MEFV gene 3'-UTR Alu repeat polymorphisms in patients with familial Mediterranean fever

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ABSTRACT

Objective. Familial Mediterranean fever (FMF), an autosomal recessively inherited autoinflammatory disorder, is caused by missense mutations in the pyrin-encoding MEFV gene. The MEFV mutations can be detected in the majority of FMF patients, but there is an important proportion of patients with the FMF phenotype who carry a single or no coding region mutation. This study aimed to investigate the promoter region and 3'-UTR polymorphisms of the MEFV gene in a group of FMF patients with no coding region mutations, to identify variations with a possible role in the regulation of MEFV expression.

Methods. The study group consisted of 289 patients with FMF and 103 ethnically-matched healthy individuals of Turkish origin. All individuals were first genotyped for the five most commonly observed mutations (M694V, M680I, V726A, E148Q and M694I). Then, the coding regions of the MEFV gene in patients carrying none of the 5 mutations were amplified and screened using single-stranded conformation polymorphism and DNA sequencing. After the exclusion of patients with mutations in exons, the promoter and 3'-UTR regions of the MEFV gene were investigated in the remainder. For the haplotype analysis, all study groups were genotyped for two of the 3'-UTR single nucleotide polymorphisms (SNP).

Results. Genotyping for five mutations revealed 186 patients (64.4%) with two mutations, 61 patients (21.1%) with one mutation, and 42 patients (14.5%) with no mutation. The carrier rate for healthy controls was found to be 10%. After screening all 10 exons in the patients with none of the 5 mutations, we identified 36 patients (12.5%) who had no coding region mutations. Analysis of the 3’-UTR region in these patients showed two Alu repeats (AluSx and AluSq), which were located in the 3’-UTR of the reference mRNA sequence. Sequencing of the 3’-UTR of the MEFV gene showed several SNPs that were clustered in 2 haplotypes. When we genotyped all study groups for two of the 3’-UTR SNPs (rs2741918 and rs450021), we observed a significant increase in the frequency of heterozygotes for the 3’-UTR haplotypes in the FMF patients with no coding region polymorphisms compared to the healthy controls (75% versus 48.5%, p=0.006, OR=3.2, 95% CI 1.4-7.4).

Conclusion. This study showed a group of 3’-UTR polymorphisms in the MEFV gene that are clustered in two haplotypes. In addition, a genetic association was observed between 3’-UTR polymorphisms and the FMF patients with no coding region mutations. These findings may suggest a role for 3’-UTR sequences in the regulation of MEFV expression.

Introduction

Familial Mediterranean fever (FMF, OMIM 249100), the most common type of hereditary autoinflammatory syndrome, is characterized by recurrent episodes of inflammation affecting the serosal membranes, joints and skin, as well as by a tendency to develop AA-type amyloidosis (1). FMF is more prevalent in the Mediterranean basin and the Middle East, mainly in individuals of Jewish, Armenian, Turkish and Arab ancestry, and it is inherited as an autosomal recessive disorder with some exceptions (1-3).

Most FMF patients carry mutations in the MEFV gene, which encodes the pyrin protein (4, 5). Pyrin, a 781-amino acid protein expressed in granulocytes, monocytes and dendritic cells, as well as in synovial, peritoneal and skin-derived fibroblasts, has been suggested to play an important role in innate immunity by acting as a negative regulator of innate and adaptive immunity in the inflammatory, serosal, and serous membranes. The MEFV gene is located on the long arm of chromosome 16, and the FMF phenotype is caused by missense mutations of the MEFV gene, which encodes the pyrin protein. The pyrin protein is a member of the pyrin family of proteins, which are involved in the regulation of innate immunity. The pyrin protein has been shown to have anti-inflammatory and anti-apoptotic effects, and to be involved in the regulation of the innate immune response.

Competing interests: none declared.
immunity by regulating the processing of IL-1β, the activation of NF-kB and apoptosis (6). More than 50 FMF-associated MEFV gene mutations have been described to date (http://fmf.igh.cnrs.fr) (7). Almost all of these variations are conservative missense mutations, and their pathogenic mechanism in the development of FMF manifestations has yet to be identified (3, 8, 9). In addition, a varying frequency of patients with the FMF phenotype who carry a single or no coding region mutation in the MEFV gene have been reported in different series (10-15).

Two recent studies showed reduced MEFV mRNA levels in patients with FMF (16, 17). A decreased MEFV mRNA level was also found to be associated with inflammation in otherwise healthy individuals who were operated on for acute appendicitis (17). In a THP-1 cell line model, Chae and colleagues demonstrated augmented IL-1β production in response to bacterial lipopolysaccharide (LPS) following the inhibition of MEFV expression using small interfering RNA (siRNA) (8). These observations may suggest that the amount of MEFV mRNA is important in the regulation of inflammation, as well as in the pathogenesis of FMF, and that some cis- or trans-acting polymorphisms could play a role in gene expression by interfering with transcription, mRNA stability or translation. Papin et al. investigated the promoter region of the MEFV gene, and identified a 243-bp region flanking the 5'-end of the coding region that had a role in MEFV expression in TNF-α treated cells (18). To date, however, no study has been carried out on the sequence variations in the 3'-untranslated region (UTR) of the MEFV gene.

In this study, we aimed to investigate the promoter region and 3'-UTR polymorphisms of the MEFV gene in a group of FMF patients with no coding region mutations, in order to identify variations with a possible role in the regulation of MEFV expression.

Patients and methods

Patients

The study group consisted of 289 independent patients with FMF (134 male, 155 female) who were being followed up at two medical schools of Istanbul University. All patients fulfilled the clinical criteria for the classification of FMF (19). Their mean ± SD age was 29.9±11.8 yrs., and the mean ± SD age at onset was 10.8±8.4 yrs. We used 103 ethnically matched healthy individuals (33 male, 70 female) as controls. The ethics committee of Istanbul Faculty of Medicine approved the study protocol, and written informed consent was obtained from all participants.

Mutation analysis

We isolated genomic DNA from venous blood using a HighPure PCR Template Preparation kit (Roche Diagnostics, Germany). In the first step of this study, all patients were screened for the 5 most frequently observed MEFV mutations using the restriction fragment length polymorphism method for the M694V, M680I, V726A and E148Q mutations, and an allele-specific oligonucleotide method for M694I as described previously (17).

In the next step, we screened the coding regions of the MEFV gene in patients carrying none of the five mutations. All ten exons of the MEFV gene were amplified using PCR with primers as described previously (4), and the PCR products were screened by single-stranded conformation polymorphism (SSCP) analysis using GeneGel Excel 12.5/24 gels and the GenePhor flatbed electrophoresis system (Amersham Biosciences, Sweden). All polymorphic samples were analyzed for variations by automated DNA sequencing.

We then investigated the promoter and 3'-UTR regions of the MEFV gene in the remaining patients who had no coding region polymorphisms. A 978-bp segment of the promoter region was amplified using a forward primer of 5'-CAGGAGTTCGAGACCAG-3' and a reverse primer of 5'-CAGGGTATCGAGACCGAG-3', employing the TAKARA LA Long Taq PCR System (Takara Co., Japan) and following the manufacturer’s instructions. We then used two sets of primers to amplify shorter overlapping segments of the 3'-UTR region: UTR1-forward 5'-AGCTGCTATGGGAAATGCAA-3' and UTR1-reverse 5'-GGTCAGGAGTTCTGAGACCGAG-3'; and UTR2-forward 5'-CGATCTCAGCTCAGTCAAC-3' and UTR2-reverse 5'-ACATGCCAAATGTTTTTCCCA-3'. The UTR1 sequences covered a 600-bp region next to the last exon (from the +86th nucleotide of the 3'-UTR to +685), and UTR2 covered a more distal segment of 700-bp 3'-UTR from +513 to +1212.

We cloned the amplified 3'-UTR sequences recovered from the agarose gel using a GelExtract kit (Qiagen, Germany). Amplified fragments of the 3'-UTR region of the MEFV gene were cloned into the pGEM-T easy vector by T-A cloning according to manufacturer's manual (Promega, USA). Mini-preps prepared using a kit (Qiagen, Germany) were tested by PCR and selected positive clones were sequenced using universal T7 and SP6 primers.

Bioinformatics

We used NM_000243.1 as the reference mRNA sequence for the MEFV gene, and the reference assembly of chromosome 16 (NC_000016) for comparisons of genomic sequences and primer design. We searched the genomic sequences for Alu repeat polymorphisms in FMF using RepeatMasker software (A.F.A. Smit, R. Hubley & P. Green RepeatMasker at http://repetmasker.org).

Statistical analysis

We compared the genotype frequencies of the 3'-UTR polymorphisms between patients and healthy controls using a χ² test and also calculated the odds ratios with 95% confidence intervals.

Results

Mutation screening

Genotyping of all study groups for MEFV poly(A) signal, with a forward primer of 5'-AAGATCCAACTCCTCCACACA-3' and a reverse primer of 5'-AACAAGGGAGAATGACGGA-3', employing the TAKARA LA Long Taq PCR System (Takara Co., Japan) and following the manufacturer’s instructions. We then used two sets of primers to amplify shorter overlapping segments of the 3'-UTR region: UTR1-forward 5'-AGCTGCTATGGGAAATGCAA-3' and UTR1-reverse 5'-GGTCAGGAGTTCTGAGACCGAG-3'; and UTR2-forward 5'-CGATCTCAGCTCAGTCAAC-3' and UTR2-reverse 5'-ACATGCCAAATGTTTTTCCCA-3'. The UTR1 sequences covered a 600-bp region next to the last exon (from the +86th nucleotide of the 3'-UTR to +685), and UTR2 covered a more distal segment of 700-bp 3'-UTR from +513 to +1212.

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Statistical analysis

We compared the genotype frequencies of the 3'-UTR polymorphisms between patients and healthy controls using a χ² test and also calculated the odds ratios with 95% confidence intervals.

Results

Mutation screening

Genotyping of all study groups for
the five mutations revealed 186 patients (64.4%) with two mutations, 61 patients (21.1%) with one mutation, and 42 patients (14.5%) with no mutation (Table I). The carrier rate for the matched healthy controls was found to be 10% (Table I).

All ten exons of the MEFV gene in those 42 FMF patients with no mutations were amplified, and the PCR products were run on a flatbed SSCP electrophoresis system. Sequencing of the polymorphic bands revealed 6 more patients with MEFV mutations: 1 patient homozygous for E167D (exon 2), 2 patients heterozygous for I591T (exon 9), 2 patients heterozygous for K695R (exon 10), and 1 patient heterozygous for A744S (exon 10). The remaining 36 patients (12.5%) comprised the study group for promoter region and 3'-UTR screening.

**Table I. Results of the first screening of FMF patients and healthy controls for five frequent mutations.**

<table>
<thead>
<tr>
<th>FMF patients (n=289)</th>
<th>Mutation</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 mutations (n=186)</td>
<td>M694V / M694V</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>M694V / M680I</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>M694V / V726A</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>M694V / E148Q</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>M694V / M694I</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>M680I / M680I</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>M680I / V726A</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>M680I / E148Q</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>V726A / V726A</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>V726A / E148Q</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>V726A / M694I</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>E148Q / E148Q</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>M694V/V726A-E148Q</td>
<td>1</td>
</tr>
<tr>
<td>1 mutation (n=61)</td>
<td>M694V / ?</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>M680I / ?</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>V726A / ?</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>E148Q / ?</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>M694I / ?</td>
<td>1</td>
</tr>
<tr>
<td>Healthy controls (n=103)</td>
<td>M694V / ?</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>M680I / ?</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>E148Q / ?</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>M694V / E148Q</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>V726A / E148Q</td>
<td>1</td>
</tr>
</tbody>
</table>

**Promoter region and 3'-UTR polymorphisms**

Investigation of the amplified 978-bp segment of the MEFV gene promoter region revealed no polymorphic site in 36 patients with no coding region polymorphism. However, analysis of the 3'-UTR region showed two Alu repeats (a 303-bp AluSx and a 305-bp AluSq), which were located in the 3'-UTR of the reference mRNA sequence, NM_000243.1. The search for the reference genomic sequence of the MEFV gene with RepeatMasker revealed several Alu repeats in introns 1, 2, 4 and 5. We also found 3 Alu repeats in the 5'-UTR of the MEFV gene outside the screening limits of the promoter region in this study: AluJo (reverse, −2370 to −2056), AluJo (forward, −1493 to −1200), and AluSx (reverse, −1141 to −837).

Sequencing of the 3'-UTR of the MEFV gene showed several SNPs, which were clustered in 2 haplotypes. We identified 2 SNPs [rs2741919 (G>A) and rs2741918 (G>A)] before the Alu sequences, 3 SNPs [rs11466051 (C>G), rs11466052 (A>G), rs11466053 (T>C)] in the first Alu repeat (AluSx), and 1 SNP in the second Alu repeat (AluSq). Because of the tri-nucleotide (ACT) insertion/deletion polymorphism in the AluSx sequence (rs34895148), we named the haplotypes as I (insertion)-haplotype and D (deletion)-haplotype (Table II). Based on the SNP findings from the coding region analysis, we determined that I- and D-haplotypes of the 3'-UTR region of the MEFV gene were in linkage disequilibrium (LD) with the previously reported haplotypes of the LD block extending from exon 3 to exon 10 (data not shown) (4, 21).

We genotyped all patients and healthy controls for rs2741918 and rs450021 using the UTR1 and UTR2 primer sets, and HphI and MspI restriction enzymes, respectively. Both SNPs were in full LD both in healthy controls and the FMF patients. Similar to the findings of an earlier study on MEFV cloning (4), the MEFV mutation genotype data showed that all the M694V mutations were associated with the D-haplotype (rs2741918-G and rs450021-G alleles) and >94% of the M680I and V726A mutations were associated with the I-haplotype (rs2741918-A and rs450021-T alleles).

We observed a significant difference in the distribution of 3'-UTR I/D haplotypes between the patients and healthy controls (Fig. 1). Because of the increased frequency of M694V mutations in FMF patients identified as having 2 mutations in the first step of the study, the D/D haplotype frequency was found to be increased in those patients compared to healthy controls (p < 0.001, OR=3.1, 95% CI 1.8-5.2). Patients with one mutation were not screened for all coding regions of the MEFV gene, and some of them may have had another MFM-associated mutation. Therefore, we did not perform any statistical analyses on this group of patients. In FMF patients who had no coding region mutations (n=36), the overall distribution of I/D haplotypes was significantly different in those patients compared to healthy controls (χ2 = 8.1, two degrees of freedom, p=0.018), and a marked increase in the frequency of I/D heterozygotes was observed in the patient group compared to healthy controls (75% vs. 48.5%, p=0.006, OR=3.2, 95% CI 1.4-7.4).

**Cloning of the genomic DNA**

For the verification of the haplotypes, we cloned the 3'-UTR region of the MEFV gene using genomic DNA from 10 FMF patients with a single or no M694V mutation. Although the clones confirmed the clustering of SNPs in the I- and D-haplotypes, we found differences between the 3'-UTR structure of the reference mRNA sequence (NM_000243.1) and that of the observed 3'-UTR haplotypes. The first five 3'-UTR SNPs (rs2741919, rs2741918, rs11466051, rs11466052, rs11466053) of the reference sequence were compatible with the D-haplotype, whereas the polymorphisms in the AluSx sequence (ACT insertion rs34895148 and rs450021) were compatible with the I-haplotype.

Sequencing of different clones from the same patient also showed a heterogeneity in the length of the poly-T sequences in both Alu repeats in each clone.
The MEFV gene 3'-UTR polymorphisms in FMF patients and healthy controls. A search for the genomic sequence of the MEFV gene revealed several Alu repeats, two of which were found to be located in the 3'-UTR region of the transcribed MEFV mRNA and 3'-UTR haplotypes involved these Alu repeats. In this study, we observed a significant increase in the frequency of 3'-UTR haplotype heterozygosity in FMF patients who had no MEFV-coding region mutations compared to healthy controls. In most of the monogenic disorders it is not unexpected to find a group of patients with no mutations in the causative genes, and this condition has usually been explained by genetic heterogeneity or phenocopying. In almost all MFM studies carried out in populations with a different genetic background, varying frequencies of patients with unidentified MEFV mutations have been reported. One of the possible explanations for these results may be differences in mutation screening methods, which did not cover the whole coding regions in all studies. Definition of the FMF phenotype may be another important contributory factor in the varying frequency of patients with no MEFV mutations. There are always exceptional cases in which making the differential diagnosis from other hereditary autoinflammatory disorders can be very difficult (22), and this may be especially true for studies conducted in populations where FMF is not prevalent. The cohort in the present study consisted of FMF patients of Turkish origin who showed a good response to colchicine treatment and no clinical features suggestive of other autoinflammatory disorders. Although we cannot rule out the possibility that some patients with other autoinflammatory conditions were included in the study group, we consider this to be very unlikely. Genetic heterogeneity could be a possible explanation for why some FMF patients have no MEFV mutations (23), and mutations or polymorphisms in as yet unknown components of the inflammasome complexes may be associated with a phenotype quite similar to FMF. However, regulatory polymorphisms or mutations affecting the expression of the MEFV gene and resulting in decreased mRNA levels may be another explanation for those FMF patients with no coding region mutations. Our findings reveal for the first time a positive genetic association between the 3'-UTR region of the MEFV gene and FMF patients with no mutations. These results suggest that there may be some regulatory polymorphisms in the haplotypes extending from exon 3 to 3'-UTR of the MEFV gene, and these polymorphisms may contribute to the FMF phenotype at least in a subgroup of patients.

Selection of a proper healthy control group is critical in the definition of a genetic association that may have functional importance. In Turkey a regional variation in the composition of healthy controls is to be expected that could affect the allele frequencies in some SNPs. In this study, one of the primary criteria in the recruitment of our healthy control group was to match the birthplace of the subjects in the disease group. The MEFV mutation carrier rate was found to be much lower than the 20% reported by Yilmaz et al. in a study conducted in Ankara, a city in central Anatolia (24). However, the main difference in the carrier rates between the patients and healthy controls was due to the higher frequency of the less penetrant E148Q mutation (12%) in the study by Yilmaz et al. (24).

Our search for the genomic segment where the MEFV gene is located shows that this region is very rich in Alu

Table II. MEFV gene 3'-UTR polymorphisms clustered in I- and D-haplotypes. Sequences amplified with UTR1 primers included rs2741919, rs2741918, rs11466051 and rs11466052 polymorphisms, and sequences amplified with UTR2 primers included rs11466053, rs34895148 and rs450021 polymorphisms.

Discussion

Investigation of the 3'-UTR region of the MEFV gene showed several SNPs clustered in two haplotypes. A search for the genomic sequence of the MEFV gene revealed several Alu repeats, two of which were found to be located in the 3'-UTR region of the transcribed MEFV mRNA and 3'-UTR haplotypes involved these Alu repeats. In this study, we observed a significant increase in the frequency of 3'-UTR haplotype heterozygosity in FMF patients who had no MEFV-coding region mutations compared to healthy controls. In most of the monogenic disorders it is not unexpected to find a group of patients with no mutations in the causative genes, and this condition has usually been explained by genetic heterogeneity or phenocopying. In almost all MFM studies carried out in populations with a different genetic background, varying frequencies of patients with unidentified MEFV mutations have been reported. One of the possible explanations for these results may be differences in mutation screening methods, which did not cover the whole coding regions in all studies. Definition of the FMF phenotype may be another important contributory factor in the varying frequency of patients with no MEFV mutations. There are always exceptional cases in which making the differential diagnosis from other hereditary autoinflammatory disorders can be very difficult (22), and this may be especially true for studies conducted in populations where FMF is not prevalent. The cohort in the present study consisted of FMF patients of Turkish origin who showed a good response to colchicine treatment and no clinical features suggestive of other autoinflammatory disorders. Although we cannot rule out the possibility that some patients with other autoinflammatory conditions were included in the study group, we consider this to be very unlikely. Genetic heterogeneity could be a possible explanation for why some FMF patients have no MEFV mutations (23), and mutations or polymorphisms
repeats. Alu repeats, the largest family of mobile elements in the human genome, has been found to be associated with various genetic disorders and to be responsible for deletions of large genomic segments (25, 26). Some Alu repeats may also play a functional role, and more than 5% of the transcribed genes have Alu repeats in their mRNA transcripts, some of which may have a regulatory function in mRNA turnover (27-30).

In conclusion, this study shows a group of 3'-UTR polymorphisms within two Alu repeats in the MEFV gene, which are clustered in 2 haplotypes, and a genetic association has been observed between the 3'-UTR polymorphisms and FMF patients with no coding region polymorphisms. These findings may suggest a role for 3'-UTR sequences in the regulation of MEFV expression. Modulation of MEFV expression through Alu repeats or other putative regulatory sequences on the extended haplotypes needs to be analyzed in functional studies.

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