

RESEARCH ARTICLE

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# Analysis of MEFV exon methylation and expression patterns in familial Mediterranean fever

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## Abstract

**Background:** MEFV mutations and decreased expression level of the gene are related to FMF pathology. DNA methylation at CpG islands is a well-known mechanism for transcriptional silencing. MEFV has a CpG island, spanning a part of the first intron and the whole of the second exon of the gene covering 998 bp region. Here, we tested the hypothesis that the MEFV transcript level in FMF patients correlates with its methylation level, and methylation, by allowing transcription silencing, has a role in FMF etiopathogenesis.

**Methods:** The study group was composed of pediatric FMF patients (N = 51) and age-gender matched healthy controls (N = 21). The relative expression level of MEFV was assessed via quantitative real-time PCR (qRT-PCR) and bisulfite sequencing (BS) was performed to analyse the methylation level quantitatively.

**Results:** MEFV expression in FMF patients were decreased compared to healthy controls ( $P = 0.031$ ). Methylation level of exon 2 of MEFV was found to be slightly higher in FMF patients compared to healthy controls (76% versus 74%) ( $P = 0.049$ ). The expression level of the MEFV was negatively correlated with the methylation level of the CpG island in both FMF and healthy controls groups (cor = -0.29,  $P = 0.041$ ) but more so in the FMF only group (cor = -0.36,  $P = 0.035$ ).

**Conclusions:** In this study, the relation between reduced MEFV expression level and FMF was confirmed. Observed slight increase in methylation in FMF patients, and correlation of methylation with expression might be indicative of its role in FMF, however a larger dataset is needed to confirm our preliminary findings.

## Background

Familial Mediterranean Fever (FMF) is an autoinflammatory disease that commonly affects Mediterranean people; mostly Turks, Jews, Armenians and Arabs. Typical symptoms of the disease are recurrent attacks of fever, and the inflammation of serosal membranes with abdominal pain [1]. MEFV is the first identified inflammatory gene, which is responsible for FMF [2,3]. Approximately 70 variations on MEFV gene are related to FMF. The most common five mutations, M694V, M694I, M680I and V726A in exon10 and E148Q in exon 2, are attributed approximately 80% of disease associated alleles in FMF patients coming from Mediterranean ancestry with clear clinical criteria [4-6].

MEFV gene encodes Pyrin/Marenostrin which is mainly expressed in neutrophils, eosinophils, cytokine activated monocytes, dendritic cells and synovial fibroblasts. Pyrin/Marenostrin has a role in inflammation by activation of caspase-1, which is responsible for the maturation of IL-1 $\beta$  and by activation of NF- $\kappa$ B. Recent studies show that Pyrin/Marenostrin is an inflammatory regulator, which acts as pro-inflammatory and anti-inflammatory [7,8]. Several different transcript isoforms were shown, MEFV-d2, MEFV-8ext, MEFV-4a, MEFV-2a, del34, del234, del2345, del7, del78, in previous studies. Full-length Pyrin/Marenostrin, 781 amino acids, is located at cytoplasm and related with microtubule and actin filaments by B30.2 domain of Pyrin/Marenostrin. Alternatively spliced form of Pyrin/Marenostrin, translated from the exon 2 lacking *MEFV-d2* transcript, which is 570 amino acids long, is located mainly at the nucleus [9-11]. MEFV gene is regulated by non-sense-mediated decay and different isoforms of MEFV transcripts have different cellular localization and functions in inflammation [12].

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It is shown that the MEFV mRNA level is decreased in FMF patients compared to healthy controls and decreased mRNA expression level was correlated with mutation number [13]. Furthermore, the MEFV expression level was found to be more decreased in FMF patients during attacks. This indicates that the lower mRNA levels of MEFV are related to inflammation [14]. Despite two previous studies, a more recent study found that the MEFV expression level was not significantly different in groups, even slightly higher in FMF patients compared to healthy controls. Further Pyrin/Marenostri levels in granulocytes were found significantly higher in FMF patients [15].

DNA methylation, the most common epigenetic mechanism, occurs at the cytosine bases in CpG islands present in the gene promoter [16]. In addition to that, methylation of CpG islands at first and second exons cause also transcriptional silencing by preventing the RNA polymerase II transcription elongation [17].

The MEFV gene contains a CpG island spanning the complete second exon and a part of first intron. Since, methylation is an important mechanism of gene regulation and the lower expression of MEFV is associated with FMF and inflammation, methylation at the second exon of MEFV might have a role in FMF pathology. In this study, we aimed to compare MEFV expression and methylation levels between FMF patients and healthy controls.

## Methods

### Sample Information

Peripheral leukocytes were obtained from 51 FMF patients (26 female, 25 male) and 21 healthy children (8 female, 13 male) who came to the Pediatric Outpatient Clinic of Cerrahpasa Medical Faculty, Istanbul University. None of the patients had attacks of serositis and fever at the time of sample collection. All FMF patients were on Colchicine. The mean age of FMF patients were  $10.3 \pm 4.02$  and healthy controls were  $8 \pm 3.71$ . The control group was chosen among children who came to the clinic either for a general check up or were diagnosed with a non-inflammatory disease. The Ethics Review Committee of Istanbul University Cerrahpasa Medical Faculty approved the study. The parents of children were informed and consent forms were fulfilled.

### Mutation Analysis

Genomic DNA was isolated from venous blood samples using DNA Isolation Kit for Mammalian Blood (Roche Diagnostics, Mannheim, Germany). To analyse the most common 5 mutations in Turkish population; E148Q, M680I, M694I, M694V and V726A, PCR-RFLP method was used [18].

### Gene Expression Analysis

Total RNA was isolated from whole leukocytes using High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany). For prevention of RNA degradation, cDNA synthesis was done immediately followed by total RNA isolation by using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). In 51 FMF patients and 11 healthy controls, probe based quantitative real-time PCR was done by LightCycler<sup>®</sup> TaqMan Master Kit (Roche Diagnostics, Mannheim, Germany) and performed at LightCycler<sup>®</sup> 2.0 system to measure MEFV mRNA level. Primer and probes were designed at Universal ProbeLibrary website of Roche <http://www.roche-applied-science.com/sis/rtPCR/upl/index.jsp?id=UP030000>. The primer set spanning to the junction of exon 4-5 and UPL Probe8 were used for quantitative real time PCR. The primer sequences were as follows; Forward: 5'-AGGAG-CAGCGATCCTATGG-3' and reverse: 5'-CAGCGCTT-CAGTTTGTTC-3'. This set detects transcripts with or without exon 2 MEFV.

The B2M gene was used as a reference gene (B2M Gene Assay-Roche Diagnostics, Mannheim, Germany). The relative expression level was calculated using  $\Delta C_T$  method ( $\Delta C_T = C_T$  of  $\beta 2M - C_T$  of MEV,  $2^{-\Delta C_T}$  value indicates the fold change between target gene and reference gene) [19].

### Methylation Analysis

Bisulfite treatment was done to obtain two different DNA sequences for methylated and unmethylated forms according to the supplier's protocol of the CpGenome DNA Modification Kit (Millipore, Billerica, MA, USA). Bisulfite sequencing (BS) was performed to measure the methylation status of second exon of MEFV gene. To amplify 41 CG dinucleotides at second exon of the gene, BS primers which are common for both bisulfite treated methylated and bisulfite treated unmethylated sequences were designed using MethPrimer software [20]. The primer sequences were as follows; Forward: 5'-GTTTTTTGGGG GAGAATAAG-3' and Reverse: 5'-TTTCCAAAACCTT CCTTCAAAT-3'. After PCR with BS primers, sequencing was performed (ABI PRISM<sup>®</sup> 3100 Analyzer, Applied Biosystems, Foster City, CA). To analyse sequencing, alignment was done using QUantification tool form Methylation Analysis (QUMA) [21] and cytosine peak height (x) and thymidine peak height (y) were measured for each CpG using ChromasPRO. Relative cytosine peak height was calculated by  $x/(x+y)$  formulas.

### Statistical Analysis

Mutation results between FMF patients and healthy controls were analysed by Chi square test and expression and methylation variations between FMF patients

and healthy controls were analysed by using Student's t-test. Correlation between expression and methylation levels was done by the Spearman correlation analysis. All statistical analyses were done using GraphPad Prism 5 software.

## Results

### Genotyping Analysis

MEFV allele frequencies were compared between FMF patients and healthy controls. The observed allele frequencies are given in table 1. Significantly, differences were found between the mutations frequencies of FMF patients and healthy controls (Mantel Haenszel chi square = 9.912 ( $P = 0.0016$ ), Odds Ratio: 2.7689 95% CI: 1.7302 < O.R. < 14.7894).

### MEFV Expression

Relative expression levels were calculated for FMF patients ( $N = 51$ ) and healthy controls ( $N = 11$ ) by the  $\Delta C_T$  method [19]. We observed that the MEFV expression level was significantly decreased in FMF patients compared to healthy controls (about 2 fold,  $P = 0.03$ ) (Figure 1). We did not find significant differences between FMF patients with mutations and FMF patients without mutations ( $P = 0.70$ ) (data not shown).

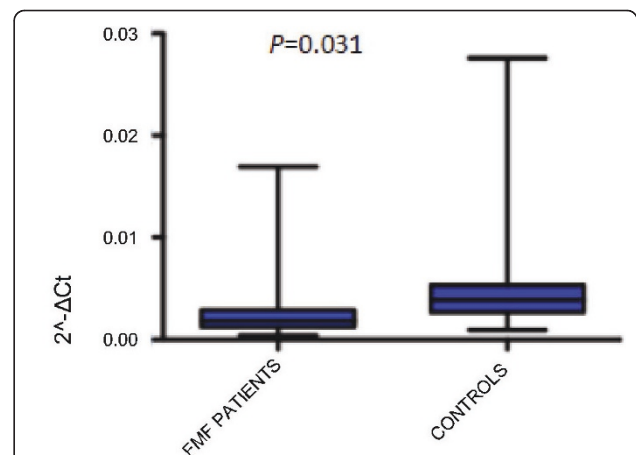
### Methylation Level of The Second Exon of MEFV

The second exon of MEFV was found to comprise high CG content (66%) and a 568 bp region containing 41 CpG dinucleotides corresponding to the whole of second exon was selected for methylation analysis.

Methylation levels of the second exon of MEFV were compared between 30 FMF patients and 21 healthy controls by bisulfite sequencing (BS). The rest of the sample could not be analysed because of methodological problems that arose during BS. It is observed that the methylation level is slightly but significantly higher in FMF patients compared to healthy controls ( $P = 0.049$ ) (Figure 2). Negative correlation between methylation and expression levels was found in both groups ( $cor = -0.29$ ,  $P = 0.041$ ), but this was higher in FMF samples-only group ( $cor = -0.36$ ,  $P = 0.035$ ) (Figure 3). Differences in methylation correlated

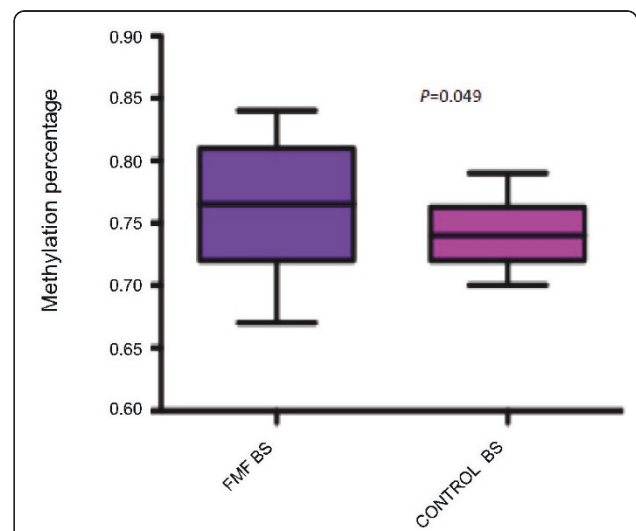
**Table 1 MEFV mutations frequency comparisons between FMF patients and healthy controls**

Variation	FMF Allele Frequency N = 102	Healthy Control Allele Frequency N = 22
M694V	45%	-
M680I	5.8%	13.6%
V726A	1.9%	4.5%
M694I	-	-
E148Q	6.8%	4.5%
<b>TOTAL</b>	<b>59.5%</b>	<b>22.6%</b>

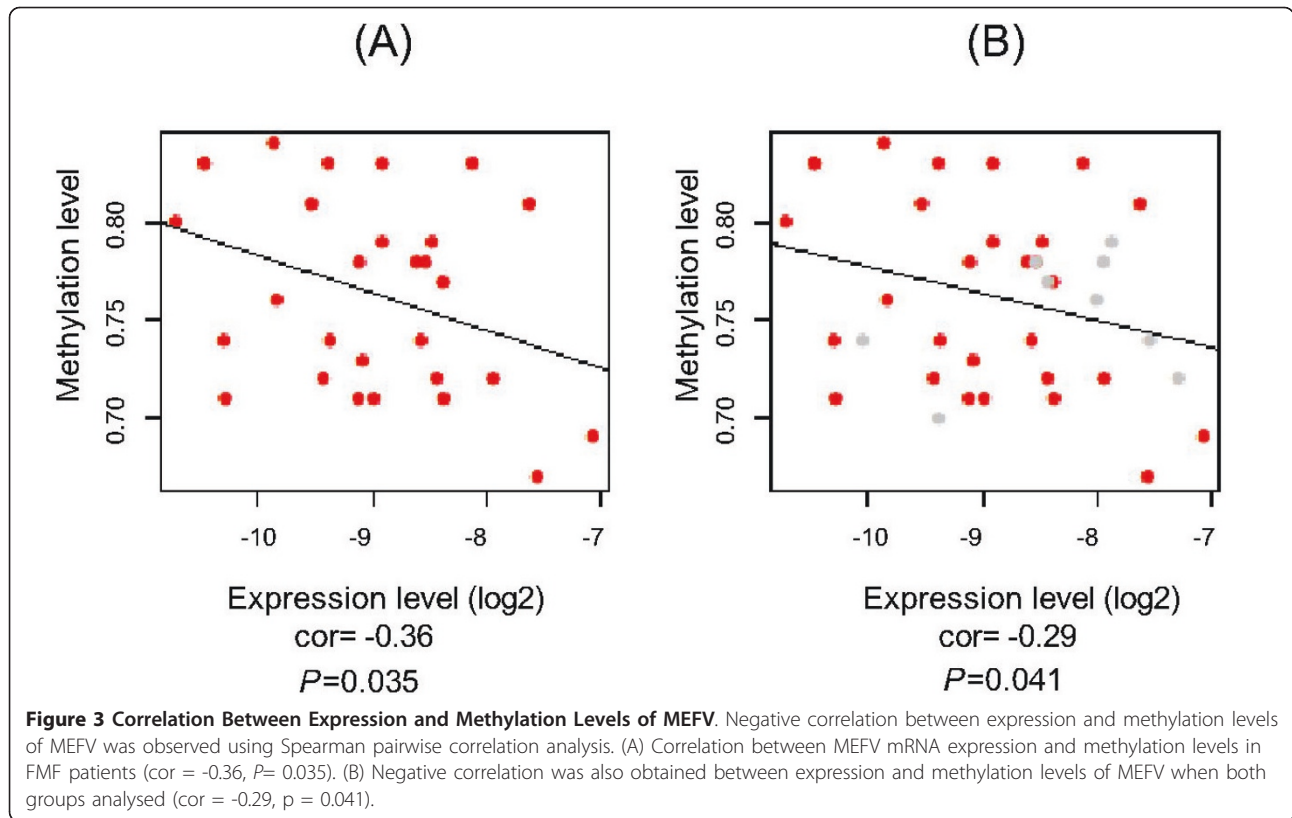


**Figure 1 Expression Level of MEFV in FMF patients and Healthy Controls.** Expression levels were measured by quantitative real time PCR analysis. RNA was obtained from peripheral leukocytes as described in materials and methods. B2M gene was used as reference gene and  $\Delta C_T$  method were used to relative quantification. MEFV mRNA level was analyzed in 51 FMF patients and 11 healthy controls. The bars represent standard deviations. Two tail Student's t-test was used to compare the MEFV expression levels. The expression was significantly lower in FMF patients compared to healthy controls ( $P = 0.031$ ).

with expression levels between FMF patients and controls can also be seen in Figure 4 where the higher MEFV expression is correlated with lower methylation in FMF group and the opposite is true for the control group.



**Figure 2 MEFV Methylation Levels in FMF Patients and Healthy Controls.** MEFV second exon methylation percentage in FMF patients and healthy controls. Bisulfite sequencing was performed in 30 FMF patients and 21 healthy controls genomic DNAs that were obtained from peripheral leukocytes. The bars represent standard deviations. Statistical analysis was done using one tail Student's t-test. Methylation level was observed slightly but significantly higher in FMF patients compared to healthy controls ( $P = 0.049$ ).



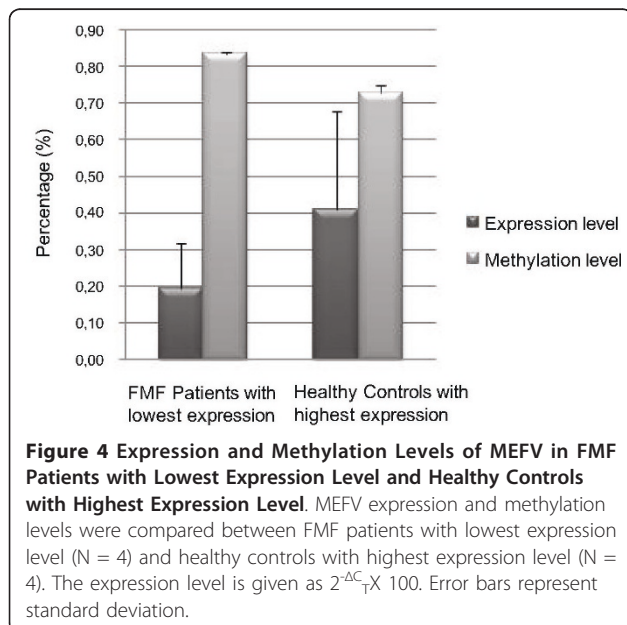
### Discussion

Bioinformatic analysis showed that the MEFV gene has high CG content (66%) at the second exon within a CpG island of 568 bp. Here we aimed to investigate the

correlation between MEFV exon 2 methylation with its expression, as methylation is a known mechanism for decreasing gene expression and MEFV mRNA expression has been known to be reduced in FMF patients.

Initially we compared the expression levels of MEFV in 51 FMF patients and 11 healthy controls. The expression level of the gene was significantly lower in FMF patients ( $\approx$  two fold,  $P = 0.03$ ). Our expression results were consistent with two previous studies [13,14]. We also compared MEFV mRNA levels between FMF patients with mutations and FMF patients with no identified mutations. Contrary to previous studies, we did not observe significant association between mutations and expression levels of MEFV in FMF patients with or without mutations; still, the expression level was slightly higher in individuals without mutations. However due to a small sample size, our study might be underpowered to detect a significant difference.

Cytokines, such as TNF- $\alpha$ , IL-2 and IL-6 are known to be associated with inflammatory diseases. The methylation status of IL-6 promoter site was found to be lower in rheumatoid arthritis patients compared to healthy controls [22,23]. In addition to the effect of methylated promoters on transcript levels, methylation of intragenic regions are also known to cause reduction in expression by preventing RNA Polymerase II elongation efficiency [17]. In this



study, for the first time to our knowledge, methylation levels of 41 CG dinucleotides at the MEFV second exon were compared between 30 FMF patients and 21 healthy controls using bisulfite sequence analysis. Methylation was found slightly but significantly higher in FMF patients than healthy controls (76% versus 74%) (one tail  $P = 0.049$ ). Negative correlation between expression and methylation was observed in all samples ( $\text{cor} = -0.29$ ,  $P = 0.041$ ), which was higher when only FMF patients were analysed ( $\text{cor} = -0.36$ ,  $P = 0.035$ ). The power of the analysis can be increased using a larger sample set.

Here we solely focused on methylation in exon 2 even if the CpG island compasses part of the first and second introns because of two reasons. One is that our hypothesis is about exon and not intron methylation as nucleosome positioning has shown to be more important in exons [24]. Secondly the majority of the CpG's are within the exon 2 (46/61) such that the first intron is 1520 bp long and it contains 11 CpG within the last 264 bp and there are 4 more CpGs within the beginning of the second intron, whereas the whole of exon 2 is occupied with CpGs. However our ongoing studies involve the examination of the intron methylation pattern as they also have been implicated in gene expression regulation [25].

We observed a wide range of standard deviations from both methylation and expression data (Figures 1 and 2). This can be caused by the fact that we used total leukocyte from FMF patients and healthy controls for analysis. MEFV expression level shows differences between leukocyte types such that there is no MEFV expression in lymphocytes. Additionally, we did not have the exact information about different leukocyte levels which varies from patient to patient, which lowers the resolution of the data. Currently we are doing our analysis on subpopulations of leukocytes i.e. monocytes and neutrophils.

MEFV gene has been shown to produce different transcripts through alternative splicing [9-11]. *In vitro* studies indicated that exon 2 deleted form (*MEFV-d2*) has a nuclear localization in leukocytes and *MEFV-d2*: *MEFV-fl* (*full length*) transcript ratio was about 3 fold higher in mononuclear cells than in polymorphonuclear leukocytes [9]. One way to test the role of exon 2 and its methylation in expression *in vitro* is to do cell transfection studies with alternative MEFV transcripts containing constructs. Full length MEFV and two MEFV variants, exon 2 fully spliced and exon 2 partially spliced (which only contain 9 CG dinucleotides as opposed to 41 CG dinucleotides) forms cloned into pCMV-CLUC vectors, along with methylated forms were transfected into HeLa cells. According to our preliminary results, the second exon fully spliced form of MEFV was the highest in expression, and the full length was the lowest in expression. Exon 2 partially spliced form of MEFV was the middle compared to full length and second exon fully spliced forms (data not shown) [26].

Furthermore we have also shown increased exon 2 spliced form in FMF patients compared to controls ( $P = 0.026$ ), which may indicate a possible connection between methylation and alternative splicing at this site [27]. These findings are in accordance with our finding that the presence of exon 2 and its methylation cause a decrease in gene expression. Further *in vitro* and *in vivo* studies with different leukocyte cell types are required to determine the effect of exon 2 methylation in gene expression in correlation with different alternatively spliced forms.

## Conclusions

In summary, we confirmed the association between reduced expression level of MEFV and FMF disease. Different methylation levels of the gene between FMF patients and healthy controls were observed, both groups having mostly methylated exon 2, but methylation was higher in FMF patients. To reveal the exact role of MEFV methylation in FMF and its relation to inflammation, we also aim to increase the sample size and analyse FMF patients during inflammatory attacks along with other auto-inflammatory and non-inflammatory disease control groups.

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## Authors' contributions

AKK carried out the molecular genetic studies, including methylation and expression analysis, did the statistical analyses, collected the sample material and drafted the manuscript. OK, NA, GH and HO have provided the clinical evaluation of the sample. GCE did the *in vitro* methylation and expression analyses. ETT participated in the design of the study, performed the statistical analysis, did the coordination, obtained the funding of the study, and helped in writing the manuscript. All authors read and approved the final manuscript.

## Competing interests

The authors declare that they have no competing interests.

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