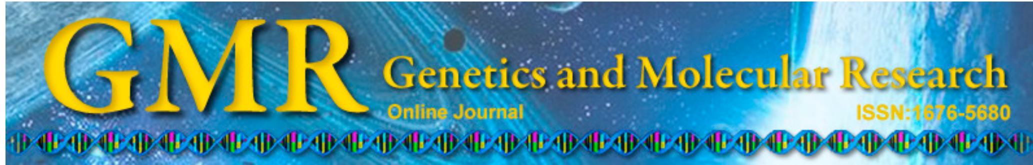

Decreased *MEFV* gene expression in rheumatoid arthritis patients

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ABSTRACT. Rheumatoid arthritis (RA) is a major cause of adult chronic inflammatory arthritis and an autoimmune disease of unknown etiology in which the inflammatory pathology involves T cell activation. Genetic mutations in the Mediterranean fever (*MEFV*) gene, encoding pyrin, influence the severity of RA, but the underlying mechanisms are not completely understood. In this study, we investigated whether the full-length *MEFV* gene (*MEFV*-fl) and the exon 2-deleted splice isoform (*MEFV*-d2) expression are associated with or responsible for the clinical conditions of RA. This study include 47 patients with RA and 47 age- and gender-matched healthy controls. Quantitative real-time polymerase chain reaction analysis was performed to examine transcriptional changes in *MEFV* gene expression from peripheral blood samples. Reverse transcription-polymerase chain reaction of peripheral blood cells revealed the downregulation of *MEFV*-fl mRNA in non-treated patients compared with healthy controls and treated patients. *MEFV*-d2 expression was not different between groups. This

is the first study to investigate the expression of *MEFV* transcript in RA. Deregulation of the *MEFV* gene is likely to result in uncontrolled inflammation as observed in RA. Therefore, downregulation of *MEFV*-fl may be involved in the pathogenesis of early-stage RA and treatment and may ameliorate *MEFV*-fl expression.

Key words: Interleukin-1β; Mediterranean fever gene expression; Mediterranean fever gene exon 2-deleted splice isoform; Pyrin; Rheumatoid arthritis

INTRODUCTION

Rheumatoid arthritis (RA) is a heterogeneous chronic autoimmune disease (Gay et al., 1993) that predominantly involves the synovial joints (Choy and Panayi, 2001). RA is characterized by 3 main symptoms: inflammation, abnormal immune response, and synovial proliferation. Gene-environment interactions appear to be important underlying causes of RA (Carmona et al., 2010; Raterman et al., 2012). Currently, no curative treatment is available, and patients must undergo a prolonged course of treatment (Carmona et al., 2010).

The Mediterranean fever (*MEFV*) gene is located on chromosome 16p13.3 and encodes a 781-amino acid protein known as pyrin or marenostrin. The gene is responsible for familial Mediterranean fever (FMF) (French FMF Consortium, 1997). Pyrin/marenostrin (P/M) is primarily expressed in neutrophils, eosinophils, cytokine-activated monocytes, dendritic cells, and synovial fibroblasts (Kireçtepe et al., 2011a). Pyrin is involved in a complex interaction with the pyrin-domain protein superfamily and lipopolysaccharides via the Toll-like receptor family and procaspase 1 activation that controls inflammation through leukocyte apoptosis (Akkoc et al., 2010). To date, approximately 80 disease-associated mutations have been identified in *MEFV* and registered in the autoinflammatory mutation database Infevers (http://fmf.igh.cnrs.fr/infevers/) (Milhavet et al., 2008). Most of these mutations are missense mutations localized to exons 2 and 10 (Rabinovich et al., 2005).

Alternative splicing and nonsense-mediated decay pathways are regulated at the post-transcriptional level in the *MEFV* gene (Grandemange et al., 2009). There are several isoforms of the *MEFV* gene, including *MEFV*-d2, *MEFV*-8ext, *MEFV*-4a, *MEFV*-2a, -del34, -del234, -del2345, -del7, and -del78, which are alternative splicing isoforms (Medlej-Hashim et al., 2010). The exon 2-lacking *MEFV*-d2 splice variant generated by an in-frame splice removal of exon 2 encodes for a 570-amino acid form, accounting for 27% of the coding sequence. Its expression pattern is similar to that of full-length *MEFV* (*MEFV*-fl). MEFV-d2 is expressed in polymorphonuclear cells, as well as in a subpopulation of peripheral blood leukocytes enriched in mononuclear cells. Full-length P/M (MEFV-fl) is located in the cytoplasm, while the MEFV-d2 transcript is primarily located in the nucleus (Papin et al., 2000). This alternative splice event may be affected by fluctuations in physiological or pathological conditions.

Decreased *MEFV* gene expression in peripheral leukocytes of FMF patients has been reported in 3 previous studies (Notarnicola et al., 2002; Ustek et al., 2007; Kireçtepe et al., 2011a,b). *MEFV* may have a modifying effect on the expression of certain inflammatory diseases such as RA (Akkoc et al., 2010). Although *MEFV* gene mutations have been described in patients with juvenile idiopathic arthritis (JIA) (Rozenbaum and Rosner, 2004), palindromic

rheumatism (Cañete et al., 2007), and RA (Rabinovich et al., 2005; Migita et al., 2008; Koca et al., 2010), the expression level of the *MEFV* gene in peripheral leukocytes in RA patients is unknown. We used a case-control design to compare *MEFV* gene expression levels between patients with RA and healthy subjects.

MATERIAL AND METHODS

Study population

In this study, we evaluated 47 RA subjects including patients receiving any disease-modifying antirheumatic drugs (N = 36) and recently diagnosed non-treated patients (N = 11) according to the American College of Rheumatology remission criteria with varying degrees of disease severity. We also examined a control group of 47 healthy, age- and gender-matched subjects without any joint diseases. Blood samples were obtained with informed written consent. Ethical approval was obtained from the University of Firat Committee on the Ethics of Research on Humans. All subjects lived in the Elazig Province. Patients were recruited from July 2011 to March 2012 and their diagnoses were confirmed according to American College of Rheumatology criteria.

RNA extraction and reverse transcription

Total RNA was extracted using the Pure Link^{TM} RNA Mini Kit (Ambion, Foster City, CA, USA) according to manufacturer instructions. Total RNA was eluted from the matrix using 25 μL RNase-free water. One microgram total RNA was reverse-transcribed into cDNAs in a total volume of 20 μL containing 10 μL 2X RT buffer and 1 μL 20X enzyme mix using a High-Capacity RNA-to-cDNA kit (PE Biosystems, Foster City, CA, USA). Samples were incubated for 60 min at 37°C, 5 min at 95°C, and then cooled at 4°C.

Quantitative real-time polymerase chain reaction (PCR)

Real-time reverse transcriptase (RT)-PCR with cybergreen chemistry was performed on 4 genes using the 7500 Fast Real-Time PCR system (PE Biosystems). Two different primer sets spanning the junctions of exons 1-3 for detecting the *MEFV*-d2 isoform and exon 4-5 for *MEFV*-fl were used to determine transcript expression levels. Levels were quantified using the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene as an internal control. The primer sequences were as follows (Kireçtepe et al., 2011a): exon 1-3 forward: 5'-CATTCAGGGAAGGC CACCAG-3', exons 1-3 reverse: 5'-TTCCTTTCATGGGAGTCCTG-3', exons 4-5 forward: 5'-AGGAGCAGCGATCCTATGG-3', exons 4-5 reverse: 5'-CAGCGCTTCAGTTTGTTTCA-3', *GAPDH* forward: 5'-AGGTCATCCCTGAGCTGAACG-3', *GAPDH* reverse: 5'-GGTGTCGCT GTTGAAGTCAGA-3'. All measurements were performed in triplicate.

The reactions were performed using a MicroAmp 96-well plate capped with MicroAmp optical caps (Perkin-Elmer, Waltham, MA, USA). The reactions were performed for 40 cycles of 10 min at 95°C, 15 s at 95°C, and 60 s at 60°C. The 10-μL reaction mixtures contained Power SYBR-Green PCR Master mix (Perkin-Elmer), 250 nM forward and reverse primer (Table 1), and standardized dilutions of all cDNA samples that were chosen to remain within the limits of the PCR standard curve.

The threshold cycle (Ct) values obtained from the 7500 Fast Real-Time PCR relative quantification software (PE Biosystems) were exported into Microsoft Excel. The amount of *MEFV*-fl and *MEFV*-d2 cDNA relative to the *GAPDH* RNA endogenous control was determined using the $2^{-\Delta\Delta Ct}$ method as described in the ABI User Bulletin Number 2.

Statistical analysis

We used the Kruskal-Wallis tests to compare means and the Spearman correlation coefficient for correlation studies. The Mann-Whitney U-test was used to evaluate differences between the normalized relative quantities of *MEFV* amplicons. Values are reported as means ± SD. Differences between groups were considered significant when the P value was less than 0.05.

RESULTS

We screened the isoform expression of *MEFV*-fl and *MEFV*-d2 and examined the associations between the expression of these genes in RA patients and healthy control subjects. The principal characteristics of patients in our study are presented in Table 1. Figure 1 shows the real-time PCR curves for quantification of *MEFV*-fl and *MEFV*-d2 mRNA levels in peripheral blood cells. The overlapping amplification curves for each cDNA sample examined in triplicate demonstrate the reproducibility of the assay. RT-PCR of *MEFV*-fl revealed a 1.5- and 1.09-fold decrease in untreated RA subjects compared with that in the control group. In treated patients, *MEFV*-d2 expression was decreased by 1.06-fold, while *MEFV*-fl was unchanged. The most important finding of this study was the 1.5-fold downregulation of *MEFV*-fl in untreated RA patients compared to controls. Other fold-decreases in treated and untreated RA patients were negligible. The results of the Mann-Whitney U-test showed that *MEFV*-fl mRNA levels were significantly lower in untreated patients than in control subjects and treated subjects (P = 0.05 and P = 0.05, respectively). However, *MEFV*-d2 mRNA levels did not significantly change in the treated and untreated groups compared to the control group (P = 0.5 and P = 0.8, respectively).

Table 1. Demographic and clinical features of patients with rheumatoid arthritis (RA) and healthy controls.

Demographic features	Treated RA (N = 36)	Non-treated RA (N = 11)	Controls (N = 47)
Age in years (min-max)	56.4 ± 1.2 (30-77)	59.81 ± 1.62 (51-81)	55.39 ± 1.5 (36-67)
Gender (female/male)	30/6	7/4	26/17
Years since RA diagnosis	8.67 ± 8.4	0.14 ± 0.16	-
Rheumatoid factor (IU/mL)	1.39 ± 2.82 (26/36)	79.63 ± 6.08 (9/12)	9.75 ± 1.64 (1/43)
Erythrocyte sedimentation rate (mm/h)	45.87 ± 2.65	48.00 ± 2.47	17.93 ± 1.61
C-reactive protein (mg/L)	5.45 ± 5.08	20.58 ± 1.02	1.07 ± 1.82
Hemoglobin (g/dL)	12.42 ± 1.47	12.86 ± 1.21	13.57 ± 1.46
Number of tender joints	10.28 ± 9.07	15.00 ± 9.11	-
Number of swollen joints	6.06 ± 6.64	8.82 ± 6.82	-
Deformed joint counts	2.64 ± 1.69	0.55 ± 1.03	-
Morning stiffness (min)	83.94 ± 93.69	92.73 ± 9045	-

Values are reported as means ± standard deviation or N (%).

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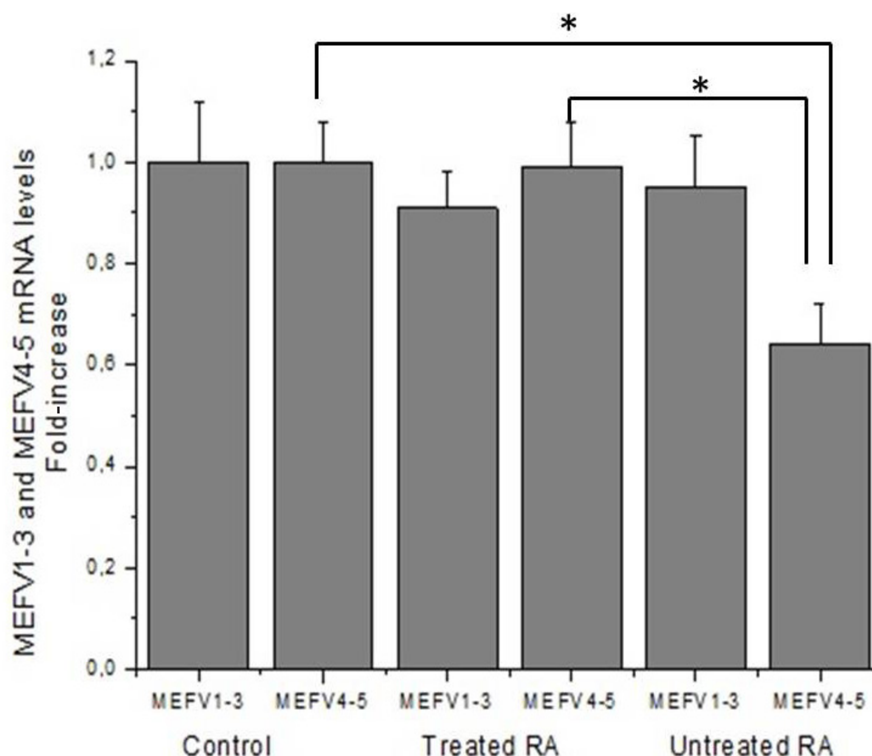

Figure 1. Real-time PCR analysis of *MEFV*-fl (exons 4-5-specific primers) and *MEFV*-d2 (exons 1-3-specific primers) isoforms of the *MEFV* gene. Analysis was performed in triplicate, and samples were normalized to *GAPDH* levels. RA = rheumatoid arthritis. Data are reported as means ± SD. *P < 0.05.

To elucidate whether decreased or inadequate *MEFV* expression is influenced by the treatment options, we performed correlation analysis for patients receiving different treatments. Of the treated patients, 29 received a combination of disease-modifying antirheumatic drug (DMARD) therapy including corticosteroids, while 8 received only 1 or a combination of methotrexate, hydroxychloroquine, sulfasalazine, and leflunomide as DMARDs. *MEFV* expression levels in treated patients receiving only DMARDs or a combination with corticosterois were not significantly different (P > 0.05).

No significant association was observed between individual traits and clinical features, such as gender, age, rheumatoid factor (IU/mL), erythrocyte sedimentation rate (mm/h), C-reactive protein (mg/L), hemoglobin (g/dL), number of tender joints, number of swollen joints, deformed joint counts, morning stiffness (min), and *MEFV* isoform expression level (P > 0.05). There was also no difference between individual traits and clinical features between treated and non-treated patients (P > 0.05).

DISCUSSION

Previous studies have generally focused on *MEFV* gene mutations rather than *MEFV* gene expression in RA patients. Mutations and variants within *MEFV* are reportedly associ-

ated with the susceptibility to a number of inflammatory rheumatic disorders, such as Behcet's disease, palindromic rheumatism, RA, and juvenile RA (Day et al., 2008; Ayaz et al., 2009; Akkoc et al., 2010). The main finding of the present study was reduced *MEFV*-fl mRNA expression in untreated RA patients compared with treated RA and control groups.

The regulation and expression of *MEFV* gene splice variants in FMF remain unclear. Increasing evidence suggests that a defect in *MEFV* expression regulation plays a role in the pathophysiology of FMF (Medlej-Hashim et al., 2010). The gene is regulated by nonsense-mediated decay, and different isoforms of *MEFV* transcripts show different cellular localization, protein-protein interactions, sub-cellular localization, or inflammatory function (Gay et al., 1993). Previous quantitative studies examining the relationship between FMF disease and *MEFV* mRNA expression showed inconsistent results. *MEFV* transcript levels decreased significantly in FMF patients compared to controls in initial reports (Notarnicola et al., 2002; Ustek et al., 2007); however, no significant difference was observed in a more recent series (Booty et al., 2009). Two recent studies reported significantly higher expression levels in *MEFV*-d2 and lower *MEFV*-fl expression in FMF patients compared to healthy controls (Kireçtepe et al., 2011a,b). Based on these reports, the *MEFV*-d2 transcript is located mainly in the nucleus (Papin et al., 2000), indicating its role as a transcription factor in the inflammation pathway in FMF diseases. To date, only 1 report has described the distribution of *MEFV* transcripts in synovial fibroblasts. RA synovial fibroblasts constitute a unique cell type that distinguishes RA from other inflammatory conditions of the joints (Huber et al., 2006). Diaz et al. (2004) reported lipopolysaccharide-induced expression of spliced *MEFV* transcripts in human synovial fibroblasts, particularly in the exon 8ext transcript. Exon 8ext transcripts represent 27% of the total message population in synovial fibroblasts. Other alternatively spliced transcripts are rare (Diaz et al., 2004). To date, the function of 8ext and its role in the pathogenesis of RA has remained unknown.

Pyrin has been implicated in the control of inflammation through leukocyte apoptosis and interleukin (IL)-1β activation (Akkoc et al., 2010). Hesker et al. (2012) reported a significant increase in the release of IL-1β in cultures derived from Mefv$^{-/-}$ mice in comparison to wild-type mice. A similar increase in IL-1β secretion may occur in response to proinflammatory proteins in RA patients. However, serum IL-1β levels in RA patients are controversial. In some studies, a correlation between IL-1β levels and disease activity has been observed (Eastgate et al., 1988; Altomonte et al., 1992), while other studies have revealed a decrease in IL-1β during therapy (Danis et al., 1992; Arvidson et al., 1997) and several studies have found no effect of treatment on RA patients (Chang et al., 1992; De Benedetti et al., 1995; Vázquez-Del Mercado et al., 1999). In all reports, IL-1β levels were generally very low during therapy. If IL-1β is considered to be downstream of *MEFV*, *MEFV*-fl downregulation may be influenced by IL-1β-mediated pathways in RA. This indicates that *MEFV*-d2 and *MEFV*-fl expression may be altered during early-stage RA and that *MEFV*-fl expression may be ameliorated after treatment in RA patients. The varying levels of *MEFV* transcript expression after treatment suggest that a link exists between treatment and isoform expression.

There were several limitations to this study. First, Kireçtepe et al. (2011b) found no significant association between mutations and the expression levels of spliced variants in FMF patients with or without mutations. They found slightly higher expression in individuals without mutations. Although we assessed *MEFV*-fl and *MEFV*-d2 variant expression in the blood, we did not examine FMF gene mutations in our study subjects. Second, as the current study was a case-control design, our results do not provide information on the time course of *MEFV*

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gene expression changes. Third, the sample size in our cohort was too small to adequately test replication, and further studies in a larger cohort are required to confirm or refute our findings.

These preliminary data raised several questions regarding the pathogenesis of RA diseases. The role of other splice isoforms of the *MEFV* gene in peripheral leukocytes or synovial fibroblasts in RA development remains unclear. In addition, follow-up studies may be conducted on RA patients with the *MEFV*-fl and *MEFV*-d2 isoforms.

In summary, we provide new information regarding the relationship between RA pathophysiology and patient phenotype, which may be modulated by varying transcript levels. Future studies should be conducted to investigate the role of these forms in other chronic inflammatory conditions such as Behçet diseases, which are known to involve IL-1β. Further analyses of the relative amounts of each transcript of the *MEFV* gene are also necessary to confirm their role in the pathogenic mechanisms underlying inflammation in RA.

Conflicts of interest

The authors declare no conflict of interest.

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