) value (0.889 and 0.721, respectively). In addition, the mean non-differentiation exact \( p \) value (after 10,000 Markov steps were done) yields a statistically significant result when PCR-RFLP allelic data are considered as genotypes (0.0446) rather than alleles (0.0590).

4. Discussion

To date, relatively little attention has been paid to the role of innate immune receptors in the pathophysiology of FMF. To the best of our knowledge, this is the first study in the literature demonstrating a clear negative association between TLR4-D299G and FMF. Thus, our findings may further support the hypothesis that FMF patients display a better defense against bacterial pathogens, providing an additional mechanism and suggesting a positive selection advantage in the area of the Mediterranean basin.

This finding may also be supported by the Hardy–Weinberg analysis, as BMDs demonstrate a statistically significant deviation, in contrast with FMF patients, who share an established equilibrium. This implies that the “normal” state might be exposed to evolutionary trends regarding the SNPs examined, which in turn reflect the hypothesized advantage of MEFV mutation carriers concerning defense against pathogens. The above finding is also supported by the statistical significance of the non-differentiation test between FMF patients and BMDs, as mentioned above.

Until now, only Ozen et al. had examined the influence of one TLR SNP, namely TLR2-R753Q, on the disease course of FMF patients [18]. In particular, they analyzed 115 patients with FMF and found that the TLR2-R753Q SNP was significantly more frequent in FMF patients (25.2%) compared with normal controls (6%). This finding was more prevalent in the 40 patients who developed secondary
amyloidosis (TLR2-R753Q frequency 37.5%) [18]. However, our study cannot confirm these interesting findings, since a quite low incidence of TLR2-R753Q SNP (allele frequency 0.9% in FMF patients and 0.6% in BMDs) was found that is in accordance with previous studies in Western populations [26,27] and different from those in Asian populations [16,18]. Similarly with other SNPs [28], this difference could be attributed to ethnic variations and might evoke significant consequences. In particular, the proportion of our patients who developed secondary amyloidosis was quite lower compared with the study of Ozen et al. (3% vs 34.7%). It is well known today that colchicine treatment prevents the development of secondary amyloidosis; however, amyloidosis development varies in different ethnic populations and it is more frequent among the people living in the eastern Mediterranean, compared with individuals living in the United States [29] or Greece [19]. It has been proposed that M694V homozygocity, male gender and the a/a genotype of serum amyloid A1 gene are possible risk factors for development of amyloidosis [1,30]. However, taking into account both the study of Ozen et al. and our findings, it is not unreasonable to speculate that another genetic factor, such as the presence of TLR2-R753Q SNP, might affect the disease course, contributing to the development of secondary amyloidosis.

Considering all of the data mentioned above, we could not exclude the possibility that our findings may result from genetic differences between Asian and Caucasian populations. Indeed, several genetic association studies have been published for several diseases and results are often conflicting. Nevertheless, taken together both our findings and the results of Ozen et al., we can speculate that FMF phenotype might be affected by the genotype of other genes participating in inflammation and/or immune responses against infections. Considering the presence of ethnic variations in the prevalence of both SNPs of immune genes and MEVF mutations, the differences of FMF phenotype, observed in different areas of Mediterranean basin, could be attributed to them. Further analysis of FMF patients from other Mediterranean areas could confirm this hypothesis.

Finally, the proposed correlation between both TLR4 SNPs and the rare MEVF-Q167D mutation, although documented using Bonferroni correction, could be accidental finding, because of the very limited subpopulation carrying the mutation (two individuals).

In conclusion, our findings indicate a negative association of TLR4-D299G with FMF. This may further support the consideration that FMF patients display a better defense against bacterial pathogens, providing an additional mechanism and suggesting a positive selection advantage in the area of the Mediterranean basin.

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