

İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

**ANALYSIS OF MEFV GENE EXPRESSION AND METHYLATION
PATTERNS IN FAMILIAL MEDITERRANEAN FEVER**

**M.Sc. Thesis by
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Biotechnology**

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**AİLESEL AKDENİZ ATEŞİ HASTALIĞI'NDA MEFV GEN
EKSPRESYONU VE METİLASYON ANALİZLERİ**

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ABBREVIATIONS

µg	: Microgram
µL	: Microliter
µM	: Micromolar
ASC	: Apoptosis-associated speck-like protein
ACTB	: Beta actin gene
B2M	: Beta 2 microglobulin
BD	: Behçet's Disease
bp	: Base pair
BS-Seq	: Bisulfite sequencing
cDNA	: Complementary DNA
CRP	: C – reactive protein
DEPC	: DiethylenePyrocarbonate
dH₂O	: Distilated water
DNA	: Deoxyribonucleic acid
DNMTs	: DNA metyhyltransferases
dNTP	: Deoxyribonucleotide
<i>E.coli</i>	: <i>Escherichia coli</i>
EDTA	: Ethylenediaminetetraacetic acid
EtBr	: Ethidium bromide
FBS	: Fetal bovine serum
FCAS	: Familial Cold Autoinflammatory Syndrome
FMF	: Familial Mediterranean Fever
HDAC	: Histone deacetylases
g	: Gram
IBD	: Inflammatory bowel disease
IFN-γ	: Interferon gamma
IL	: Interleukin
LPS	: Lipopolysaccharide
M	: Molar
MCS	: Multiple cloning site
MEFV	: Mediterranean Fever gene
min.	: Minute
miRNA	: Micro RNA
mL	: Mililiter
mM	: Milimolar
mRNA	: Messenger Ribonucleic Acid
MWS	: Muckle Wells syndrome
NF-κB	: Nuclear Factor Kappa B
ng	: Nanogram
nM	: Nanomolar
NMD	: Nonsense-mediated decay
NOMID	: Neonatal Onset Multisystem Inflammatory Disease
PBS	: Phosphate buffered saline

PCR	: Polymerase Chain Reaction
Pen/Strep	: Penicillin/Streptomycin
pmol	: Picomole
Pol II	: RNA polymerase II
RA	: Rheumatoid arthritis
ra siRNA	: Repeat associated siRNA
RNA	: Ribonucleic Acid
SAM	: S-adenosylmethione
sec.	: Second
siRNA	: Small interference RNA
snRNA	: Small nuclear RNA
snoRNA	: Small nucleolar RNA
SNP	: Single nucleotide polymorphism
TAE	: Tris acetate EDTA
TNF	: Tumor necrosis factor
tRNA	: Transfer RNA
UPL	: Universal probe library
UV	: Ultra violet

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ANALYSIS OF MEFV GENE EXPRESSION AND METHYLATION PATTERNS IN FAMILIAL MEDITERRANEAN FEVER

SUMMARY

MEFV is the first identified autoinflammatory gene for Familial Mediterranean Fever (FMF). FMF mainly affects people of Mediterranean ancestry, generally Turks, non-Ashkenazi Jews, Armenians and Arabs. The most common MEFV mutations are M694V, M680I, M694I, V726A and E148Q, which are attributed to about 70% of disease-associated alleles. Among these mutations, E148Q, located at the second exon of MEFV, is associated with a milder phenotype of the disease. Although the expression level of gene is lower in other mutant allele carriers, it is shown that E148Q mutation increases the expression level of MEFV.

Bioinformatic analysis of MEFV gene has shown the presence of CpG island spanning the second exon and a part of first intron of gene covering a 998 bp region. E148Q mutation occurs within this CpG island. DNA methylation is an important epigenetic regulation which affects gene expression patterns. Since the lower expression level of MEFV is known to be associated with acute inflammation, we wanted to know whether the methylation in the second exon of MEFV is correlated with the expression of the mRNA as well as its relevance to FMF disease. We studied 51 child FMF patients from Cerrahpaşa Medical Faculty department of Pediatrics and 11 healthy controls from the same department. The methylation levels of the second exon of MEFV gene were quantified by MethyLight technique, which is a probe-based real-time PCR method using the genomic DNA as a template. Expression levels were quantified using real-time PCR methods from blood RNA samples. MEFV cDNA reporter gene constructs containing exon 2 variants, were also transfected in HeLa cells, and expression levels in vitro were determined.

In this study, 33 of 51 FMF patients had mutations (59.4% mutant allele) and four of 11 healthy controls had mutant alleles (22.7% mutant allele). These results show significant difference between the mutation frequency in FMF patients and healthy controls as expected ($p=0.0016$, Odds Ratio: 2.7689). Our expression results indicate that, compared to healthy controls ($p=0.031$), mRNA levels were decreased two-fold in FMF patients. In accordance, methylation status of second exon of MEFV were slightly higher in FMF patients compared to healthy controls, but there were no significant differences between the two groups. On the other hand, in vitro studies indicated increased expression from the construct lacking exon 2, compared to the one with parts of the exon 2 ($p=0.0013$), which indicate the importance of methylation interference with mRNA expression.

AİLESEL AKDENİZ ATEŞİ HASTALIĞINDA MEFV GEN EKSPRESYONU VE METİLASYON ANALİZLERİ

ÖZET

MEFV, Ailevi Akdeniz Ateşi (AAA) hastalığı ile ilişkilendirilmiş ilk otoinflammatuar gendir. AAA çoğunlukla Türkler, aşkenaz olmayan yahudiler, Ermeniler ve Araplar gibi akdeniz kökenli toplumlarında görülmektedir. En sık görülen mutasyonlar, M694V, M680I, M694I, V726A ve E148Q hastalıkla ilişkili allellerin yaklaşık %70'ini oluşturmaktadır. Bu mutasyonların içinden MEFV geninin ikinci ekzonunda bulunan E148Q mutasyonunun hastalığın daha hafif bir şekilde geçirilmesiyle ilişkili olduğu görülmüştür. E148Q mutasyonu dışındaki mutasyonları taşıyanlarda MEFV gen ifade seviyesinin daha az olmasına karşın E148Q mutasyonu taşıyanlarda MEFV gen ifadesinin arttığı gösterilmiştir.

MEFV geni için yapılan bioinformatik analizler sonucunda, genin ikinci ekzonunu ve birinci intronun bir kısmını içeren 998 bp uzunluğundaki bölgeye yayılmış olan bir CpG adacığı tespit edilmiştir. E148Q mutasyonu bu CpG adacığı içerisinde bir bölgede gerçekleşmektedir. DNA metilasyonu gen ifadesini etkileyen önemli epigenetik mekanizmalardan birisidir. Düşmüş olan MEFV gen ifadesi miktarının akut inflammasyonla ilişkili olduğu bilinmektedir. Bu nedenle biz MEFV geninin ikinci ekzonunda ki metilasyonun gen ifadesini düzenleyip düzenlemediğini ve hastalığın gelişiminde etkin olup olmadığını bilmek istiyoruz. Cerrahpaşa Tıp Fakültesi, Çocuk Sağlığı ve Hastalıkları anabilim dalına başvuran 51 FMF hastası ve 11 sağlıklı kontrolde çalışma yapıldı. Probe kullanılarak yapılan tam zamanlı polimeraz zincir reaksiyonunu (qRT-PCR) temel alan bir metod olan “MethyLight” yöntemi ile genomik DNA kalıp olarak kullanılarak, ikinci ekzondaki metilasyon miktarı belirlenmiştir. Kandan elde edilen RNA’dan, MEFV gen ifade seviyesi tam zamanlı PZR yöntemi kullanılarak belirlenmiştir. Ayrıca ikinci ekzon varyantlarını içeren MEFV cDNA raportör gen konstarktı HeLa hücrelerine transfekte edildi ve *in vitro* olarak ekspresyon seviyesi belirlendi.

Bu çalışmada 51 FMF hastasından 33 tanesinin (%59.4 mutant allel), 11 sağlıklı kontrolden 4 tanesinin (%22.4 mutant allel) mutant alleli taşıdığı gösterildi. Bu sonuçlar beklenildiği gibi iki grup arasında anlamlı bir fark olduğunu göstermektedir ($p=0.0016$, Odds Ratio: 2.7689). Sonuçlar AAA hastalarında, sağlıklılara oranla yaklaşık iki kat azalmış MEFV ekspresyon miktarını göstermektedir ($p=0.031$). FMF hastaları ve sağlıklı kontrollerde metilasyon durumuna bakıldığında, iki grup arasında istatistiksel olarak anlamlı bir fark bulunmamasına karşın, FMF hastalarında metilasyonun sağlıklılara göre biraz daha yüksek olduğu gözlemlendi. Buna karşın *in vitro* çalışmalarda, ikinci ekzonu içermeyen MEFV cDNA ile transfekte edilmiş hücrelerde, ikinci ekzonu içeren hücrelere oranla, ekspresyonun anlamlı olarak arttığı gösterildi ($p=0.0013$). Bu sonuçlar MEFV geninin 2. ekzonundaki metilasyonun, gen ifade seviyesi üzerindeki önemini göstermektedir.

1. INTRODUCTION

1.1 MEFV Gene and Familial Mediterranean Fever Disease

MEFV (MEditerranean FeVer) gene is an autoinflammatory gene which has been introduced as candidate gene for FMF. International FMF Consortium identified MEFV (MEditerranean FeVer) by positional cloning [1]. Same year, the region of 60 kb and four different mRNA's, which comprise MEFV, were reported by The French FMF Consortium [2]. MEFV gene is located on chromosome 16p between D16S80 and D16S283 and consists 10 exons (Figure 1.1). MEFV encodes 3.7 kb transcripts, which are mainly expressed in neutrophils, eosinophils, cytokine activated monocytes, dendritic cells and synovial fibroblasts [3]. The expression of MEFV is increased by proinflammatory agents which are interferon γ (IFN- γ), tumor necrosis factor α (TNF- α), lipopolysaccharide (LPS) and interleukin 1 β (IL-1 β) [4]. Several different isoforms of MEFV were shown previous studies. One of them is MEFV-fl, which encodes full-length pyrin, and other isoform is MEFV-d2 which produce 2nd exon spliced form of pyrin [5]. Another form of MEFV is MEFV-8ext involving a part of the following intron, which encodes truncated protein. In addition, similar forms of MEFV, MEFV-4a and MEFV-2a have part of following introns [6]. It is shown that full-length pyrin, which consists of 781 amino acids is located cytoplasm and related with microtubule and actin filaments by B30.2 domain of pyrin. Alternatively spliced form of pyrin, which is 570 amino acids long is located mainly at the nucleus [7].

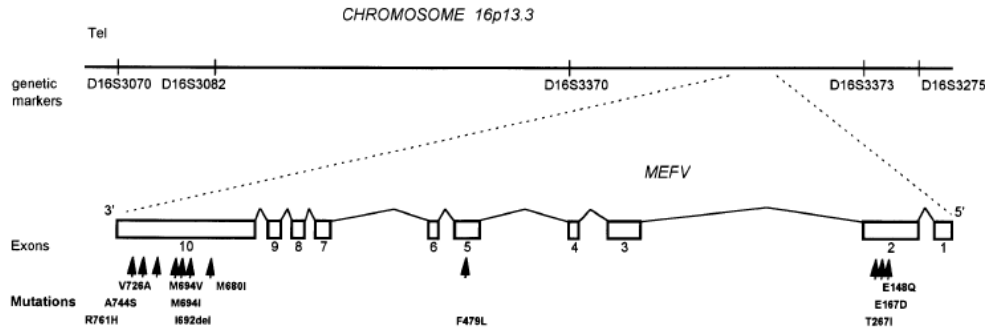


Figure 1.1 : Schematic presentation of MEFV gene (17)

Familial Mediterranean Fever (FMF) is the most common inherited periodic fever syndrome, also known as autoinflammatory disorder. The disease affects people of Mediterranean ancestry, mostly Turks, non-Ashkenazi Jews, Armenians and Arabs [3]. The frequency of FMF in Turkish population is 1/1073; however, it increases to 1/395 in the interior regions of Turkey [8]. The carrier frequency in Mediterranean population is between 1/8 and 1/16 [9]. Typical symptoms of FMF disease are recurrent attacks of fever, inflammation of peritoneum, synovium or pleura, accompanied by abdominal pain. The major complication in untreated cases is development of amyloidosis which is generally affecting kidneys and sometimes adrenals, intestine, spleen, lung and testis [10]. Diagnosis is based on clinical criteria, family history and laboratory results such as leukocytosis, elevated C – reactive protein (CRP) and sedimentation rate during attacks. Additionally, MEFV mutations are analyzed to support diagnosis. Colchicine is the only effective treatment to relieve FMF attacks and to prevent amyloidosis [11].

1.2 Function of MEFV Gene and Its product; Pyrin

1.2.1 MEFV Mutations

Until today, 187 sequence variations are identified on MEFV gene. 73 of these variations have been reported to be associated with FMF [12]. Mutations are located in exons 1, 2, 3, 5, 9 and 10 of MEFV. Two mutational hot spots have been identified: one in exon 2 and one in exon 10 (Figure 1.2). The most common mutations which are attributed to 70% of disease associated alleles are; M694V, M694I, M680I, V726A and E148Q [13]. However, E148Q mutation is located at the

40.5% of the patients have nine different MEFV variations. E148Q was found to be the most common mutation with frequency of 38.1% of mutated alleles [16, 17]. Additionally, Rabinovich *et. al.* found that 17% of RA patients have common MEFV mutations with the high frequency E148Q mutation (12/98 patients). After these evidences, it is thought that MEFV gene has a major role in the inflammatory pathway [20].

1.2.2 MEFV Expression

Several gene expression studies have reported the association between MEFV mutations and MEFV expression levels. In 2002, Notarnicola *et. al.* studied the relationship between mRNA level of MEFV gene and MEFV mutations. They found lower mRNA levels of MEFV in FMF patients compared to healthy controls and intermediate mRNA levels in healthy carriers. Besides, they showed that M694V mutation is associated to low MEFV expression levels and MEFV mRNA level is also decreased by increasing mutation numbers. The FMF patients with E148Q mutation who have milder symptoms showed higher mRNA levels than other patients [21]. A second study, by Ustek *et. al.* was confirmed the previous study results; decreased mRNA levels of MEFV in FMF patients compared to healthy controls. Besides, they showed that the expression levels of MEFV gene is more reduced in FMF patients during attacks. They indicated that the lower mRNA levels of MEFV are related to inflammation [22].

Booty *et. al.* analyzed the allelic expressions of MEFV in three different groups from 8 FMF patients and found that the expression levels of the gene in healthy controls and patients with one mutation and patients with two mutations are not significantly different. Despite of the previous two studies, MEFV expression was found slightly higher in the FMF patients than healthy controls. To confirm these results, they examined pyrin levels in granulocytes by Western blot analysis and found significant increase in pyrin levels between FMF patients and healthy controls. On the other hand, they did not find any differences between patients with 1 and 2 mutations [22].

Several alternatively spliced forms of MEFV gene were shown recent studies. Alternative splicing and nonsense-mediated decay (NMD) have role at the post-transcriptional regulation of gene expression. Grandemange *et. al.* showed that NMD regulates the expression of MEFV by inhibition of NMD using CHX which is

protein translator inhibitor or siRNA. They also investigated that alternative spliced forms of MEFV encodes different protein isoforms which exists different cellular localization [23].

1.2.3 The Gene Product of MEFV – Pyrin/Marenostrin

The translational product of MEFV is called pyrin/marenostrin. Pyrin protein consists of five domains which are (I) N-terminal PYRIN domain (also known as PYD, PAAD, DAPIN), (II) bZIP transcription factor basic domain, (III) B-Box zinc domain, (IV) α -helical (coiled-coil) domain (CC), (5) C-terminal B30.2 domain (PRYSPRY) which corresponds tenth exon of MEFV gene (Figure 1.3). All domains are functional in the course of inflammation through apoptosis, cytokine secretion, regulation of transcription and cytoskeletal signaling [22].

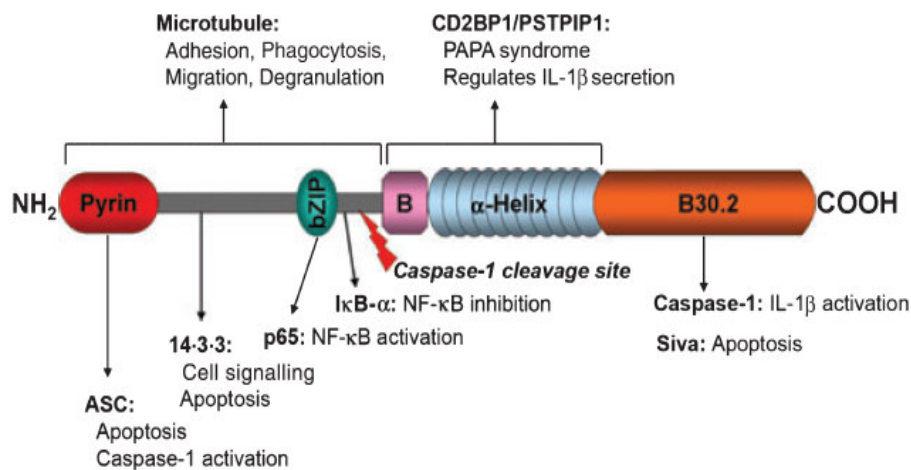


Figure 1.3 : The structure of pyrin protein. Interacting proteins are also shown [20]

PYD domain consists of 90 amino acids at the N-terminal of pyrin protein. It is a member of a superfamily, which includes caspase recruitment domain (CARD), death domain (DD) and death effector domain (DED). About 20 proteins, which have role in inflammation or apoptosis, contain the PYD domain. Pyrin protein has homotypic interaction with other proteins, which have PYD domain by PYD/PYD interaction [4, 24, 25]. Apoptosis-associated speck-like protein (ASC) is one of the proteins that interact with pyrin by PYD/PYD interaction. ASC protein, which is the adaptor protein in inflammasome, has PYD domain at the N-terminal and CARD domain at the C-terminal that binds to caspase-1. Pyrin has role in activation of

caspase-1, which has proteolytic activation of precursor cytokines such as IL-1 β , IL-18, IL-33 and maturation of IL-1 β , which has role as pyrogenic cytokine through interaction with ASC protein (Figure 1.4). In addition, it is shown that pyrin activates NF- κ B through regulation of caspase-1 [26, 27].

Another protein in the inflammasome structure that interacts with ASC protein is cryopyrin (also known as NALP3, NLRP3, CATERPILLER 1.1) which is a member of NALPs family. Cryopyrin has three domains, which are effector-binding domain at the N-terminal (PYD domain), nucleotide binding site (NBS) domain of the NACHT subfamily (NACHT domain) and leucine-rich repeat domain at the C-terminal (LRR domain). Mutation of cryopyrin cause three autoinflammatory diseases; Muckle Wells syndrome (MWS), Familial Cold Autoinflammatory Syndrome (FCAS) and Neonatal Onset Multisystem Inflammatory Disease (NOMID). Cryopyrin has function in regulation of IL-1 β through binding to ASC via PYD-PYD interaction as well as pyrin protein (Figure 1.3) [28].

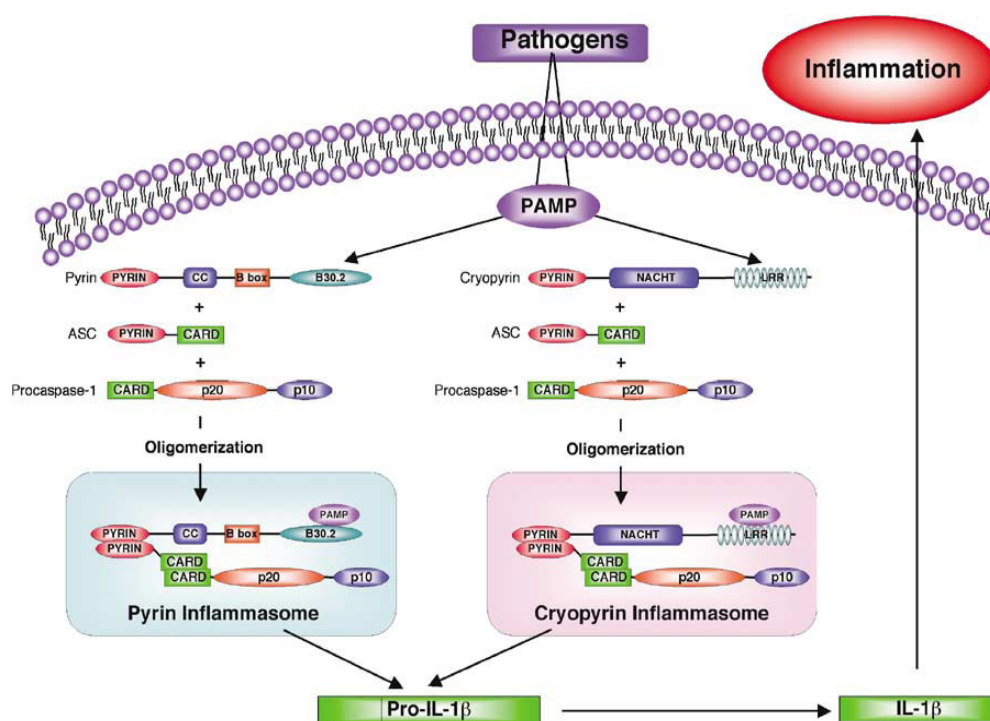


Figure 1.4 : Pyrin and Cryopyrin have role in inflammation through affecting IL-1 β

Another domain of the pyrin protein is B30.2 which correspond the 10th exon of MEFV gene that the mutational hot spot region (M694V, M680I, V726A, M694I).

It is shown that B30.2 domain of pyrin binds to caspase-1, the inflammasome proteins and IL-1 β directly. The p10 and p20 subunits of caspase-1 bind to the B30.2 domain of full-length pyrin strongly and inhibits the conversion of inactive form of IL-1 β to active form [29]. MEFV mutations at the 10th exon (M694V, M680I and V726A) cause less interaction between B30.2 domain and p10 and p20 subunits of caspase-1 and induction inflammation through preventing IL-1 β activation (Figure 1.5). Chae *et. al.* showed that pyrin is a substrate of caspase-1 which cleaves pyrin at Asp330 to make 330 residue N-terminal fragment which includes PYD and bZIP domain and activates NF- κ B without ASC and 451 residue C-terminal fragment. Caspase-1 cleaves mutant pyrin more effective than wild type pyrin. However, pyrin function has not been clear, all these studies show that pyrin acts as both pro-inflammatory and anti-inflammatory [30].

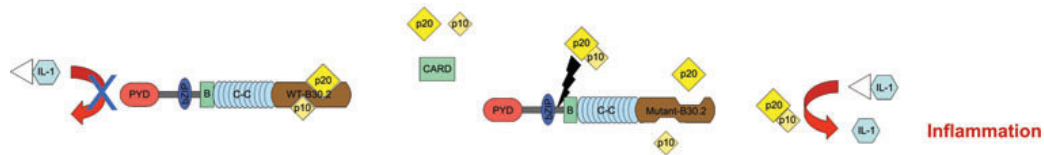


Figure 1.5 The direct interaction between B30.2 domain of pyrin and p10 and p20 domains of caspase-1 [28]

These studies showed that there is association between mRNA level of MEFV and FMF, as well as mutation of MEFV. However, there are many studies about MEFV gene and pyrin protein, mRNA studies are very deficient. Transcription level of gene is changed via DNA methylation as well as gene mutations.

1.3 Epigenetic Mechanisms

Epigenetic is described as functionally changing of genome without nucleotide sequence differences (*Waddington, 1942*). These changes include DNA methylation, histone modifications and non-coding RNAs. Epigenetic changes, which are generally reversible have role on cellular mechanisms such as development, differentiation, X-chromosome inactivation, imprinting, T-cell function and gene silencing [31].

1.3.1 DNA Methylation

DNA methylation is the most common epigenetic mechanism. In eukaryotic organisms, DNA methylation occurs at the cytosine bases in the CpG islands which describe as genomic region that contain CpG clusters with CG dinucleotides above 55%. Most of the CpG dinucleotides are condensed at the promoter of gene [32]. In addition to methylation of CpG islands, it is showed the methylation in non-CG context [33]. Binding of the methyl group to cytosine base is catalyzed by DNA methyltransferases (DNMTs). Methyl group is transferred from S-adenosylmethionine (SAM) to fifth carbon of cytosine nucleotide by DNMTs (Figure 1.6). Various DNMTs were identified which are DNMT1, DNMT2, DNMT3a and DNMT3b. DNMT1 has role at the replication fork to copying methylation pattern of parental DNA to synthesized DNA in replication. DNMT3a and DNMT3b carry out de nova methylation at the unmethylated area. Nevertheless, DNMT2 has only weak DNA methyltransferase activity in vitro, it methylates tRNA efficiently [34].

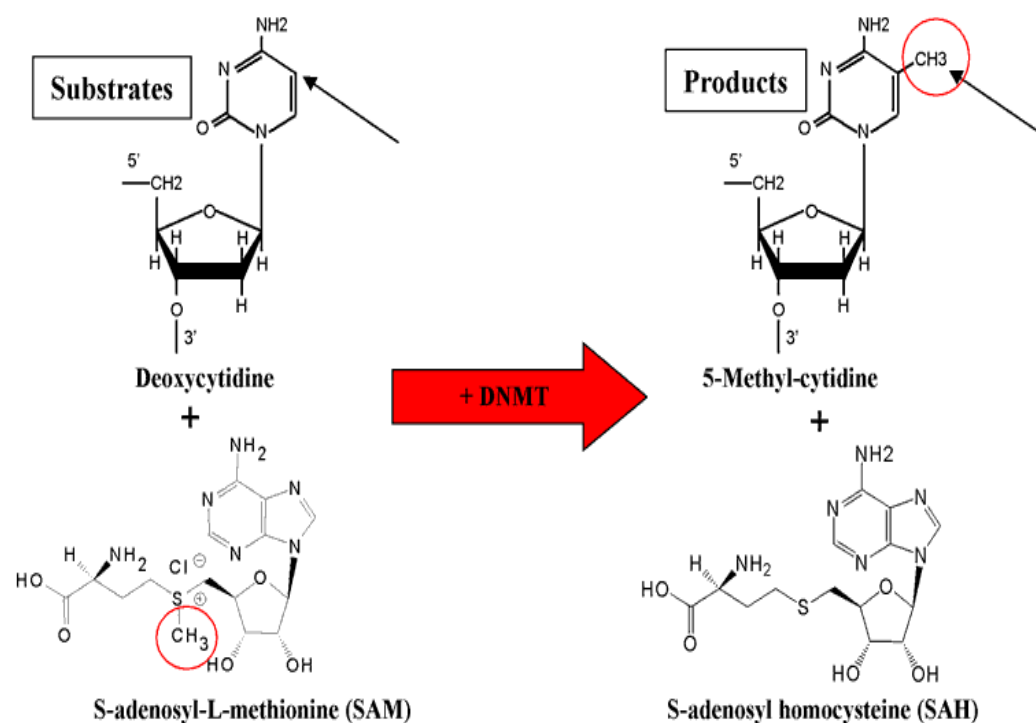


Figure 1.6 : Methylation of cytosine by DNMT

1.3.2 Histone Modification

Another epigenetic change is histone modification, which is acetylation, methylation, phosphorylation and ubiquitylation. The most studied histone modifications are acetylation and methylation. Five types of histone proteins, which are H1, H2A, H2B, H3 and H4, make nucleosome and 146 bp DNA surround it to form chromatin structure [35]. Normally, electrostatic attraction between positively charged histone protein and negatively charged DNA causes chromatin compaction and reduces gene expression. Addition of acetyl group by histone deacetylases (HDAC) to histone tails conduce to open confirmation of chromatin and induce gene expression via changing charge of histone protein. It is shown that histone modifications and DNA methylation are associated each other and indicate chromatin remodeling mechanism, thus gene expression is changed by alteration of packaging of chromatin structure (Figure 1.7) [36].

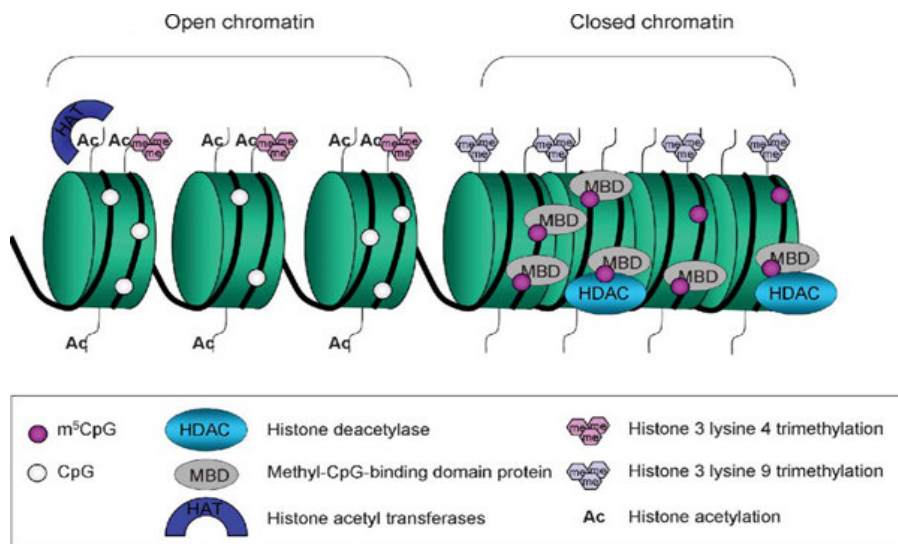


Figure 1.7 : Histone modifications

1.3.3 Non-Coding RNAs

In addition to DNA and chromatin modification, non-coding RNAs (nc-RNAs) which are not translated to proteins have important roles at the regulation of expression. They affect transcriptional and post-transcriptional gene silencing. Non-coding RNAs contain micro RNAs (miRNAs), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), repeat associated siRNAs (rasiRNAs) [31]. Recent

studies show the association between ncRNA disruptions and human diseases. miRNA expression was found abnormal in some cancers, such as liver, colon, breast, hematopoietic and brain cancers or in Alzheimer's disease and cardiovascular diseases. Also, there is a relationship between the loss of specific miRNA loci and Prader-Willi syndrome which is induced by the loss of imprinting on chromosome 15q11-q13 [37].

1.3.4 Regulation of Gene Expression by DNA Methylation

Gene expression is commonly regulated via DNA methylation in mammalian cells. Methylated CpG islands are generally located at the promoter region of gene and are normally unmethylated. DNA methylation of promoter region regulates gene expression via directly inhibiting binding of transcription factor or repressing chromatin remodeling [38]. Recent studies show that abnormal methylation pattern at the promoter site is relevant to expression changing which cause to develop some diseases, such as cancer, autoimmune diseases [31, 39]. For example, hypomethylated promoter of tumor suppressor gene is associated with hepatocellular cancers [40], breast cancer [41]. In addition to promoter methylation, recent studies emphasize the importance of intron or exon CpG islands methylation at the regulation of transcription [42, 43]. It is shown that the second exon of bcl-2 gene is methylated abnormally in colorectal cancer patients [44].

In normal cell, DNA methylation occurs CpG islands at the repetitive genomic regions, such as satellite DNA, transposable elements [39]. The effect of intragenic methylation to transcription efficiency was studied by Lorincz *et. al.* and they found that the transcription efficiency decreases slightly at the methylated region downstream of promoter, compared to unmethylated region. In addition, RNA polymerase II (Pol II) was decreased at the methylated region downstream of promoter, despite that there was no difference of Pol II at the methylated or unmethylated region of promoter site. As a result, they suggested that methylation at the downstream of promoter does not affect transcriptal initiation but decreases transcriptional elongation [45].

Also, Lister *et. al.* showed the methylation pattern at the non-CG context by new single-base-resolution DNA methylation map. They carried out bisulfate sequencing (BS-Seq) at the human embryonic stem cells and fetal fibroblasts. They observed that

99.98% of methylated cytokines are located at the CpG island in fibroblast. Besides that they detected abundant DNA methylation at the non-CG context which is 25% of all methylcytosines. They suggested that methylation of cytosine at the non-CG context might be a general feature of human embryonic cell. In addition, they showed the absence of methylation status at the non-CG context while conserved methylation at the CpG island at the differentiation of the embryonic stem cell. Methylations at the non-CG context are reappeared at the pluripotent stem cell [45].

Recently, the effects of epigenetic mechanisms on inflammatory responses are began to be questioned. In T cell, normally methylated region at the promoter of the IL-2 gene is demethylated shortly for increase IL-2 expression. Also, H3 acetylation occurs at the IL-4 and IL-13 gene cluster while differentiation of CD4 T cells to T helper 2. Epigenetic variability at the genes which produce inflammatory molecules or repress the producing of inflammatory molecules regulates immune or inflammatory responses [36]. These results are suggested the importance of epigenetic mechanisms at the inflammatory diseases. For example, Neil *et. al.* found one unmethylated CpG pattern at the IL-6 gene in RA patients, compared to healthy control. It is shown that methylation of this CpG site inhibits binding of nuclear proteins to DNA. They suggested that hypomethylation of the CpG site enables the binding of transcriptional factors to specific region at the DNA, thus expression of IL-6 are increases [31].

1.4 Aim of the Study

Some studies indicate aberrant MEFV expression may cause the pathology in FMF. Since methylation is an important mechanism in regulating expression, methylation of the CpG island at the second exon of MEFV gene might have a role in FMF pathology. The aim of this study was to compare methylation levels of second exon of MEFV gene in FMF patients and healthy controls. Also we wanted to correlate methylation with expression levels within and between groups. Additionally, in vitro studies were done using pCMV-C-Luc reporter assay to determine expression level of MEFV by using two different constructs, which are fully and partially spliced forms of second exon of MEFV and their methylated forms. Those constructs were transfected into HeLa cell lines and their transcription levels were quantified luminometrically.

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2. MATERIAL AND METHODS

2.1 Materials and Laboratory Equipment

2.1.1 Used Equipments

The laboratory equipment used in this study is listed in Appendix A.

2.1.2 Used Chemicals, Enzymes, Markers and Kits

The chemicals, enzymes and markers used are given in Appendix B with their suppliers. The compositions and preparation of buffers and solutions are given in Appendix C. The kits used and their suppliers are given in Appendix D.

2.2 Sample Selection

The study group was composed of FMF patients who came to Pediatric Polyclinic of Cerrahpaşa Medical Faculty, Istanbul University. The control group was chosen among children who came to the clinic either for a general check up or diagnosed with a non – inflammatory disease. The age of the children in the patients or healthy group is between 2 to 17.

The Ethics Review Committee of Istanbul University Cerrahpaşa Medical Faculty approved the study. The parents of children were informed and permission forms were fulfilled. After that, the blood samples were collected in 4 cc vacuum tubes containing EDTA and were kept at 4 °C for maximum two hours for prevention of RNA degradation.

2.3 DNA Isolation from Whole Blood

The DNA isolation was done by DNA Isolation Kit for Mammalian Blood (Roche). The isolated DNAs were used for genotyping and methylation analysis.

For DNA isolation, approximately 3 ml blood sample was used. 9 ml Red Blood Cell Lysis Buffer was added to blood sample and the mixture was shaken at room temperature for 10 minutes. The tubes containing mixture were centrifuged at 875 X g for 10 minutes. After centrifugation, the red supernatant was removed carefully and white pellet was homogenized by vortex. 1.5 ml White Cell Lysis Buffer was added on white pellet and was incubated at 37 °C for 15 minutes. The samples were transferred to the sterile tubes. 780 µl Protein Precipitation Solution was added to each sample and mixed about 15 second by vortex. The samples were centrifuged at 12.000 X g for 10 minutes. The supernatant, which contained DNA, was transferred 15 ml falcon tube. 2 volumes absolute ethanol was added to supernatant and mixed gently until the liquid remained no cloudy. Precipitated DNA strand was transferred by sterile pipette tip from absolute ethanol to 1 ml 70% ethanol and inverted several times. The sample was centrifuged at 875 X g for 5 minutes and the supernatant was discarded. After DNA pellet was dried at room temperature, 300 µl TE buffer was added to DNA pellet and incubated at 65 °C for about 30 minutes. The samples were kept 4 °C for short term and -20 °C for long-term storage.

DNA concentration was calculated by the NanoDrop® ND-1000 UV-Vis Spectrophotometer. DNAs were diluted to 50 ng/µl for working aliquots.

2.4 RNA Isolation from Whole Human Blood

Total RNAs of the groups were used for gene expression analysis. Prevention of RNA degradation in vacuum tube with EDTA, RNA was tried to be isolated immediately. Because of that blood samples in tubes were kept at 4 °C for maximum 3 or 4 hours. The tubes and pipette tips used in RNA isolation were made RNase free by DEPC treatment. Total RNA was isolated from whole human blood by High Pure RNA Isolation Kit (Roche).

1 ml Red Blood Cell Lysis Buffer was added to a sterile 1.5 ml reaction tubes. 500 µl peripheral blood was added into tubes and mixed. The tubes were shaken at room temperature for 10 minutes. Then the tubes were centrifuged at 500 X g for 5 minutes. After centrifugation, red supernatant was removed carefully with sterile pipette. Again 1 ml Red Blood Cell Lysis Buffer was added to white pellet and mixed by *flicking*. The tubes were again centrifuged at 500 X g for 3 minutes and the supernatant was removed carefully. 200 µl PBS was added into white pellet and

resuspended. Also 400 µl Lysis/Binding Buffer was added and mixed by vortex for 15 seconds. The sample was transferred to filter tube and centrifuged at 8.000 X g for 15 seconds. After centrifugation, fluid was discarded. For each sample 90 µl DNase incubation buffer and 10 µl DNase I were mixed in a separate tube. The mixture was added into filter tube and incubated at room temperature for 15 minutes. After incubation, 500 µl Wash I Buffer was added into filter tube and centrifuged at 8000 X g for 15 seconds and the fluid were discarded. Then 500 µl Wash II Buffer was added into filter tube and centrifuged at 8.000 X g for 15 seconds and the fluid were discarded. 200 µl Wash II Buffer was added again into filter tube and centrifuged at maximum speed for 2 minutes and the fluid was discarded with collection tube. The filter tubes were inserted 1.5 ml microcentrifuge tubes and 70 µl Elution Buffer was added. The tubes were centrifuged at 8.000 X g for 1 minute. The eluted RNAs were used for cDNA synthesis and stored at -80 °C for later usage.

2.5 cDNA Synthesis

Because of easily degradation of RNA, cDNA was used for MEFV expression analysis with Real-Time PCR. For prevention of RNA degradation, cDNA synthesis was done followed total RNA isolation by using Transcriptor First Strand cDNA Synthesis Kit (Roche).

1 µl of 50 pmol/µl anchored oligo (dT) primer, 5 µl of PCR grade dH₂O were added into sterile PCR tubes. 7 µl total RNA isolated previously was added to tubes and mixed by pipettes. Tubes were placed in a thermal cycler and denatured at 65 °C for 10 minutes. After denaturation, mixture was taken on ice immediately. 4 µl 5X Transcriptor Reverse Transcription reaction buffer, 0.5 µl 40 U/µl Protector RNase inhibitor, 2 µl dNTP mix (10 mM each), and 0.5 µl 10 U/µl Transcriptor Reverse Transcriptase were mixed separate tube and added to mixture containing RNA samples. For inactivation of Reverse Transcriptase, the samples were incubated at 55 °C for 30 minutes and at 85 °C for 5 minutes in thermal cycler. The reaction was stopped by placing the tubes on ice. Synthesized cDNAs were stored at -20 °C for longer periods.

2.6 Genotyping

2.6.1 Polymerase Chain Reaction (PCR)

The target sequences within second and tenth exon of MEFV were amplified by Polymerase Chain Reaction (PCR). For PCR reaction, isolated genomic DNA was used as a template. General PCR mix which was used to amplify target DNA sequence is as in table 2.1.

Table 2.1 General PCR mix

Stock Concentration	PCR Ingredient	Volume	Final Concentration
10 X	MgCl ₂ free TaqBuffer	2 µL	1 X
25 mM	MgCl ₂	1.5 µL	1.875 M
2.5 mM	dNTP Mix	0,32 µL	40 µM
10 pmol/µL	Forward Primer	1 µL	0.5 µM
10 pmol/µL	Reverse Primer	1 µL	0.5 µM
-	dH ₂ O	8.7 µL	-
5 U/ µL	Taq Polymerase	0.2 µL	0.05 U/µL
5 M	Betain	4 µL	1 M
50 g/ µL	Template DNA	2 µL	100 ng/µL
TOTAL		20 µL	

2.6.2 Oligonucleotide Primers

Six oligonucleotide primers were used to amplify target sequences of MEFV which contain five variant site; M694V, M680I, V726A, M694I and E148Q. The primers were selected from previous studies. The sequences of primers are given table 2.2.

Table 2.2 Oligonucleotide primers

Variation	Primer Sequence	Amplicon Size
M694V	F-5'-TACTGGGTGGTGAT*CAT-3' R-5'-AGGGCTGAAGATAGGTTGAA-3'	215 bp
M680I	F-5'-TGTATCATTGTTCTGGGCTCT-3' R-5'-AGGGCTGAAGATAGGTTGAA-3'	360 bp
V726A	F-5'-TGTATCATTGTTCTGGGCTCT-3' R-5'-AGGGCTGAAGATAGGTTGAA-3'	360 bp
M694I	F-5'-TGTATCATTGTTCTGGGCTCT-3' R-5'-CTGGACGCCTGGTACTCATTTTTT*C-3	195 bp
E148Q	F-5'-ATATTCCACACAAGAAAACGGC-3' R-5'-GCTTGCCCTGCGCG-3'	244 bp

2.6.3 PCR Conditions

PCR conditions were optimized previous studies. Except of E148Q PCR, all PCR conditions were similar. Only annealing temperatures were different in the conditions because of using different primers. PCR programs for each SNP are given in table 2.3, 2.4 and 2.5.

Table 2.3 PCR program for M694V, M680I and V726A

PCR Phase	Temperature	Time	Repeat
Initial Denaturation	96 °C	5 min.	1
Denaturation	96 °C	30 sec.	
Annealing	55 °C	30 sec.	35
Extension	72 °C	30 sec.	
Final Extension	72 °C	10 min.	1
Final Hold	4 °C	∞	

Table 2.4 PCR program for M694I

PCR Phase	Temperature	Time	Repeat
Initial Denaturation	96 °C	5 min.	1
Denaturation	96 °C	30 sec.	
Annealing	59 °C	30 sec.	35
Extension	72 °C	30 sec.	
Final Extension	72 °C	10 min.	1
Final Hold	4 °C	∞	

Table 2.5 PCR program for E148Q

PCR Phase	Temperature	Time	Repeat
Initial Denaturation	96 °C	5 min.	1
Denaturation	96 °C	30 sec.	
Annealing	64 °C	30 sec.	5
Extension	72 °C	30 sec.	
Denaturation	96 °C	30 sec.	
Annealing	62 °C	30 sec.	35
Extension	72 °C	30 sec.	
Final Extension	72 °C	10 min.	1
Final Hold	4 °C	∞	

DNA fragment lengths, which amplified PCR, were between 195 – 360 bp. These PCR products can be seen at 1% agarose gel. 1% mini or midi agarose gels were prepared for detection of PCR products. For mini gel preparation, 0.5 g agarose was added to 50 ml 1 X TAE buffer and 0.25 µl ethidium bromide (EtBr) was added. For midi gel preparation, 2 g agarose and 0.75 µl EtBr were added to 200 ml 1 X TAE buffer. 5 – 6 µl PCR product and 1 µl 6 X loading dye (Fermentas) were mixed and loaded into wells of the gels. MassRuler DNA ladder low range (Fermentas) was used as standard in gel electrophoresis. The gels were run at 120 V for about 40 min. and analyzed under UV light with transilluminator. The pictures of gels were taken with UV PhotoMW software.

Table 2.6 Restriction enzyme mix

PCR Phase	Repeat
10 X Restriction Buffer	2 µL
dH ₂ O	5 µL
Restriction Enzyme	1 µL
Amplicon	12 µL

The mixture were kept at 37 °C for overnight and after that for inactivation of restriction enzyme, incubated at 80 °C for 30 minutes. Used restriction enzymes and expected fragment sizes were given in the table 2.7.

Table 2.7 Used restriction enzymes and expected fragment sizes

Variation	Restriction Enzyme	Fragment Size
M694V	<i>Pag I</i>	Wild Type: 2 fragments (200 – 15 bp) Mutant: 1 fragment (215 bp)
M680I	<i>Hinf I</i>	Wild Type: 2 fragments (126 – 234) Mutant: 1 fragment (360)
V726A	<i>Alu I</i>	Wild Type: 1 fragment (360) Mutant: 2 fragments (320 – 40)
M694I	<i>Mbo II</i>	Wild Type: 1 fragment (195) Mutant: 2 fragments (182 – 13)
E148Q	<i>Ava I</i>	Wild Type : 3 fragments (92 – 83 – 69) Mutant: 2 fragments (161 – 83)

After restriction enzyme digestion, PCR products were run on agarose gel. According to product sizes 2%, 3.5% and 4% agarose gels were prepared. To analyze M680I and V726A SNPs 2%, M694V and M694I 3.5%, E148Q 4% agarose gels were used. 12 µl PCR products were mixed with 2 µl 6 X loading dye and loaded into wells of gels. The gels were run at 120 V for about 40 minutes and band sizes were seen under UV light.

2.7 Expression Analysis

2.7.1 Real-Time PCR for Quantification of MEFV Expression

The real – time PCR was done by LightCycler[®] TaqMan Master Kit (Roche) and performed at Roche LightCycler[®] 2.0 instrument. Primer and probes were designed at Universal ProbeLibrary website of Roche (www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=UP030000). B2M gene was used as reference gene. For standard curve preparation, cDNA sample were used in 4 different concentrations, as undiluted, 1:10 diluted, 1:100 diluted and 1:1000 diluted forms. Real – Time PCR mixtures and Real – Time PCR program are given table 2.8, table 2.9 and table 2.10.

Table 2.8 Real – time PCR mixture for MEFV

Ingredient	Concentration	Volume	Final Concentration
TaqMan Master Mix	5 X	4 µL	1 X
MEFV Forward Primer	10 µM	0.2 µL	250 nM
MEFV Reverse Primer	10 µM	0.2 µL	250 nM
UPL Probe #8	10 µM	0.2 µL	100 nM
dH ₂ O	-	10.4 µL	-
cDNA Template	-	5 µL	-

Table 2.9 Real – time PCR mixture for B2M as reference gene

Ingredient	Concentration	Volume	Final Concentration
TaqMan Master Mix	5 X	4 µL	1 X
B2M Primer Mix	10 µM	0.2 µL	250 nM
UPL Reference Gene Assay	10 µM	0.2 µL	100 nM
dH ₂ O	-	10.6 µL	-
cDNA Template	-	5 µL	-

Table 2.10 Real – Time PCR program for MEFV and B2M

Analysis Phase	Temperature	Time	Repeat
Pre-Incubation	95 °C	10 min.	1
Denaturation	95 °C	10 sec.	
Annealing	60 °C	30 sec.	45
Extension	72 °C	1 sec.	
Cooling	40 °C	30 sec.	1

2.7.2 Relative Quantification and Statistical Analysis

The comparative C_T (also known as $\Delta\Delta C_T$) method is used to calculate relative gene expression. This method is a mathematical model for calculation of gene expression and does not require the standard curve. The $\Delta\Delta C_T$ method is useful to assay large number of samples. It should only use when the PCR efficiencies of the target and reference genes are relatively equivalent. For each sample, the ΔC_T value was calculated by subtraction C_T value of target gene from C_T value of reference gene ($\Delta C_T = C_T$ of $\beta 2M$ - C_T of MEFV). $2^{-\Delta C_T}$ value indicates the fold change between target gene and reference gene [44].

The $2^{-\Delta C_T}$ value of FMF patients group and healthy control group were analyzed statistically by using *Student's t test*, Chi square analyses using SPSS statistical analyses program (v.16).

2.8 In Vitro Expression Analysis

MEFV cDNA constructs containing exon 2 variants were previously cloned by our group. Two different spliced transcripts of MEFV were cloned into phCMV-C-Luciferase (phCMV-C-Luc) which is a reporter vector to quantify the binding activity *in vivo* through using luciferase assay. These vectors were transfected to HeLa cells.

Stock cells were taken from -80 °C and melted at room temperature. Cells were transferred into 10 ml 37 °C warmed 10% DMEM centrifuged at 3000 rpm for 5 minutes. 10% DMEM consists 44.5 mL DMEM, 0.5 mL L-Glutamin, 0.1 mL penicillin/streptomycin (Pen/Strep) and 5 mL fetal bovine serum (FBS). HeLa cells

were cultured for two days in 37 °C and 5% CO₂ incubator. 50,000 cells were seeded into per well at 24 well tissue culture plate one day before transfection. 1 µg plasmid DNA was diluted in the DMEM and 3 µl Transfast (Promega) was added. The mixture was incubated at room temperature for 15 minutes. Each transfection was performed as triplicates. The medium was removed from cells and transfection mixture was added. The cells were incubated for 90 minutes in 37 °C and 5% CO₂ incubator. After incubation, the transfection mixture was removed and 500 µl growth media was added into each well and cells were incubated. 48 hours after transfection luminometrical measurement was done by Dual-Luciferase® Reporter Assay System (Promega). The activities of firefly (*Photinus pyralis*) and renilla (*Renilla reniformis*) luciferases were measured consecutively. 60 µl 1X passive lysis buffer was added into each well and the cells were lysed. 50 µl LAR II was added into one well of luminometer plate and 50 µl lysate was loaded into the well. Firefly luciferase luminescence was measured. 50 µl Stop-Glo® reagent was added and renilla luciferase luminescence was measured immediately. The procedure was performed for each sample and results were reported.

2.9 Methylation Analysis

2.9.1 CpG Island Analysis of MEFV

CpG island of MEFV gene was analyzed by using CpG island searcher software of University of Southern California (<http://cpgislands.usc.edu>). An illustration of the program screen is given in figure 2.1.

Select the lower limit values

%GC ☐ 50% ☐ 51% ☐ 52% ☐ 53% ☐ 54% ☒ 55% ☐ 56% ☐ 57% ☐ 58% ☐ 59% ☐ 60%

ObsCpG/ExpCpG ☐ 0.60 ☐ 0.61 ☐ 0.62 ☐ 0.63 ☐ 0.64 ☒ 0.65 ☐ 0.66 ☐ 0.67 ☐ 0.68 ☐ 0.69 ☐ 0.70

Length ☐ 200bp ☐ 300bp ☐ 400bp ☒ 500bp ☐ 600bp ☐ 700bp ☐ 800bp ☐ 900bp ☐ 1000bp ☐ 1100bp ☐ 1200bp

Gap between adjacent islands ☒ 100bp ☐ 150bp ☐ 200bp ☐ 250bp ☐ 300bp

Sequence

Figure 2.1: Illustration of CpG island software

2.9.2 Bisulfite Treatment

For quantify of DNA methylation level, unmethylated cytosine is converted into uracil by bisulfite treatment method whereas methylated cytosine is not converted. Consequently, two different DNA sequences, for methylated and unmethylated, were made. Bisulfite treatment was done according to protocol by supplier of the EZ DNA Methylation – Gold™ Kit (Zymo Research).

Inception amount of DNA is important because of the efficiency of bisulfite conversion. 1 µg DNA was dissolved in 20 µl dH₂O and used for each reaction (recommended 1 µg to 2 µg). 130 µl CT Conversion Reagent was added to 20 µl DNA sample and mixed by flicking. The tube was placed in thermal cycler and incubated as following; 98 °C for 10 minutes and 64 °C for 2.5 hours. Preferentially, the sample can stored at 4 °C for 24 hours. 600 µl M – Binding Buffer was added to a Zymo – Spin™ IC Column tube and the sample was loaded into the Zymo – Spin™ IC Column tube which contain the M – Binding Buffer and mixed several times. The tube was centrifuged at maximum speed for 30 seconds and flow-through was discarded. 100 µl M – Wash Buffer was added to column and centrifuged at maximum speed for 30 seconds and flow-through was discarded. 200 µl M – Desulphonation Buffer was added to column and sample was incubated at room temperature for 15 – 20 minutes. After incubation, the tube was centrifuged at maximum speed for 30 seconds. 200 µl M – Wash Buffer was added to column and centrifuged at maximum speed for 30 seconds and this step is repeated again. Column was placed into 1.5 ml microcentrifuge tube. 10 µl M – Elution Buffer was added to the column matrix and tube was centrifuged at maximum speed for 30 seconds. The DNA was stored at – 20 °C for later use or – 80 °C for long-term use.

2.9.3 Real-Time PCR for Quantification of MEFV Methylation

MethyLight assay was used to measure methylation status of second exon of MEFV gene [45]. LightCycler® TaqMan Master Kit from Roche and performed at Roche LightCycler® 2.0 instrument were used for this study [46]. Probes and primers were designed Universal ProbeLibrary website of Roche (www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=UP030000). Bisulfite treated DNA was used as a template. For each sample, One set of primer was designed for methylated form

of DNA sequence. In addition, ACTB primers were used as reference gene, which were designed both methylated and unmethylated form of DNA sequences. To confirm the efficiency of bisulfite treatment, genomic DNAs were performed for each sample. For standard curve preparation, fully methylated DNA sample was used four different concentrations. Also General Real – Time PCR mixture and program are given in the table 2.11 and table 2.12 below.

Table 2.11 : Real – time PCR mixture

Ingredient	Concentration	Volume	Final Concentration
TaqMan Master Mix	5 X	4 µL	1 X
Methylated Forward Primer	10 µM	1 µL	500 nM
Methylated Forward Primer	10 µM	1 µL	500 nM
UPL Probe #7	10 µM	0.4 µL	200 nM
dH ₂ O	-	10.6µL	-
cDNA Template	-	5 µL	-

Table 2.12 : Real-Time PCR program

Analysis Phase	Temperature	Time	Repeat
Pre-Incubation	95 °C	10 min.	1
Denaturation	95 °C	10 sec.	
Annealing	60 °C	30 sec.	45
Extension	72 °C	1 sec.	
Cooling	40 °C	30 sec.	1

2.9.4 Relative Quantification and Statistical Analysis

The relative quantification and statistical analysis were done same way as expression analysis. The comparative C_T method is used to calculate methylation level and FMF patients group and healthy control group results were analyzed statistically by using *Student's t test*, Chi square analyses using SPSS statistical analyses program (v.16).

3. RESULTS

3.1 Demographic Data of the FMF Patients

Blood samples were collected from 51 FMF patients and 11 healthy controls. The FMF patients group consists of 50% of female and 50% of male. None of the patients had attacks of serositis and fever at the time of samples collection. The healthy control group was composed of mostly male (81%) and had a mean age of $8\pm3,6$. Clinical information of FMF patients are shown at the table 3.1.

Table 3.1 : Clinical information of FMF patients

FMF Patients (N=51)	% Percentage
Female	50 %
Male	50 %
Mean Age	10 ± 4 (range 2-20)
Amyloidosis	-
Usage of Colchicine	100 %
Response to Colchicine	100 %

3.2 Genotype Analysis Results

The genotype analysis was done by comparing agarose gel results with the expected restriction enzyme digestion patterns and allele counting. PCR and restriction enzyme results of sample for each mutation analysis are given Appendix E.

3.3 Statistical Mutation Analysis

MEFV allele frequencies were compared between FMF patients and healthy controls. The observed allele frequencies are given in table 3.1. Significantly, differences were found between the mutations frequencies of FMF patients and healthy controls.

Table 3.2 : Frequency of five common MEFV mutations in FMF patients and healthy control

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 %
*TOTAL	59.5%	22.6%

*Mantel Haenszel chi square= 9.912 (p= 0.0016), Odds Ratio: 2.7689

95% CI: 1.7302< O.R. <14.7894

3.4 Expression Analysis Results

Singleplex PCR reactions for MEFV and β 2M were done separately. The standard curve settings were done successfully and the error rates of the Real-Time PCR reaction were about 1%, which is among acceptable value, and the efficiencies of the reactions were about two, which are given in table 3.2. The standard curves of MEFV and β 2M are given in figures 3.10 and 3.11 below.

Table 3.3 : Error rates and efficiencies of MEFV and B2M real-time PCR reactions

	MEFV	B2M
Error Rate	0.01	0.004
Efficiency	1.98	2.4

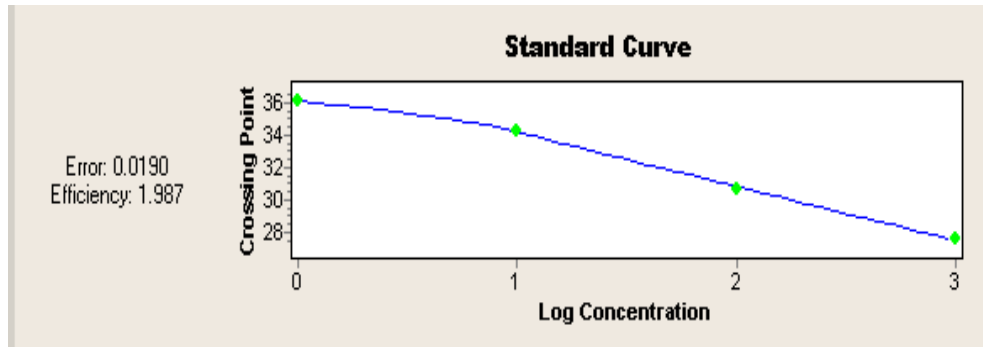


Figure 3.1 : Standard curve for MEFV

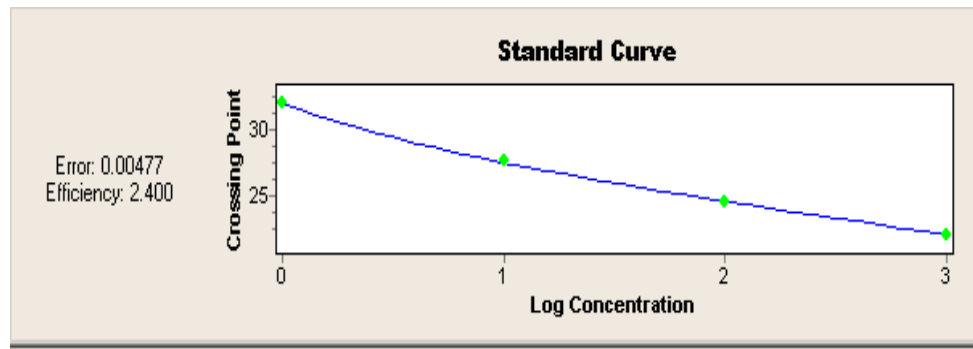


Figure 3.2 : Standard curve for B2M

Relative expression level was calculated for each sample by Δ CT method. In the overall data, the lowest mRNA levels was observed in the FMF patient with M694V and M680I heterozygous mutant genotype (0.00041 Relative Expression Units) and highest mRNA level was found in the healthy control with no mutation (0.02758 Relative Expression Units). When we compared the expression level results from 51 FMF patients and 11 healthy controls, we observed two fold decrease in mRNA levels of MEFV gene in FMF patients compared to healthy controls ($p=0.031$). The graph shows the relative expression level of MEFV in FMF patients and healthy controls are given figure 3.12.

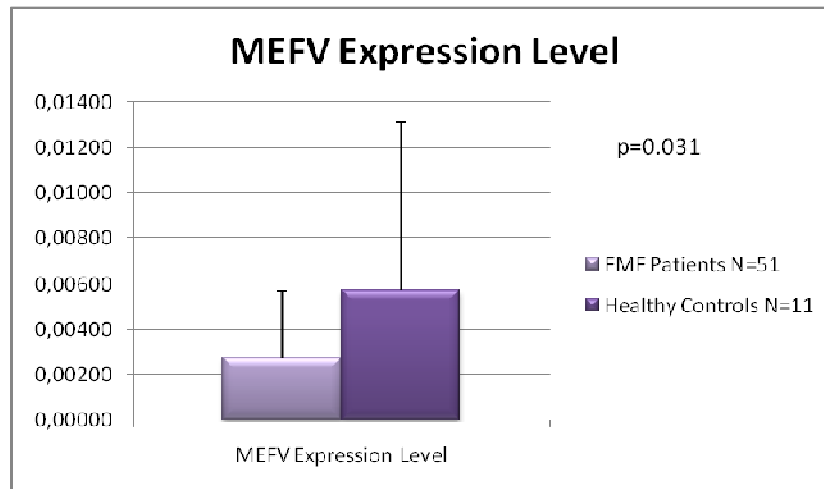
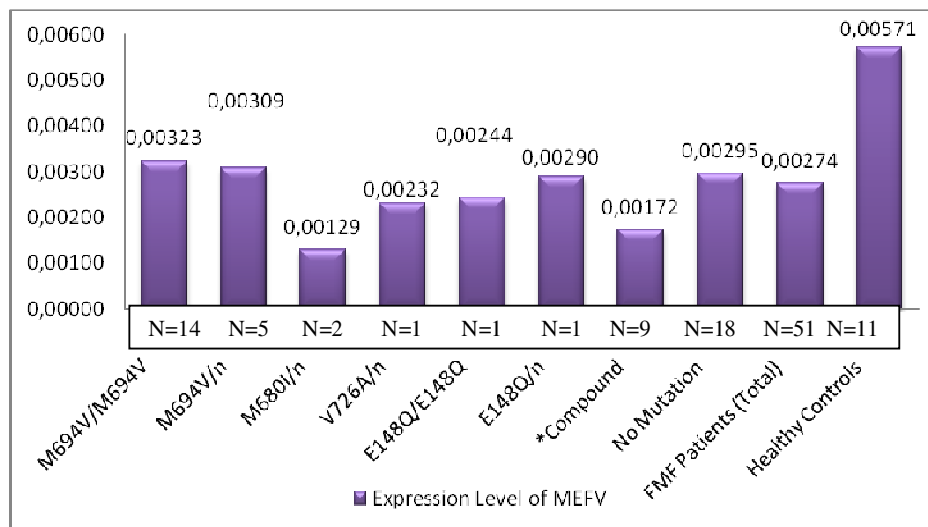


Figure 3.3 : Comparison of expression level of MEFV in FMF patients and healthy controls

In additionally, MEFV variants of M694V, M680I, V726A and E148Q were separately analyzed for expression levels. The relative expression of MEFV mRNA level was lower in FMF patients with M694V/n than healthy controls but higher than FMF patients with another mutations or without mutation (N=3, 0.00353 relative expression level). The graph showing expression levels of MEFV gene among FMF patients group is shown in figure 3.13.



*M694V/n-M680I/n (3), M694V/M694V-E148Q/n (3), M694V/n-E148Q/n, M694V/M694V-M680I/n, M694V/n-V726A/V726A

Figure 3.4 : Comparison of expression level of MEFV in FMF patients with mutations and without any mutations

The relative expression of MEFV was also compared between FMF patients with mutations and FMF patients without mutations. There is no significantly difference between two groups ($p= 0.70$). Also, expression levels were compared between the healthy controls with mutations and without any mutations. Significantly difference was not found also in these two groups ($p= 0.59$). The graphs are shown in figure 3.14 and 3.15.

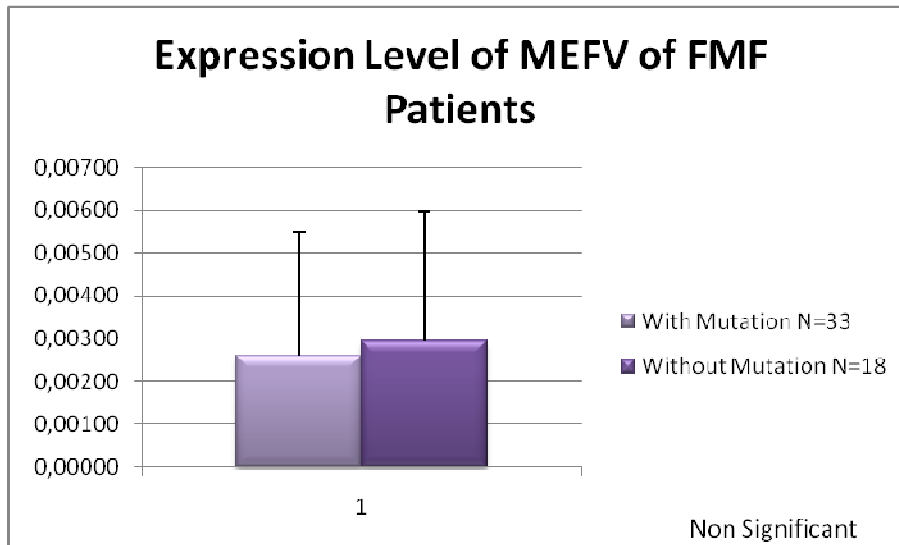


Figure 3.5 : Comparison of expression level of MEFV in FMF patients with mutations and without mutations

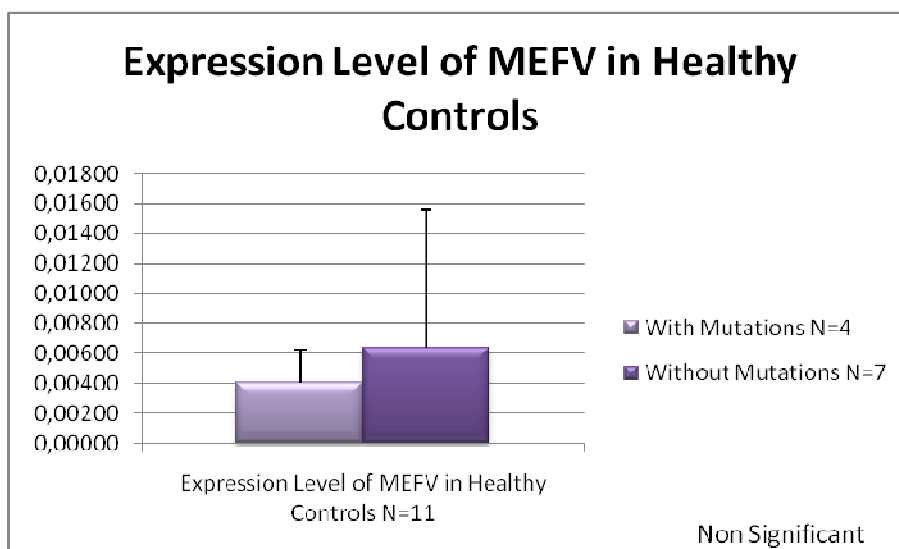


Figure 3.6 : Comparison of expression level of MEFV in healthy controls with mutations and without mutations

3.5 CpG Island Analysis Results

MEFV gene has a 51 % CG content overall genomic DNA and this value is 59% at the cDNA. The second exon of MEFV was found to contain high CG content (66%). When MEFV gene was analyzed for CpG island (CpG island searcher software of University of Southern California, <http://cpgislands.usc.edu>) a 998 bp CpG island was found at the second exon of MEFV gene (Figure 3.16). Within this CpG island, 568 bp region containing 41 CpG sites which corresponds to the second exon of gene was selected for methylation analysis. CpG sites are given in Appendix F.

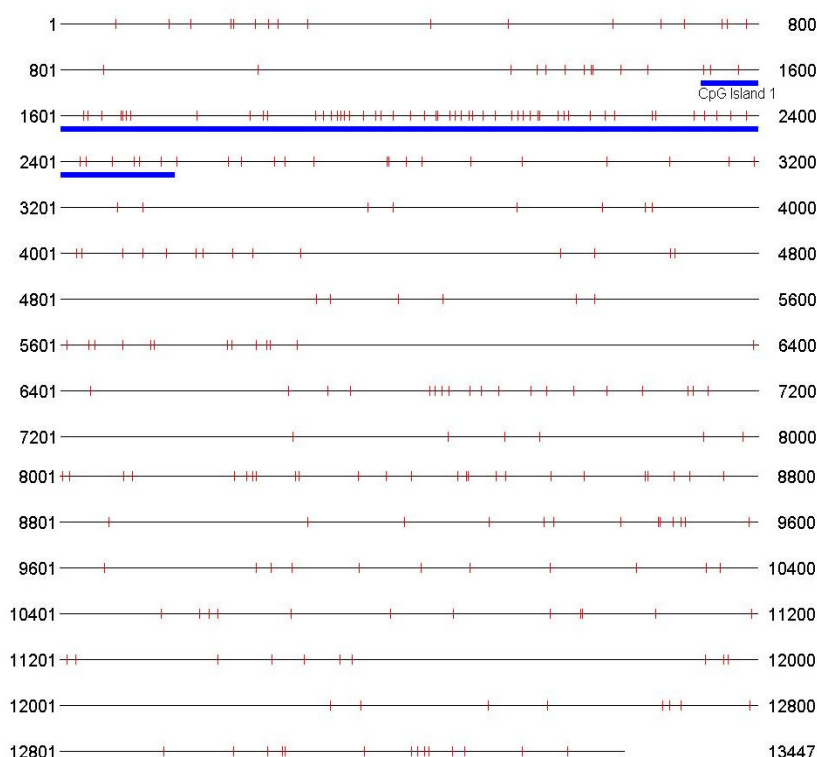


Figure 3.7 : CpG island on the MEFV genomic DNA sequence

3.6 In Vitro MEFV Expression Analysis

Two exon 2 variants of MEFV which are fully spliced and alternatively spliced forms were transfected into HeLa cells to analyze expression level of MEFV *in vivo*. In addition, methylated forms of these two constructs were analyzed. For each sample light units of firefly and renilla were proportioned to calculate expression level of MEFV. About two fold increased MEFV expression level was observed in the fully spliced form of second exon of MEFV gene to compare partially spliced

form. Approximately two fold increase in MEFV expression level in second exon fully spliced form of MEFV compared to second exon partially spliced form of MEFV. In addition, methylated form of these constructs were analyzed and we observed that methylation of MEFV cDNA decreases the expression level of MEFV gene. The graph is given figure 3.17.

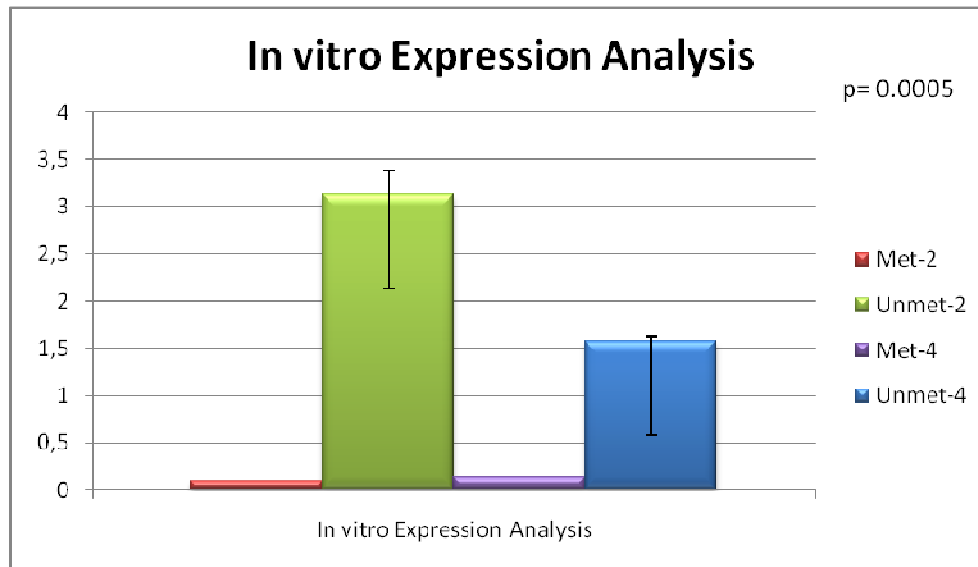


Figure 3.8 : In vitro expression levels of exon2 variants of MEFV

3.7 Real-Time Results for Methylation Status

Methylation levels of MEFV gene were compared between FMF patients and healthy control groups. Bisulfite treated DNA was used as a template to calculate MEFV methylation level and ACTB level which is used as an internal control. Real-Time PCR were done with primers and probes, which are specific to methylated sequences. Genomic DNA was used as a control for bisulfite treatment efficiency in each reaction. Fully methylated DNA (Millipore) was used in three different concentrations, as undiluted, 1:10 diluted and 1:100 diluted forms to prepare standard curve. The error rates of the Real-Time PCR reaction were about 1% for target gene and 0.1% for reference gene and the efficiencies of the reactions were about two for both gene. The standard curves of MEFV and ACTB are given in figures 3.18 and 3.19 below.

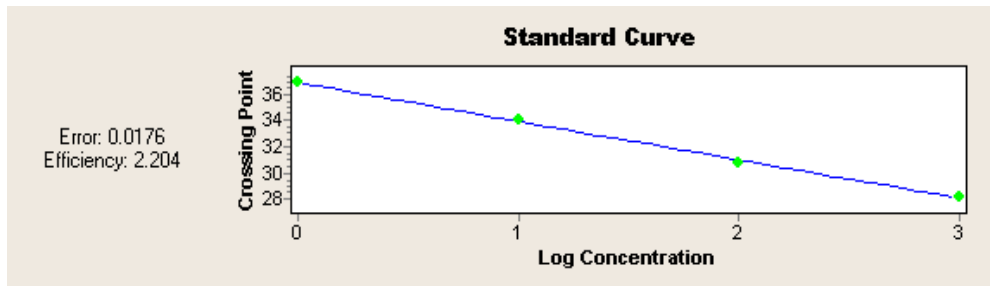


Figure 3.9 : Standard curve for MEFV in MethyLight experiment

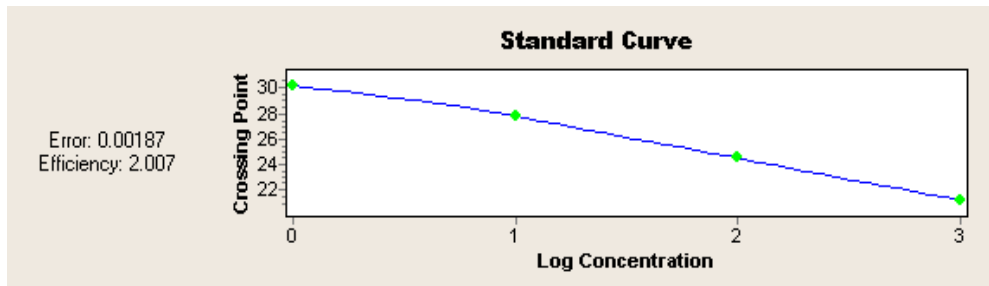


Figure 3.10 : Standard curve for ACTB in MethyLight experiment

Bisulfite treatment was seen to be efficient, since no amplification at the genomic DNAs of each sample. The methylation levels of samples were calculated by comparing to reference gene (ΔC_T method) in 34 of 51 FMF patients and 10 of 11 healthy controls. The $2^{-\Delta C_T}$ value of FMF patients group and healthy control group were analyzed statistically by using *Student's t test*. There was no significantly difference methylation status of MEFV between FMF patients and healthy controls ($p=0.53$, Std. Dev: 1,06). The graph of methylation status of MEFV is given figure 3.20.

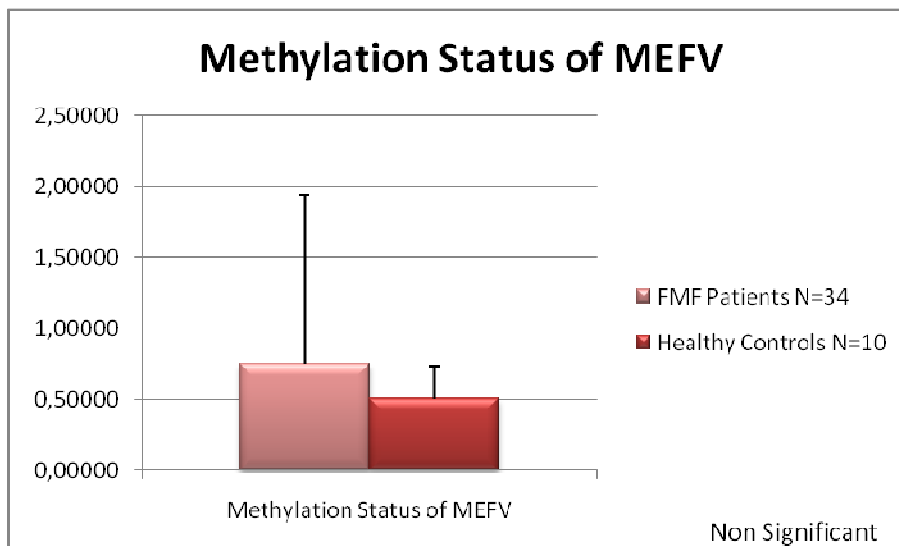
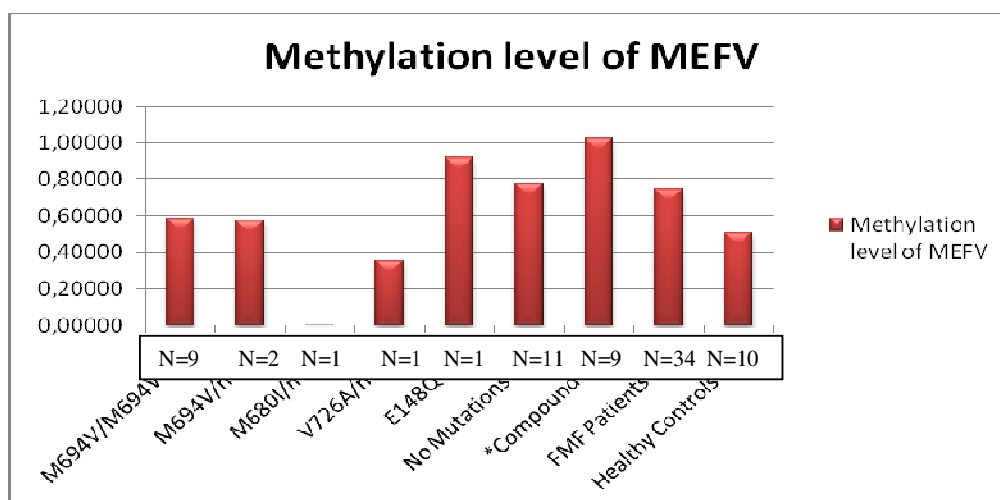


Figure 3.11 : Comparison of methylation levels of MEFV in FMF patients and healthy controls

Methylation levels of MEFV were also compared between FMF patients with different mutations and healthy controls. Methylation level of MEFV in FMF patients with M694V/M694V, M694V/n, E148Q/n and compound mutations and without mutations were higher than healthy children. On the other hand, in FMF patients with M680I/n and V726A/n genotype, the methylation levels of MEFV were lower compared to healthy controls (Figure 3.21).



*M694V/n-M680I/n (3), M694V/M694V-E148Q/n (3), M694V/n-E148Q/n, M694V/M694V-M680I/n, M694V/n-V726A/V726A

Figure 3.12 : Comparison of methylation levels of MEFV in FMF patients with different mutations and healthy controls

MEFV methylation level was compared between FMF patients with mutations and FMF patients without mutations and healthy controls with mutations and without mutations. In two analysis, there are no significantly differences were found. The graphs are given in figure 3.22 and 3.23.

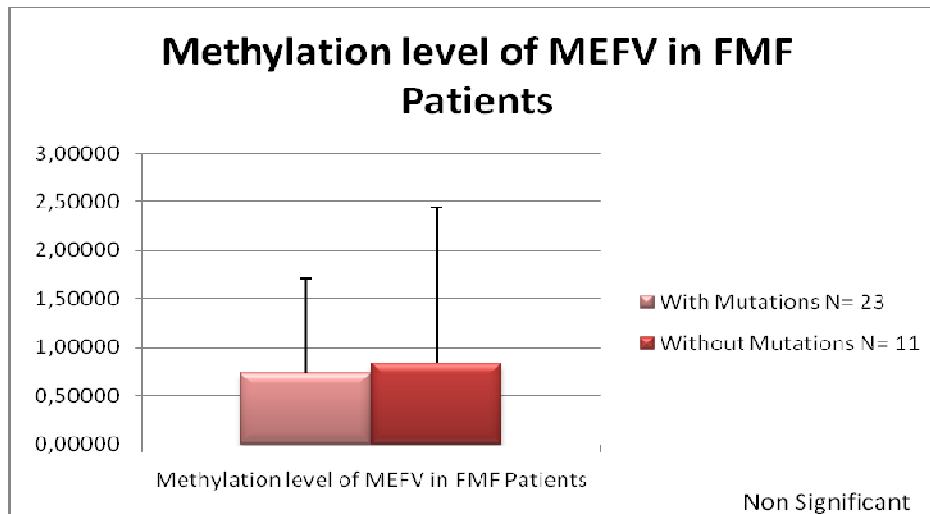


Figure 3.13 : Comparison of methylation levels of MEFV in FMF patients with mutations and FMF patients without any mutations

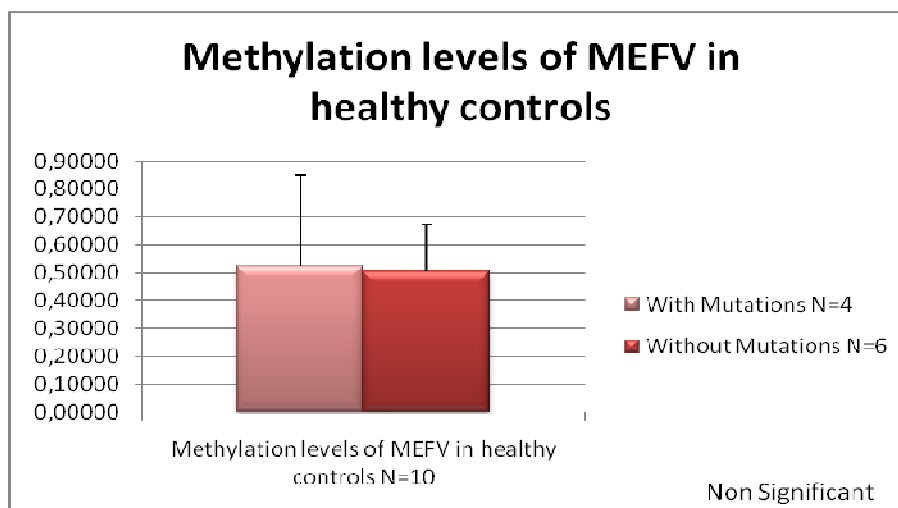


Figure 3.14 : Comparison of methylation levels of MEFV in healthy controls with mutations and without any mutations

Methylation status of second exon of MEFV was compared between FMF patients with mutations and without any mutations and healthy controls with mutations and without mutations. Methylation status of MEFV was found approximately 1,5 fold higher in FMF patients than healthy controls (Figure 3.23).

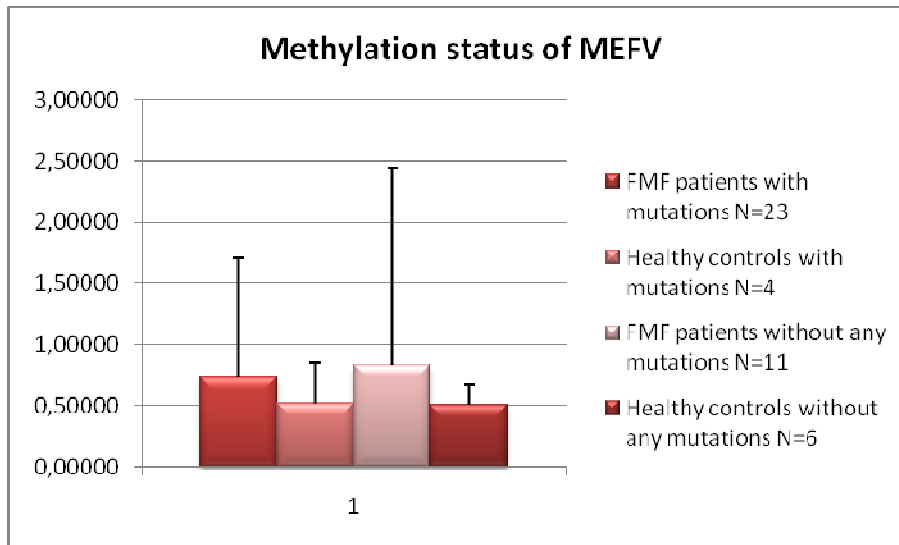


Figure 3.15 : Methylation status of MEFV

4. CONCLUSION AND DISCUSSION

The gene for FMF, MEFV, encodes the protein pyrin that is known to have a function in inflammation and apoptosis. However, its role for anti-inflammation or pro-inflammation is still under question; moreover there are conflictory results for the possible function of mutations on its mRNA expression.

In this study, we wanted to investigate the expression and methylation patterns of MEFV gene. Its expressional patterns in FMF and healthy controls, raises the question of whether methylation has a role in its differing expressional patterns because of the presence of a CpG islands in the second exon of MEFV. Previous studies showed that methylation in the exons of the gene can alter the expression by preventing transcriptional elongation (i.e. Lorincz *et. al.* 2004).

The present study shows that the expression levels of MEFV gene were significantly lower in FMF patients than in healthy controls ($p=0.031$). Our results are consistent with two previous expression studies (*Notarnicola et. al.*, 2002 and *Ustek et. al.*, 2007). On the other hand, *Booty et. al.*, 2009 showed that there is no significant difference in MEFV mRNA expression between FMF patients and healthy controls. We also compared the MEFV expression levels between FMF patients with mutations and FMF patients with no identified mutations and between healthy controls with mutations and healthy controls without mutations. Contrary to *Notarnicola's* study (2002), we did not observe significantly association between mutations and expression levels of MEFV in neither groups; still, the expression levels were slightly higher in individuals without mutations. When we analyzed the MEFV expression levels according to the type of mutations, the highest expression levels of gene was associated to M694V mutations, which is the opposite of the previous two results in literature (*Notarnicola et. al.*, 2002 and *Ustek et. al.*, 2007).

Methylation status of second exon of MEFV gene were not found significantly different between FMF patients and healthy controls ($p=0.53$). However the

methylation status in FMF patients were approximately 1.5 fold higher than healthy controls. When we compared the methylation levels of MEFV between FMF patients with mutation and FMF patients without mutations, we cannot find a significant difference ($p= 0.93$), however, methylation level were found slightly higher in FMF patients without any mutations. Accordingly, the low expression levels of MEFV may be related to the higher methylation status of the gene in FMF patients, hence more conclusive data is needed to prove the role of methylation on the pathophysiology of FMF.

In this study, we could not find significant association between methylation status of second exon of MEFV and expression levels of the gene. As a further aspect, the number of samples will be increased to obtain more statistically accurate results. As shown in the study of *Ustek et. al., 2007*, there is a significant difference in the relative expression of the MEFV mRNA using the primers covering 2-3 exons between the asymptomatic and inflammatory periods. However no such difference was observed when the primers for 7-10 exons are used. Therefore, the expression levels of the second exon fully spliced form of MEFV and of the full-length MEFV can be compared in future. In addition, the methylation status of MEFV gene in asymptomatic and inflammatory periods of FMF patients requires a further analyses.

Finally, in vitro cell culture studies can be done in the THP-1 cells, which are human monocytic cell line, to compare the expression levels of three variants of MEFV, which are fully spliced form of second exon, partially spliced form of second exon and full-length form of MEFV.

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APPENDICES

APPENDIX A : Equipment

APPENDIX B : Chemicals, Enzymes and Markers

APPENDIX C : Buffers

APPENDIX D : Used Kits

APPENDIX E : Sample PCR and Restriction Enzyme Results

APPENDIX F : CpG Sites on Second Exon of MEFV gene

APPENDIX A

EQUIPMENT

Centrifuges	Sigma 1-13 B. Braun International Allegra 25R Centrifuge Beckman Coulter
Electrophoresis equipments	E – C mini cell primo EC320
Gel Documentation System	UVI PhotoMW Version 99.05 for Windows
Pipettes	Gilson Pipetman 20 µL 200 µl, 1000 µl
pH meter	Mettler Toledo MP220
Spectrophotometer	PerkinElmer Lambda25 UV/VI Spectrometer
Thermo cycler	Applied Biosystems GeneAmp PCR System 2700 Corbett PalmCycler Techne FTGENE 5D
Transilluminator	Biorad UV Transilluminator 2000
Vortex	Herdolph Reax top

APPENDIX B

CHEMICALS

Agarose	AppliChem
Boric acid	Amresco
dNTP	Fermentas
EDTA	AppliChem
Ethanol	Riedel-de Haën
EtBr	Amresco
MgCl₂	Fermentas
Primers	IDT DNA
Tris Base	Amresco
10X PCR Buffer	Fermentas

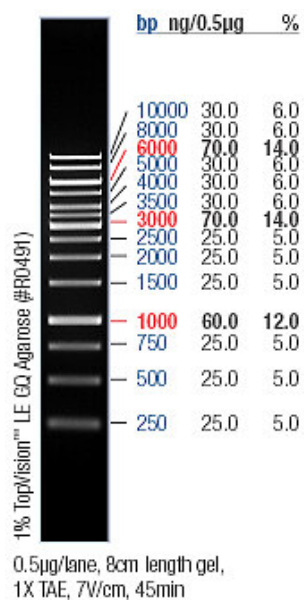
ENZYMES

<i>AluI</i>	Fermentas
<i>AvaI</i>	Fermentas
<i>HinfI</i>	Fermentas
<i>MboII</i>	Fermentas
<i>PagI</i>	Fermentas
Taq DNA Polymerase	Fermentas

MARKERS

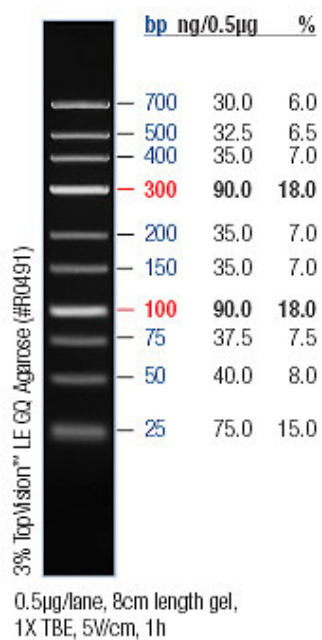
Gene Ruler™ 1 kb DNA Ladder

Fermentas



Gene Ruler™ DNA Ladder Low Range

Fermentas



APPENDIX C

BUFFERS

TE Buffer

Tris base 10 mM

EDTA 1 mM

Add ddH₂O to 1 liter and adjust the pH to 8.0

TAE (Tris-Acetic Acid-EDTA) Buffer (10X)

Tris base 108 g

Glacial acetic acid 50 mL

EDTA 40 mL (0.5 M, pH 8.0)

Add ddH₂O to 1 liter and adjust the pH to 8.0

Mini Agarose Gel (1%)

Agarose 0.5 g

TAE buffer (1X) 50 mL

Add 1.5 µL EtBr (final concentration: 0.5 mM) before pouring the gel into tray.

Midi Agarose Gel (1%)

Agarose 1.5 g

TAE buffer (1X) 150 mL

Add 4.5 µL EtBr (final concentration: 0.5 mM) before pouring the gel into tray.

APPENDIX D

USED KITS

DNA Isolation	DNA Isolation Kit for Mammalian Blood (Roche)
RNA Isolation	High Pure RNA Isolation Kit (Roche)
cDNA Synthesis	Transcriptor First Strand cDNA Synthesis Kit (Roche)
Bisulfite Treatment	EZ DNA Methylation Kit (Zymo)
Real-Time PCR	LightCycler TaqMan Master Mix (Roche)

APPENDIX E

SAMPLE PCR AND RESTRICTION ENZYME RESULTS

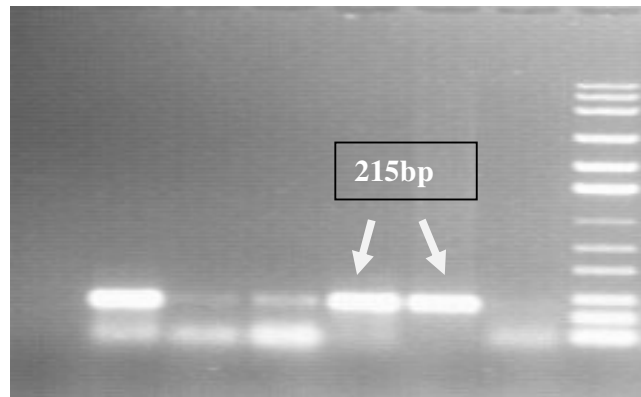


Figure C.1 : M694V PCR result

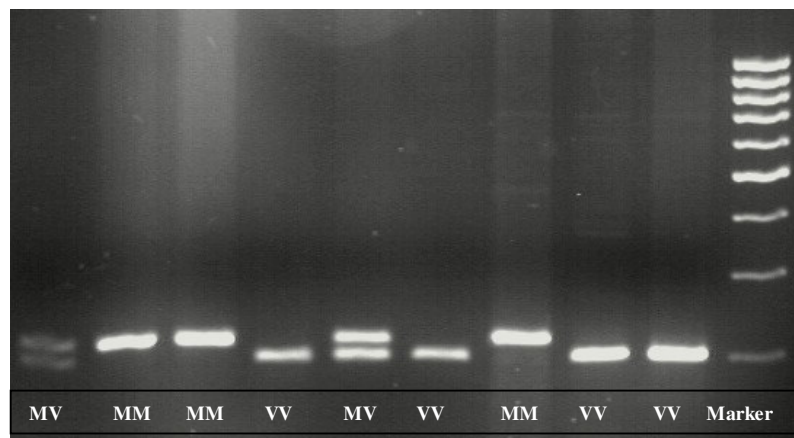


Figure C.2 : M694V restriction enzyme digestion result

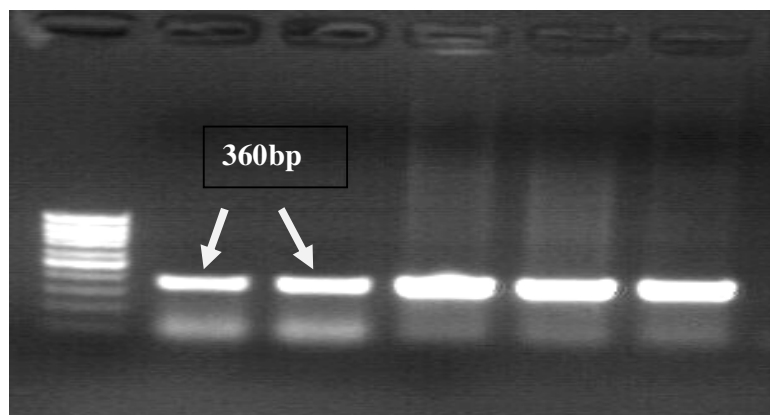


Figure C.3 : M680I and V726A PCR results

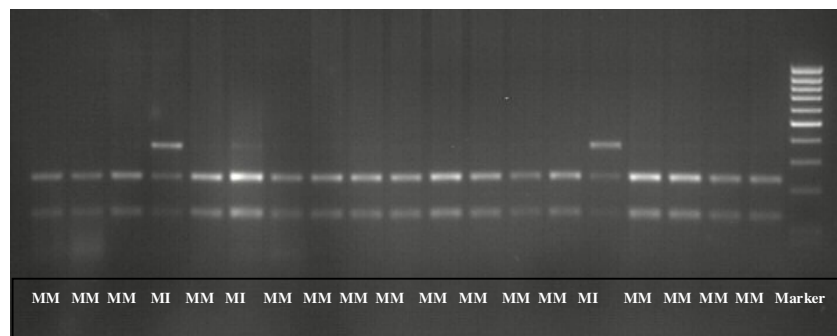


Figure C.4 : M680I restriction enzyme digestion result

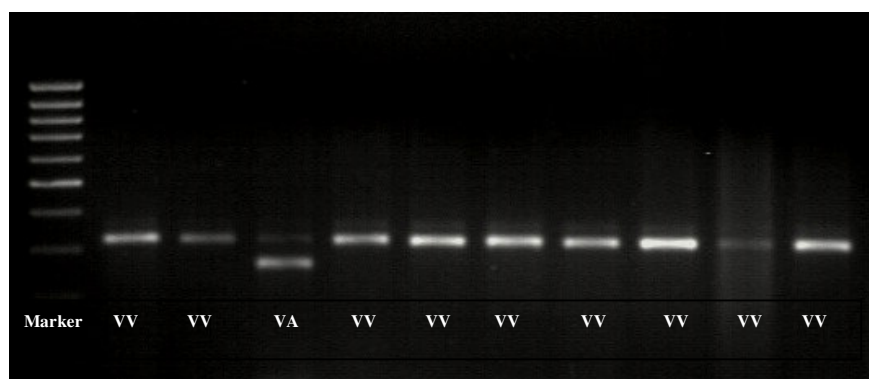


Figure C.5 : V726A restriction enzyme digestion result

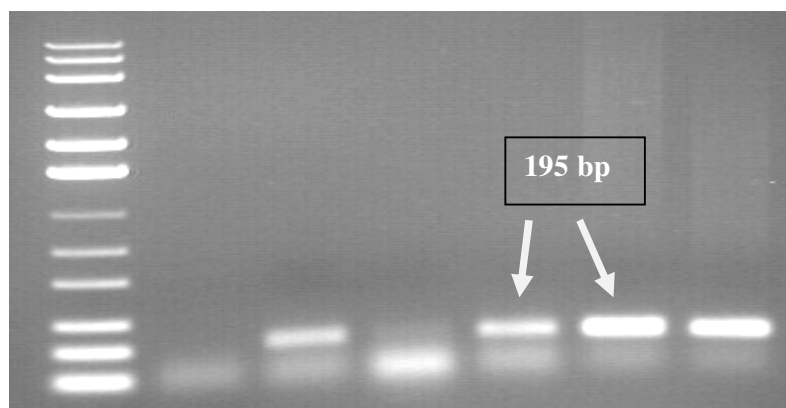


Figure C.6 : M694I PCR result

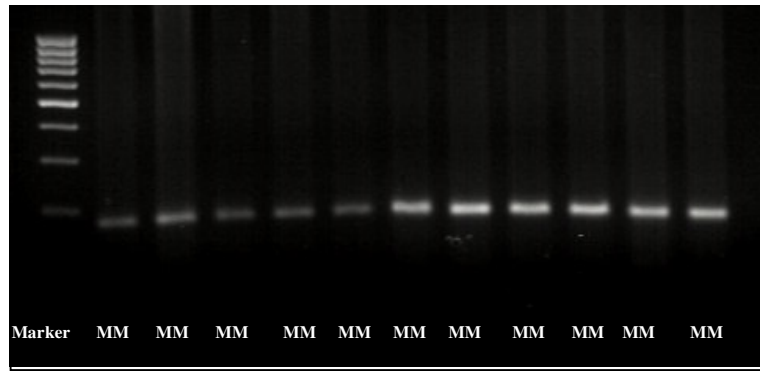


Figure C.7 : M694I restriction enzyme digestion result

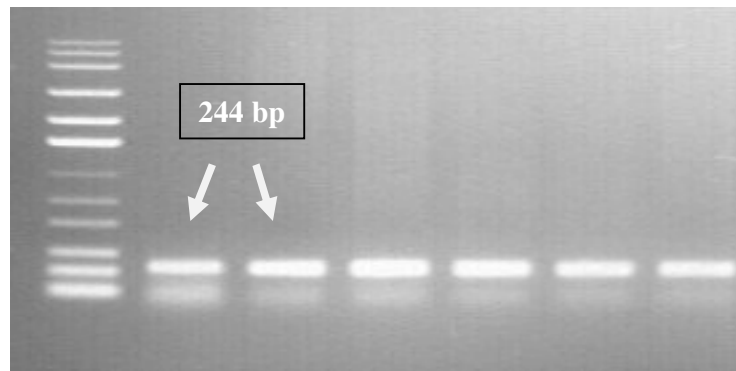


Figure C.8 : E148Q PCR result

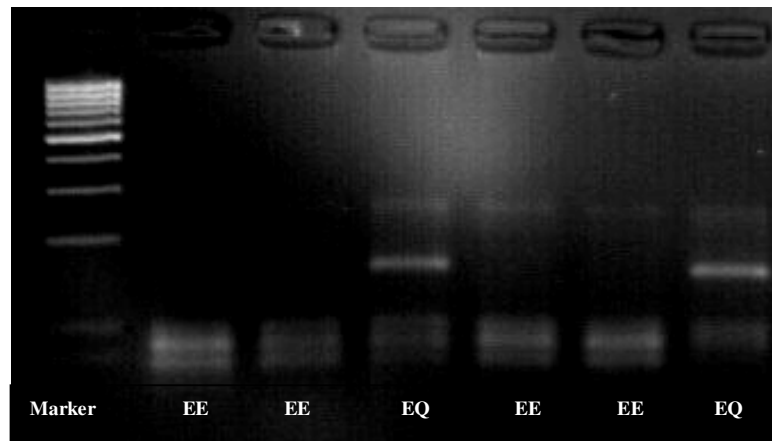


Figure C.9 : E148Q restriction enzyme digestion result

APPENDIX F

CpG SITES ON Second Exon of MEFV

Methylated	GTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTAGATTATTT CG AGGGGA CG	60
Unmethylated	GTTTTTGGGGGAGAATAAGTTTAGGAGTTTGAAGATTTAGATTATTTGAGGGGAATG	60
	*****	*
Methylated	AGGGGA CG GTTTT CG GT CG TACGGGG CG GAGTTGTTAGTTTG CG GTGTAGTTAGTT CG	120
Unmethylated	AGGGGAATGGTTTTTGGTTGTATGGGGTGGAGTTGTTAGTTTGGTGTAGTTAGTTTG	120
	*****	*****
Methylated	AGGT CG GGAGGGGGTTGT CG AGGAAGTTTTTGAAGTAA CG TAGAGAGAAGGTTT CG GAGG	180
Unmethylated	AGGTTGGGAGGGGGTTGTTGAGGAAGTTTTTGAAGTAAATGTAGAGAGAAGGTTTGGAGG	180
	*****	*****
Methylated	GTTTGGAC CGCG TAGGGTAAGTTT CG GATT CG GAGTT CG GTTTTGT CG GG CG GGAGAAGTT	240
Unmethylated	GTTTGGATGTGTAGGGTAAGTTTTGGATTTGGAGTTTGGTTTTGTTGGGTGGGAGAAGTT	240
	*****	*****
Methylated	T CG GTTTTTGTAGGG CG TTAGAGGGGGTTAGGT CG AGGTT CG GTT CG GTAGAAA CG TTA	300
Unmethylated	TTGGTTTTTGTAGGGTGTAGAGGGGGTTAGGTTAGGTTTGGTTGTGTAGAAATGTTA	300
	* *****	*****
Methylated	GTTT CGCG GGGAGGTTGTAGGGGTGG CG GGGG CG TTT CG GGGTAGAAGGAGTGTAGGT	360
Unmethylated	GTTTTGTGGGAGGTTGTAGGGGTGGTGGGGGTGTTTGGGGTAGAAGGAGTGTAGGT	360
	**** *	*****
Methylated	TTTT CG AAGTGTATTGTTTT CG GGAAAGAT CG GATTTAGAAGTTTTGAGGTTATTATTT	420
Unmethylated	TTTTTGAAGTGTATTGTTTTGGGAAAGATGTGATTTAGAAGTTTTGAGGTTATTATTT	420
	**** *	*****
Methylated	TTATAGGGGAGAAGC CG TT CG TAAATTTAGAAATTTTTTGGATTTTAGAGGAAAAGATAG	480
Unmethylated	TTATAGGGGAGAAGGTGTTGTAAATTTAGAAATTTTTTGGATTTTAGAGGAAAAGATAG	480
	*****	*****
Methylated	TTG CG AATTTGGATT CG GTAATAGAATTT CG GGTAAGGTTTATTT CG GATGGAGGGGTAT	540
Unmethylated	TTGTGAATTTGGATTGGTAATAGAATTTGGGTAAGGTTTATTTGGATGGAGGGGTAT	540
	*** *****	*****
Methylated	TTG CG GATTTGAAGGAAGGTTTTTGAAA	568
Unmethylated	TTGTGGATTTGAAGGAAGGTTTTTGAAA	568
	*** *****	

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