İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

THE IMMUNE GENETIC PROPERTIES OF THE HEALTHY ELDERLY: ANALYSIS ON COMMON MEFV MUTATIONS AND GENE EXPRESSION

M.Sc. Thesis by

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Department : Advanced Technologies

Programme: Molecular Biology-Genetics and Biotechnology

JANUARY 2008

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Date of submission : 24 December 2007

Date of defence examination: 21 January 2008

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JANUARY 2008

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

SAĞLIKLI YAŞLILARIN İMMÜN-GENETİK ÖZELLİKLERİ: YAYGIN MEFV MUTASYONLARININ VE GEN İFADESİNİN ANALİZİ

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Tezin Enstitüye Verildiği Tarih : 24 Aralık 2007
Tezin Savunulduğu Tarih : 21 Ocak 2008

Tez Danışmanı : Yard.Doç.Dr. Eda TAHİR TURANLI
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OCAK 2008

ACKNOWLEDGEMENTS

I would like to express my deep graditude to my supervisor Assist. Prof. Dr. Eda TAHİR TURANLI for the inspiring guidance, support and the opportunities she provided me. It was a great pleasure for me to carry on this thesis under her supervision. I want to thank her for encouraging me to establish this thesis, which could not have been accomplished without her.

I would also like to thank to Prof. Dr. Hasan YAZICI for implementing and supporting this project; Prof. Dr. Tanju BEĞER, Prof. Dr. Deniz ERDİNÇLER, and Dr. Aslı ÇURGUNLU for clinical evaluation of the healthy elderlies.

I would like to thank to Dr. Murat BOLAYIRLI for his meticulous work in biochemical tests and the collaborating nurses from Geriatrics polyclinic of Cerrahpaşa, Gül ÖZKESKİN and Nefise YURTSEVER for their kind help in collecting the blood samples.

I would like to thank to Scientific Research Projects of Istanbul University (project no. 505/050552006) for the financial support they provided for this project.

I would also like to thank to the authorities of TUBITAK (2228 programme) and gratefully acknowledge the financial support they provided me for my graduate studies.

I would like to thank my lab partners, Esra KARACA, Gökçe ÇELİKYAPI, and Selçuk DAŞDEMİR for their collaboration and help in setting up our lab from ground. It was an invaluable experience for me to work with them.

My dearest being Mert YENTÜR, thanks for always being there for me in the last couple of years, your love and support meant the world to me. And I thank God for enabling me to achieve all that I have achieved, and I thank to my parents Pakize and Mehmet Ali KARAMAN and my brother Ekin KARAMAN for their endless love, patience and having every confidence in my ability to succeed.

2007, Fall **Sinem KARAMAN**

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ABBREVIATIONS

FMF : Familial Mediterranean Fever
MEFV : Mediterranean Fever (gene)
RNA : Ribonucleic Acid
IL : Interleukin
BD : Behçet's Disease
UC : Ulcerative Colitis
RA : Rheumatoid Arthritis
PAN : Polyarteritis Nodosa
RF : Rheumatoid Factor
IgM : Immunoglobulin M
IgG : Immunoglobulin G
CRP : C-Reactive Protein
hsCRP : High-sensitivity C-Reactive Protein
PCR : Polymerase chain Reaction
mRNA : Messenger Ribonucleic Acid
rRNA : Ribosomal Ribonucleic Acid
GAPDH : Glyceraldehyde-3-Phosphate Dehydrogenase
LNA : Locked Nucleic Acid
DNA : Deoxyribonucleic Acid
EDTA : Ethylenediaminetetraacedic acid
RE : Restriction Enzyme
ETOH : Ethanol
cDNA : Complementary DNA

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THE IMMUNE GENETIC PROPERTIES OF THE HEALTHY ELDERLY: ANALYSIS ON COMMON MEFV MUTATIONS AND GENE EXPRESSION

SUMMARY

Familial Mediterranean Fever (FMF) is an autosomal recessive disease that commonly affects people of Mediterranean heritage. It is typically characterized by attacks of serositis observed in the abdomen and accompanied by fever and elevated acute-phase molecules. Five mutations in MEFV (the gene for FMF) namely, E148Q, M680I, M694V, M694I, and V726A, make up for approximately 70% of the disease associated alleles. However, two main observations suggest that MEFV gene is involved in inflammatory pathway in general and is not only specific to FMF; 1) increased MEFV mutation frequencies in Behçet's disease (BD), ulcerative colitis (UC), rheumatoid arthritis (RA), and polyarteritis nodosa (PAN) which are chronic inflammatory diseases, 2) FMF protein product pyrin is involved in the anti-inflammatory regulation pathway.

In recent studies, relationships between MEFV mutations and MEFV expression levels are frequently investigated. In one of these studies, M694V mutation carriers were shown to have lower MEFV expression (in both healthy but carrier controls and FMF patients), and the expression level drops even further when two mutant alleles of M694V are present. In another study, it is shown that the mRNA levels of MEFV drop dramatically when the patients have FMF attacks. The researchers concluded that the decreased MEFV mRNA level is related with acute inflammation, and one might indicate that the lowered expression of MEFV may be responsible for the attacks.

A method in studying the association between those genes, which play a role in inflammatory response such as MEFV and inflammation, can be the study of the frequency of such genes in the elderly population who have not experienced diseases related to FMF or rheumatoid arthritis with chronic inflammation. Here, the hypothesis tested is the less frequent occurrence of inflammatory gene mutations in the elderly population who have lived a healthy life without inflammatory disease as compared to general population. So the aim of this study was to reveal any statistical difference in the frequencies of five most common mutations reported in MEFV gene, between a population of healthy elderly and historical controls representing the normal population. For this purpose, genotyping was done for five mutations, and the results were first compared with historical controls and then subgroup analyses for inflammation markers such as; RF (Rheumatoid Factor) and hsCRP (high sensitive

C-Reactive Protein) positivity versus mutation distribution were done. Furthermore, a gene expression analysis was done with real-time PCR, in order to reveal any association between mutations and expression level of MEFV. Comparison between the mRNA levels of MEFV was done between mutation carriers, wild type allele carriers and FMF patients with known MEFV mutations.

SAĞLIKLI YAŞLILARIN İMMÜN-GENETİK ÖZELLİKLERİ: YAYGIN MEFV MUTASYONLARININ VE GEN İFADESİNİN ANALİZİ

ÖZET

Ailesel Akdeniz ateşi (AAA), yaygın olarak Akdeniz toplumlarını etkileyen otozomal çekinik bir hastalıktır. Genelde karında görülen serozit atakları ve buna eşlik eden ateş ve yükselmiş akut-evre molekülleriyle karakterizedir. MEFV (ailesel akdeniz ateşi geni) geninde bulunan beş mutasyon, yani E148Q, M680I, M694V, M694I, ve V726A, hastalıkla alakalı alellerin yaklaşık %70'ini oluşturur. Ancak, iki gözlem MEFV geninin enflamatuar yolizinde bulunduğunu ve AAA'ya has olmadığını gösterebilir 1) Kronik iltihaplı hastalıklardan, Behçet Hastalığı (BH), ülseratif kolit (CH), romatoid artrit (RA) ve poliarterit nodoz (PAN) da MEFV mutasyon sıklığının artmış olması, 2) MEFV geninin ürünü pirin proteinin anti-enflamatuar regülasyon yolizinde rol alması.

Son zamanlarda yapılan çalışmalarda MEFV'deki mutasyonlarla MEFV'nin gen ifadesi seviyesi arasındaki ilişkiler sıkça incelenmeye başlanmıştır. Bu çalışmalardan birinde M694V mutasyonunu taşıyan kişilerde (hem sağlıklı taşıyıcılarda, hem de FMF hastası taşıyıcılarda) MEFV gen ifadesinin daha düşük olduğu bulunmuştur, ayrıca iki M694V mutasyonunu taşıyan kişilerde gen ifade seviyesinin çok daha fazla düştüğü gözlenmiştir. Başka bir çalışmada ise FMF hastalarının MEFV mRNA seviyelerinin ataklar esnasında, ataksız geçen döneme kıyasla çok daha düşük olduğu bulunmuştur. Araştırmacılar, MEFV'nin mRNA seviyesinin akut iltihapla bağlantılı olduğu sonucuna varmışlardır. Buradan düşük MEFV mRNA üretiminin atakların sorumlusu olabileceği ileri sürülebilinir.

MEFV'nin iltihapla alakası gibi, iltihap yanıtında rol alan genlerin arasındaki ilişkinin analizinde kullanılabilecek metodlardan biri, daha once AAA veya Romatoid Artrit gibi kronik iltihaplı hastalık yaşamamış sağlıklı yaşlılarda bu genlerin sıklığının araştırılması olabilir. Burada sınanan varsayım iltihaplı hastalığa yakalanmayanlarda iltihapla ilgili genlerin serbest toplumda beklenen sıklıktan daha az sıklıkta olmasıdır. Dolayısıyla bu çalışmanın amacı, bir sağlıklı yaşlı grubu ile normal popülasyonu temsil eden tarihsel kontroller arasında, MEFV'nin en yaygın beş mutasyonunun sıklıkları arasında istatistiksel olarak bir farklılığın varolup varolmadığının ortaya çıkartılmasıdır. Bu amaçla, bu beş mutasyon genotiplendi ve sonuçlar önce tarihsel kontrollerle kıyaslandı, daha sonra da iltihabı işaret eden faktörlerden Romatoid Faktör ve C-Reaktif Protein seviyelerinin pozitivitesine karşılık mutasyon dağılımlarının altgrup analizleri yapıldı. Ayrıca, Gerçek-Zamanlı Polimeraz Zincir Reaksyonu ile mutasyonlar ve gen ifadesi seviyesi arasındaki olası

ilişkiler analiz edildi. MEFV mRNA seviyeleri mutasyon taşıyıcıları, mutasyonsuz alel taşıyıcıları ve bilinen mutasyonları taşıyan AAA hastaları arasında yapıldı.

1. INTRODUCTION

1.1 Familial Mediterranean fever, MEFV gene and its product PYRIN/Marenostrin

Familial Mediterranean Fever (FMF) is an autosomal recessive disease that mainly affects people of Mediterranean ancestry. It is one of the most common periodic fever syndromes. FMF is typically characterized by attacks of serositis usually in the abdomen; chest and joints accompanied by fever and elevated acute-phase molecules [1-3]. The frequency of FMF in Turkish population is determined as 1 in 1073; however, this value increases to 1 in 395 in the interior regions of Turkey [4].

In 1997, the International FMF Consortium found a region of 115-kb on chromosome 16 via positional cloning, and declared it as the candidate region for FMF. MEFV (Mediterranean Fever) gene was identified within this region as the gene for FMF [5]. In the same year, but as a part of a separate study, French FMF Consortium deduced the region to 60 kb and identified four genes including MEFV within this region [6]. MEFV gene is found on 16p13.3, between D16S80 and D16S283, it is made up of 10 exons and it encodes an RNA product of 3.7-kb. The MEFV protein is called pyrin or marenostrin, and unspliced form of pyrin consists of 781 amino acid residues [5,6]. Pyrin is expressed predominantly in neutrophils, eosinophils and cytokine-activated monocytes. There is an alternative spliced form of pyrin which has its 2^{nd} exon spliced, and is 570 amino acids long [7]. Full-length pyrin is mainly found in the cytoplasm, while the spliced form is found in the nucleus. Papin suggest that pyrin is a nuclear protein and the alternative splicing of this protein regulate the action of mutant and wild type pyrins via altering their localization [7]. Mansfield reported the co-localization of full-length pyrin with the cytoskeletal proteins, namely microtubules and the actin cytoskeleton [8]. Pyrin includes four defined domains from N terminus to C terminus; (1) the PYD domain,

(2) the B-Box, (3) coiled-coil (CC) segments, and (4) the 160 − 170 amino acid B30.2 domain. The B30.2 domain overlaps with the 10th exon of MEFV gene and majority of the mutations observed in FMF patients lie within this region [9]. Recently, the B30.2 domain of pyrin is suggested to interact directly with Caspase-1, (Figure 1.1) and a decrease in interaction is shown related with the 10th exon mutations (M680I, M694V, or V726A) [10]

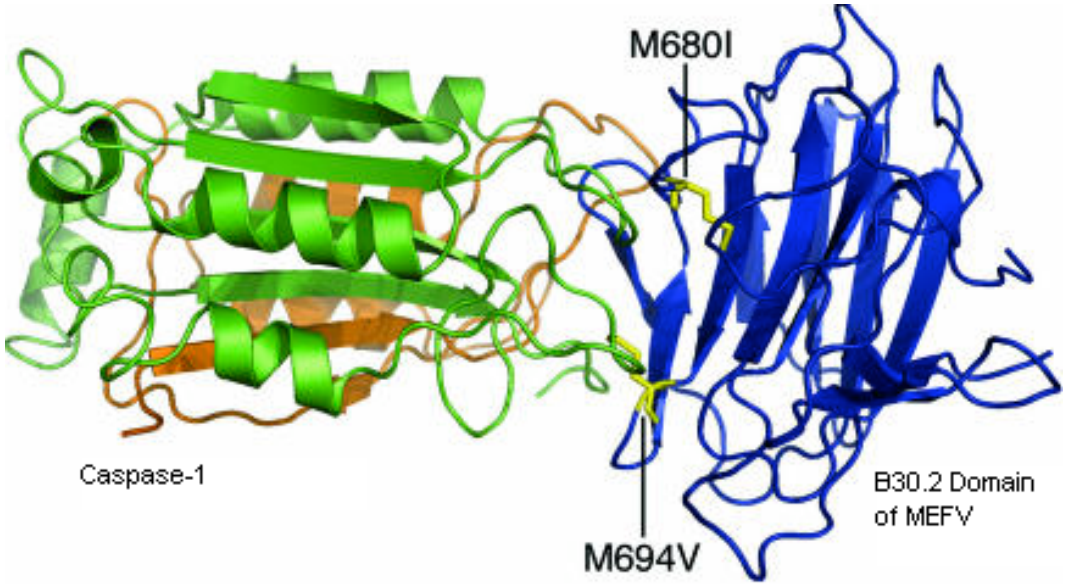

Figure 1.1 Pyrin B30.2 domain and suggested interaction with Caspase-1 [10]

Pyrin involves in innate immune response. It is upregulated by anti-inflammatory signals such as IL-10 (Interleukin 10), IL-4, (Interleukin 4) as well as proinflammatory signals such as Lipopolysaccharides (LPS). Pyrin is thought to be a suppressor of inflammation [5,6] because it uses its PYD domain to interact with ASC adapter protein to suppress IL-1β (Interleukin 1β) processing via interfering with caspase-1 activation [9]. When functional pyrin is expressed, it binds to ASC protein and inhibits the binding and activation of caspase-1. (Figure 1.2) But if pyrin is expressed in low levels or is non-functional, the caspase-1 is activated and the pathway leads to IL-1β processing and formation of active IL-1β, which causes the inflammation and the fever by stimulating the thermoregulatory center in the central nervous system [9,11].

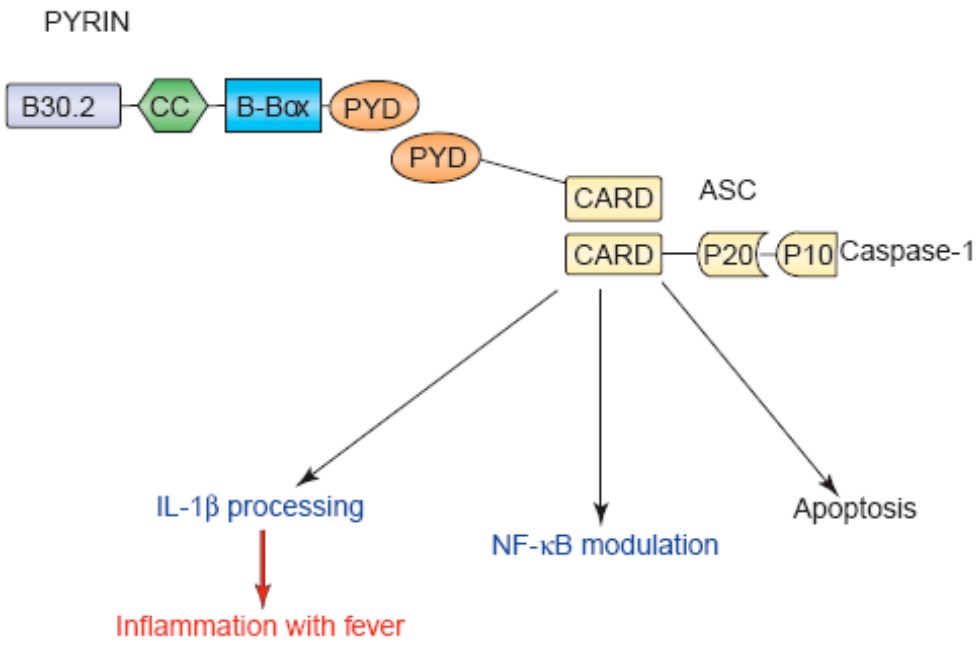

Figure 1.2 Domains of pyrin & Pyrin- PYD domain and interaction with ASC [9]

1.2 MEFV mutations

Since the cloning of the MEFV gene in 1997 [5,6], about 40 disease-associated mutations have been identified [12]. Five mutations in MEFV gene, E148Q, M680I, M694V, M694I, and V726A, make up for at least 70% of the disease associated alleles [13]. Some of these mutations and their localization within the MEFV gene is shown in Figure 1.3 below. The causes and resulting changes (if any) of five most common mutations are as follows: a codon change of GAG to CAG at position 148 creates the E148Q mutation in exon 2 and causes the loss of an Ava*I* restriction site. Another codon change of ATG to ATC at position 680 creates the M680I mutation in 10^{th} exon, and causes Hinf*I* restriction site loss. The codon change of GTT to GCT at position 726 creates the V726A mutation in 10^{th} exon, and causes Alu*I* restriction site creation. The codon change of ATG to GTG at position 694 creates the M694V mutation in 10^{th} exon, whereas the change in the same codon is from ATG to ATA, it creates the M694I mutation [14].

FMF is especially predominant in Turkish, Non-Ashkenazi Jew and Armenian populations, which are of the Mediterranean ancestry. The approximated carrier

frequencies are relatively higher in these populations (1/6 in Turkish and Armenian, 1/7 in Non-Ashkenazi Jew population) [15]. According to Yılmaz *et al.* the carrier frequency of normal Turkish population is 20% (1/5), and the frequencies of mutations in this normal control group are; M694V = 1.5%, M680I = 2.5%, V726A = 1%, M694I = 0% and E148Q = 6%, N=100, 200 chromosomes. [16] Another study of analyzing the mutation frequency of healthy Turkish population revealed the mutation frequencies as; M694V = 3.06%, M680I = 0%, V726A = 2.04%, M694I = 0% and E148Q = 2.04%, N=49, 98 chromosomes [17].

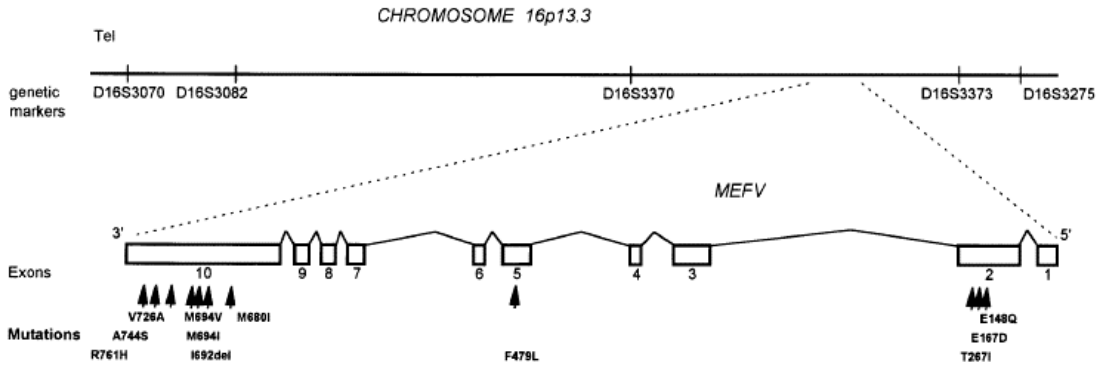

Figure 1.3. Schematic representation of MEFV gene, exons and loci of important mutations [14]

1.3 Chronic Inflammatory Diseases and MEFV Mutations

Pyrin is thought to be an anti-inflammatory regulator found in many granulocytes. Mutated forms of pyrin, which cannot function properly, are frequently found in Familial Mediterranean fever patients, (74% of FMF chromosomes in usual cases consist of five most common mutations in MEFV) [13] and since pyrin is in an inflammatory regulation pathway the MEFV mutations can contribute to the occurrence of other chronic or periodic inflammatory diseases. Increased MEFV mutation frequencies are shown in Behçet's disease [18,19], ulcerative colitis (UC) [20], rheumatoid arthritis (RA) [21], polyarteritis nodosa (PAN) [22] which are known chronic and periodic inflammatory diseases. MEFV mutations are found more frequently in BD patients (26% carried one mutant allele of either M694V, M680I or V726A in the study of Atagunduz *et al.*; M694V = 2.6%, V726A = 2.6%, E148Q =

5.2% in the study of Touitou *et al*.) than the control groups (9.1% carried one mutant allele of either M694V, M680I or V726A in the study of Atagunduz *et al*.; M694V = 0%, V726A = 0%, E148Q = 2.2% in the study of Touitou *et al*) and these mutations are reported to be a susceptibility factor for Behçet's disease. In Giaglis *et al.*'s study, a group of 25 untreated UC patients - as subjects, and two control groups; 28 RA patients and 65 normal individuals, were investigated for MEFV mutations in exons 2 and 10. The RA control group was used as an internal control group, as it represents a disease that has an unaltered level of inflammation unlike the periodic inflammation states observed in UC. It is shown that 28% of UC patients carry at least one MEFV mutation; whereas, only 3.5% of RA control group and 1.5% of normal control group carry MEFV mutations in exon 2 and 10. Moreover, 57% of mutation carrier UC subjects had inflammatory arthritis, in contrast with the mutation free UC patients, none of whom had inflammatory arthritis. The group concluded that MEFV mutations may have a role in the alteration of inflammatory response [20]. Furthermore, Rabinovich *et al.* reported that the median severity score of RA was significantly higher in the mutation carrier group than the non-carrier group ($p = 0.0005$). It is also stated in the same study that the E148Q mutation in MEFV gene is an independent modifier of RA symptoms [21]. Another study that supports the correlation between the prevalence of MEFV mutations and inflammatory diseases was published by Yalcinkaya *et al.* in 2007. This study scrutinized the MEFV mutation frequencies in childhood polyarteritis nodosa patients. 6 common mutations of MEFV (M694V, M680I, V726A, E148Q, M694I and K695R) were investigated in 29 PAN patients. 38% of these 29 PAN patients (n=11) were shown to carry at least one MEFV mutation, and three of these were M694V homozygous mutants. The researchers implied that the MEFV mutations might become a susceptibility factor via inducing a proinflammatory state and increasing the immune response to streptococcal infections.

1.4 MEFV Gene Expression Levels

Recent studies on revealing the effects of MEFV mutations on the MEFV mRNA levels have given important results. Notarnicola *et al.* studied the effects of different mutations on MEFV gene expression, and found relations between the type of the

mutation and difference in expression level. More specifically, the healthy control group without the mutation had the highest MEFV expression, healthy but carrier group had intermediate expression; but FMF patients with mutations had dramatically dropped expression levels of MEFV transcript (P = 0.00001). These findings proposed that there is a direct relationship between the number of mutations in MEFV and decrease in mRNA amount. Furthermore, the studies showed that there is also significant difference between non-patient carriers and mutation free-controls (P = 0.008). The M694V mutation was revealed to be the most effective mutation in decreasing the expression level of MEFV mRNA. This data was both confirmed for FMF patients with M694V, and for healthy carriers. The study implies that the pathology in FMF might be caused by the aberrant MEFV expression [23].

Another study on MEFV expression levels was conducted by Ustek *et al*., which ascertained the changes in the expression of MEFV in normal and acute inflammation/FMF-attack phases. They also had a control group of 17 normally healthy individuals who just were having operation because of acute appendicitis. The results confirmed the findings of Notarnicola *et. al*. But additionally, this study showed even more decreased MEFV mRNA levels in the blood samples collected during the FMF attacks compared to the asymptomatic phases of the patients, which may indicate that the cause of the attacks being lowered expression of MEFV in the first place. The researchers concluded that the decreased MEFV mRNA level is connected with acute inflammation [24].

1.5 Inflammation Markers

1.5.1 Rheumatoid Factor

Rheumatoid Factors (RF) are autoantibodies that are usually produced in particular inflammatory diseases, mainly in Rheumatoid Arthritis (RA). On the other hand, RF production in the normal elderly people, in immunized normal people and in individuals with chronic inflammation or autoimmune conditions is also reported [25,26]. The production of RFs are accompanied by destructive joint inflammation in RA disease. RFs are produced in two forms: IgM (Immunoglobulin M) and IgG

(Immunoglobulin G) and bind to the relevant epitopes in the Fc site of Immunoglobulin G's (IgGs). These RFs are found in the bound form with autologous IgG in the synovia of RA patients. Moreover, higher RF values have shown a positive relationship with the arthritis severity and progressive joint disintegration [25].

1.5.2 C-Reactive Protein

C-reactive protein (CRP) is an acute-phase pentamer protein that is composed of five identical protomers, synthesized by the liver. It was first recognized by William S. Tillet and Thomas Francis in 1930s while observing the immune response of pneumococcal pneumonia patients. Normal concentration in blood serum is 0.8 mg/L. CRP level is majorly controlled via transcriptional regulation and its expression is upregulated with IL-6, both reported from *in vivo* and *in vitro* studies. The contribution of IL-1β to upregulation of CRP expression is immense when it is combined with IL-6, because IL-1β enhances the translational level of regulation unlike IL-6. Thus, the level of CRP dramatically increases with inflammation since its expression depends on proinflammatory signals. As a result, CRP levels show the burden of inflammation [27]. In clinical investigation the CRP levels are measured with hsCRP (high sensitivity-C reactive Protein) test [27,28].

1.6 Real Time PCR

Real Time PCR (Polymerase Chain Reaction) is a powerful tool both to observe the behavior of the ongoing PCR reaction and make absolute or relative quantification of specific targets. Absolute quantification is employed to reveal the original copy number of the target in the samples via a comparing the signal strength with a standard curve. On the other hand, in relative quantification; the amount of target template is compared to a control sample [29]. The relative quantification Real-Time PCR systems usually use relative fold change method for analyzing differences in gene expression. In this method a calibrator is selected among the samples and its expression is designated as 1X. The relative expression levels are obtained by first normalizing the expression levels via dividing the "Target gene" value with "Reference gene" value, and then dividing this normalized expression values of each

sample with the normalized calibrator value. The resulting values give the relative expression levels of the samples, such as an n fold change in expression according to the 1X sample. Both of the reference and target mRNA amounts are calculated from the standard curves prepared by 1/1, 1/10 and 1/100 dilutions of the calibrator sample. There are two points of great importance; firstly the calibrator sample should have the "normal" expression of the target gene. Secondly, it is very important to have sufficient amount of calibrator sample, which is used in every reaction [30].

1.6.1 Selection of the Endogenous Control

The main principle of including an endogenous control in a Real-Time PCR reaction is to have an internal constant (an mRNA with invariable expression) simply to normalize the reactions for the amount of RNA used in the Reverse Transcription reaction. Many housekeeping genes account for good internal controls because their expression is hardly altered. Moreover, the internal control gene's expression should also not be altered with any tested parameter in the experiment [31]. Relative gene expression evaluations can be applied safely, if the endogenous control is chosen as a gene with higher expression than the target gene. Some of the good internal controls for Real time PCR are; β-actin, β_2-microglobulin and rRNA. GAPDH (Glyceraldehyde-3-Phosphate-Dehydrogenase) was also used frequently as internal controls but it is shown that the expression of GAPDH is increased in proliferating cells, so it is not reliable and should not be used especially in cancer research [31,32].

1.6.2 Properties of the Probes

Probes used in this study were selected from Universal ProbeLibrary of Roche. These probes were hydrolysis probes that recognized sequences from human β_2-microglobulin and MEFV mRNA. The probes were pre-labeled by the supplier with a reporter dye FAM and a dark quencher dye. The probes were 8-9 bases long LNA (Locked Nucleic Acid) probes. LNA has a special chemistry, which allows very short oligonucleotides to be efficient in hybridization within real-time PCR studies. Because of the unusual chemistry of LNA, the annealing temperature of the probes are unexpectedly high for such short oligonucleotides. Another advantage of LNA

probes is that they are highly sequence specific, which makes them excellent for single-base mismatch recognition [33].

These probes are selected within exon spanning regions on the MEFV and β_2-microglobulin mRNAs. Since the probes are extremely short, the specificity of the reaction has to be achieved also with the primer sets used. The software available on the website of Universal ProbeLibrary (www.universalprobelibrary.com) helps researchers to select a set of specific primers in addition to a probe from the Universal ProbeLibrary that will provide the finest results [33].

1.7 Aim of the Study

The aim of this study was to analyze the frequency of five most common mutations observed in MEFV (Mediterranean Fever) gene in a population of healthy elderly, to test if there is any statistical difference in their frequency occurrence from the normal population. A negative association was expected between healthy ageing and MEFV mutation frequencies. The genotyping results were compared with historical controls and also subgroup analyses for inflammation markers such as; RF (Rheumatoid Factor) and hsCRP (high sensitive C-Reactive Protein) value differences were done. Furthermore, in order to reveal any association between mutations and expression level of MEFV, a gene expression analysis was done with quantitative real-time PCR; the comparison was done between mutation carriers, wild type allele carriers and FMF patients with known MEFV mutations. Findings from this thesis are expected to reveal that, MEFV gene is not only a gene that causes FMF but rather a central gene in inflammatory response and the mutations in MEFV might disrupt the protein function or its expression level, causing imbalance in anti-inflammatory pathway and be the cause of many different chronic inflammatory diseases.

2. MATERIALS and METHODS

2.1. Materials and Laboratory Equipment

2.1.1. Used Equipments

The laboratory equipment used during this project is listed in Appendix A.

2.1.2. Used Chemicals, Enzymes, Markers and Buffers

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

2.1.3. Used Kits

The kits used and their suppliers are given in Appendix D.

2.2. Selection Criteria of the Healthy Elderly

The study group is selected from the patients who come to general health check to the Geriatrics Polyclinic of Cerrahpaşa Medical Faculty. The patients were selected into the study group if they have satisfied the “healthy elderly criteria” based on the following aspects:

1. The patient should be over 60.
2. The patient should not have a history of any chronic inflammatory disease.
3. The patient should be free of any acute or chronic inflammation at the time of the study.

4. The patient should not have any other disease than hypertension, osteoporosis, osteoarthritis.

5. The patient's functional capacity should be sufficient.

2.3 Collection and Storage of Blood Samples

The peripheral blood samples are collected from the patients, who came to the Geriatrics Polyclinic of Cerrahpaşa Medical Faculty, selected according to the healthy elderly criteria defined previously. The blood samples were collected in vacuum tubes containing EDTA. The samples were kept at −20 ^{o}C for short term storage (1-2 weeks), and in −80 ^{o}C for longer terms (several months) of storage.

2.4 DNA Isolation from Human Whole Blood

The DNA isolation was done by Magtration System 8Lx Instrument, with the kit provided by the supplier (Precision System Science). Approximately 4 mL of blood sample was used for each isolation. The isolated stock DNA was kept at -20 °C.

2.5 DNA Amount, Purity and Working Solution Calculations

The concentration and purity of the isolated DNA is calculated by using the absorbance values measured at 260, 280 and 320 nm. The concentration of the DNA is calculated with the equation 2.1 and the purity of the DNA samples are calculated with the equation 2.2 given below:

$$DNA\ Concentration\ (ng/\mu L) = (A_{260} - A_{320}) \times 50 \times Dilution\ Factor \quad \textbf{(2.1)}$$

$$DNA\ Purity = \frac{(A_{260} - A_{320})}{(A_{280} - A_{320})} \quad \textbf{(2.2)}$$

In order to have a set of DNA samples that have the same DNA concentration (50 ng/µL), dilutions from the stock DNA are prepared (working solutions). The necessary amount of stock DNA solutions are calculated for 500 µL of 50 ng/µL working solutions (approximately 25 µg DNA).

2.6. Polimerase Chain Reaction (PCR)

PCR was used to amplify the target DNA sequences on MEFV gene which contained the related SNP regions. For this purpose the isolated genomic DNAs of the healthy elderly group were used as templates. A standard mixture of PCR (except from the primers) was used to amplify the related sequences of all five mutations.

Table 2.1 Standard PCR mix

Ingredient	**Stock Concentration**	**Volume**	**Final Concentration**
Taq Buffer	10X	2 µL	1X
$MgCl_2$	25 mM	1.5 µL	1.875 mM
Forward Primer	10 pmol/µL	1 µL	0.5 µM
Reverse Primer	10 pmol/µL	1 µL	0.5 µM
dNTP mix	2 mM	0.4 µL	40 µM
Taq Polymerase	5 U/µL	0.2 µL	0.05 U/µL
dH_2O	–	7.9 µL	–
Template DNA	50 ng/µL	2 µL	100 ng
Q Solution	5X	4 µL	1X
FINAL		20 µL	

The performance of the PCR reaction is affected from the purity of the template DNA as well as the amplicon size (length) and content (GC%).

2.6.1 Template DNA

Some of the isolated DNA samples contained heme and magnetic bead contamination. In order to purify such contaminated samples, the following procedure was followed:

- 0.52 mL of protein precipitation solution (DNA Isolation Kit for Mammalian Blood, Roche) was added to each 1 mL of impure sample.
- The mixture was vortexed for 25 seconds.
- The mixture was centrifuged at 10.000 rpm for at least 10 minutes for precipitation of the proteins.
- The supernatant was transferred to another tube, the volume of the supernatant was measured while transferring.
- Absolute (100%) ETOH was added on the supernatant as 2X volume of the supernatant.
- The new mixture was centrifuged at 2000 rpm for 10 minutes, the ETOH was cleaned with a micropipette.
- The pellet was resuspended in 600 μL of 70% ETOH, without vortexing (for further cleaning of the DNA).
- The mixture was centrifuged at 12.000 rpm for 5 minutes for DNA precipitation.
- The ETOH was cleaned and the pellet was left for drying.
- The dried pellet was resuspended in ~400 μL TE buffer.

2.6.2 Oligonucleotide Primers

The oligonucleotide primers used in this study were selected from previous studies [34,35] and are given in the table 2.2 below. They are confirmed on Amplify 3X

software [36] that they actually bind to the related sequence on MEFV gene. The efficiency of binding and the amplicon sizes are also determined with this software.

It is also important to analyze the primer sets for dimer formation. The hairpin, heterodimer and self dimer analysis of the primer sets are done and confirmed with the SciTools on the IDT DNA website [37].

Table 2.2 Oligonucleotide primers

SNP	**Primer Sequence**	**Amplicon Size**	**Reference**
MEFV E148Q	F-5'-ATATTCCACACAAGAAAACGGC-3' R-5'-GCTTGCCCTGCGCG-3'	244 bp	[33]
MEFV M680I	F-5'-TGTATCATTGTTCTGGGCTCT-3' R-5'-AGGGCTGAAGATAGGTTGAA-3'	360 bp	[34]
MEFV M694V	F-5'-GCTACTGGGTGGTGAT*CAT-3' R-5'-AGGGCTGAAGATAGGTTGAA-3'	215 bp	[34]
MEFV M694I	F-5'-TGTATCATTGTTCTGGGCTCT-3' R-5'-CTGGACGCCTGGTACTCATTTTT*C-3'	195 bp	[34]
MEFV V726A	F-5'-TGTATCATTGTTCTGGGCTCT-3' R-5'-AGGGCTGAAGATAGGTTGAA-3'	360 bp	[34]

2.6.3 PCR Cycle Conditions

The PCR cycle conditions were modified according to the desired amplicon. Except from the PCR conditions of E148Q mutation, all the other mutations' PCR cycle conditions were similar (Table 2.3). The only differences in the conditions were in annealing temperatures, caused by the usage of different primer sets.

Table 2.3 General PCR cycle conditions

Repeat Number	Degree	Time	Phase
1	94 °C	5 minutes	Initial Denaturation
35	94 °C	30 seconds	Denaturation
	Variable	30 seconds	Annealing
	72 °C	30 seconds	Extension
1	72 °C	10 minutes	Final Extension

2.6.4 PCR Optimization

The PCR reactions usually need to be optimized for higher efficiency, amplification of the correct target and determination of appropriate annealing temperature for different primer sets. In this thesis, Mg^{2+} titration, Touchdown PCR and Gradient PCR methods were applied to optimize the PCR conditions.

2.6.5 Magnesium (Mg^{2+}) Titration

Mg^{2+} is the cofactor of Taq DNA polymerase, as well as many DNA polymerases. The amplification efficiency changes within a scale of low to high concentrations of Mg^{2+}. Frequent observations about the effect of Mg^{2+} concentration on PCR include the decrease in amplicon amount with low concentrations, and unspecific amplification of non-target regions with high concentrations. In order to obtain the appropriate amount of Mg^{2+} for a PCR reaction, Mg^{2+} titration should be applied. This procedure is usually done by setting up PCR mixtures that only differ in Mg^{2+} concentration. A used procedure for determination of Mg^{2+} concentration of M694I PCR is given in table 2.4 below.

Table 2.4 Magnesium titration

Ingredient	**1.5 mM**	**1.875 mM**	**2.0 mM**	**2.5 mM**	**3.0 mM**
$MgCl_2$ (25 mM)	**1.2 µL**	**1.5 µL**	**1.8 µL**	**2.4 µL**	**3.0 µL**
ddH_2O	**12.2 µL**	**11.9 µL**	**11.6 µL**	**11.3 µL**	**11.0 µL**
F. Primer	1 µL	1 µL	1 µL	1 µL	1 µL
R. Primer	1 µL	1 µL	1 µL	1 µL	1 µL
10X buffer	2 µL	2 µL	2 µL	2 µL	2 µL
Taq	0.2 µL	0.2 µL	0.2 µL	0.2 µL	0.2 µL
dNTP	0.4 µL	0.4 µL	0.4 µL	0.4 µL	0.4 µL
Template	2 µL	2 µL	2 µL	2 µL	2 µL

2.6.6 Gradient PCR

When appropriate annealing temperature is not known for a primer set, gradient PCR might be used to determine the optimum annealing temperature for a PCR reaction. In gradient PCR, the thermo cycler is set in such a way that the wells in the thermo cycler block have a gradient of temperature from high to low for the annealing phase of the cycles. So, a set of identical PCRs can be run in the same block with different annealing temperatures, which saves time and prevents multiple usage of the thermo cycler for the same optimization. When the PCR products are viewed with agarose gel electrophoresis, the PCR mixture that has the nearest annealing temperature to the optimum temperature should give the best band intensity. In this thesis, the gradient PCR was used to optimize the annealing temperature of M694I PCR, a gradient from 65 to 55 was used and the best bands were observed in 59 °C, and this temperature is used as the annealing temperature of M694I PCR for the rest of the studies.

2.6.7 Optimization with Q Solution and Betaine

A significant amount of PCRs did not work with standard magnesium titrations, annealing temperature adjustments or purification of the template DNA. Q solution from Qiagen was used as a PCR enhancer for such reactions in this thesis. It was frequently used in M694V PCRs and in almost all E148Q PCRs. Q solution is an additional solution used in PCR that comes with commercial DNA polymerases of Qiagen. Another PCR enhancer, Betaine, which is also frequently used in PCRs as GC melts, was used in the absence of Q solution. The optimized PCR mixtures with appropriate concentrations of Q solution and Betaine is given in the Table 2.5 below.

Table 2.5 PCR conditions with Q Solution and Betaine

Ingredient	**Stock Concentration**	**Volume**	**Final Concentration**
Taq Buffer	10X	2 µL	1X
$MgCl_2$	25 mM	1.5 µL	1.875 mM
Forward Primer	10 pmol/µL	1 µL	0.5 µM
Reverse Primer	10 pmol/µL	1 µL	0.5 µM
dNTP mix	2 mM	0.4 µL	40 µM
Taq Polymerase	5 U/µL	0.2 µL	0.05 U/µL
dH_2O	–	7.9 µL	–
Template DNA	50 ng/µL	2 µL	100 ng
* Q Solution	5X	**4 µL**	1X
* Betaine	5 M	**4 µL**	1 M
FINAL	20 µL		

2.7 PCR Conditions of MEFV-E148Q Mutation

The PCR conditions for the E148Q PCR is shown in the table 2.6 below.

Table 2.6 PCR conditions for E148Q

Repeat Number	Degree	Time	Phase
1	96 °C	5 minutes	Initial Denaturation
5	96 °C	30 seconds	Denaturation
	64 °C	30 seconds	Annealing
	72 °C	30 seconds	Extension
35	96 °C	30 seconds	Denaturation
	62 °C	30 seconds	Annealing
	72 °C	30 seconds	Extension
1	72 °C	10 minutes	Final Extension

2.8 PCR Conditions of MEFV-M680I, V726A and M694V Mutations

The PCR conditions for the M680I, V726A and M694V PCRs were the same and are shown in the table 2.7 below.

Table 2.7 PCR conditions for M680I, V726A and M694V

Repeat Number	Degree	Time	Phase
1	94 °C	5 minutes	Initial Denaturation
35	94 °C	30 seconds	Denaturation
	55 °C	30 seconds	Annealing
	72 °C	30 seconds	Extension
1	72 °C	10 minutes	Final Extension

2.9 PCR Conditions of MEFV-M694I Mutation

The PCR conditions for the M694I PCR is shown in the table 2.8 below.

Table 2.8 PCR conditions for M694I

Repeat Number	Degree	Time	Phase
1	94 °C	5 minutes	Initial Denaturation
35	94 °C	30 seconds	Denaturation
	59 °C	30 seconds	Annealing
	72 °C	30 seconds	Extension
1	72 °C	10 minutes	Final Extension

2.10 Agarose Gel Electrophoresis of PCR Products

The right percentage of an agarose gel is important for observing the PCR bands. In this thesis, all of the PCR products' amplicon sizes are in a range between 195 to 360 bp, which can be seen on a 1% agarose gel. In order to observe the PCR products of the E148Q, M680I, V726A, M694I and M694V mutations, 1% mini or midi gels are prepared. Mini gels are prepared with 0.5 g agarose and 0.5 µg/mL ethidium bromide added into 50 mL of 1X TBE buffer, which is diluted from 10X stock TBE. Similarly, %1 midi gels are prepared with 1.5 g agarose and 0.5 µg/mL ethidium bromide added into 150 mL of 1X TBE buffer. 6 µL of PCR product is mixed with 1 µL 6X loading dye and loaded into the wells. Gene ruler 1 kb marker (Fermentas) is used as DNA ladder in order to calculate the lengths of the PCR products. The gels are run in 1X TBE buffer, at 120V with power supplier, for at least 30 minutes. The gels are observed under UV light with a transilluminator, and pictures are taken with UV PhotoMW software.

2.11 Restriction Enzyme Digestion of PCR Products

The restriction enzyme digestions of the PCR products were done according to the protocol provided by supplier of the enzymes. Restriction enzyme digestions were also performed and confirmed in electronic environment with EnzymeX software

[38]. The general protocol is given in table 2.9 and the restriction enzymes and expected fragment sizes are given in table 2.10 below.

Table 2.9 Restriction Enzyme Digestion mixture

Ingredient	**1X amount**
10X reaction buffer	2 µL
ddH_2O	5 µL
Restriction Enzyme (10 U/µL)	1 µL
Amplicon	12 µL
Final Volume	**20 µL**

The restriction enzyme mixture containing PCR products were kept at 37 °C overnight for digestion. Digestion, and the RE are inactivated by application of the mixtures to 80 °C for 30 minutes.

Table 2.10 Restriction Enzymes used for each mutation and expected fragment sizes.

SNP	**Restriction Enzyme**	**Amplicon (bp)**	**Expected Fragments**
E148Q	Ava*I*	244	Wild Type: 3 fragments (92+83+69) Mutant: 2 fragments (161+83)
M680I	Hinf*I*	360	Wild Type: 2 fragments (126+234) Mutant: 1 fragment (360)
M694V	Pag*I*	215	Wild Type: 2 fragments (200+15) Mutant: 1 fragment (215)
M694I	Mbo*II*	195	Wild Type: 1 fragment (195) Mutant: 2 fragments (182+13)
V726A	Alu*I*	360	Wild Type: 1 fragment (360) Mutant: 2 fragments (320+40)

2.12 Agarose Gel Electrophoresis of Restriction Enzyme Digested PCR Products

The right percentage of the agarose gel is important for observing the restriction enzyme digested bands correctly. In this thesis, the RE digested bands are observed in agarose gels of concentrations between 1% to 4%, according to the size of the

products and the minimum band size difference to be discriminated. E148Q restriction enzyme digested products were viewed in 4%, M680I and V726A restriction enzyme digested products were viewed in 2%, M694I and M694V restriction enzyme digested products were viewed in 3.5% mini or midi agarose gels. In all of the restriction analyses except from E148Q, 12 µL of RE digested PCR product is mixed with 2 µL of 6X loading dye and loaded into the wells. The gels are run in 1X TBE buffer, at 120V, for at least 30 minutes. The gel pictures are taken with UV PhotoMW software.

2.13. Genotyping

The genotyping is done by analyzing the agarose gel photos of the RE digested PCR products for the five most common mutations observed in MEFV gene (E148Q, M680I, M694V, M694I, V726A). This was done separately and blindly by myself and by my advisor Dr Eda Tahir Turanlı. Each gel included two positive controls, one heterozygote carrier and one homozygote mutant. Low range marker (Fermentas) was added to decide the band sizes along with positive controls. The expected band patterns were decided via EnzymeX software, and these patterns are shown in figures 2.1 (E148Q), 2.2 (M680I), 2.3 (M694V), 2.4 (M694I) and 2.5 (V726A) below.

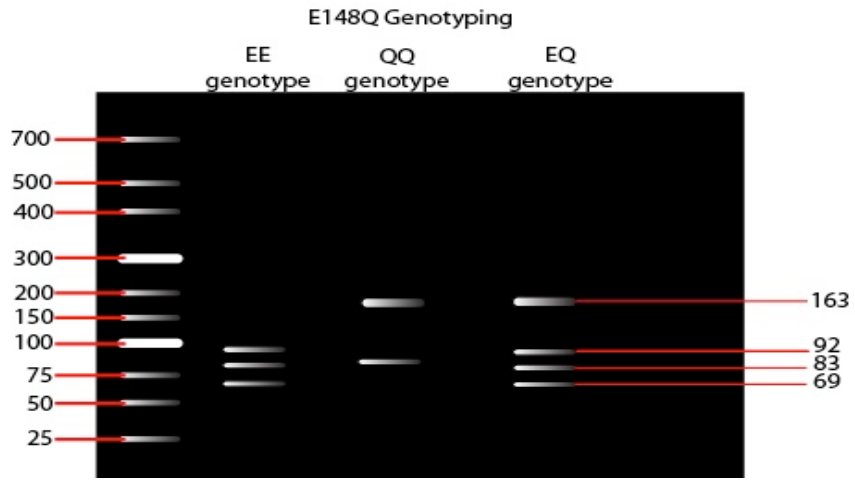

Figure 2.1 E148Q expected restriction enzyme fragments sizes and genotyping

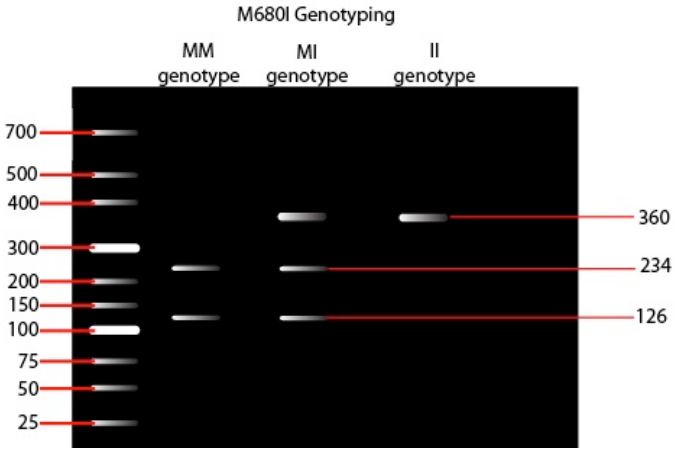

Figure 2.2 M680I expected restriction enzyme fragments sizes and genotyping

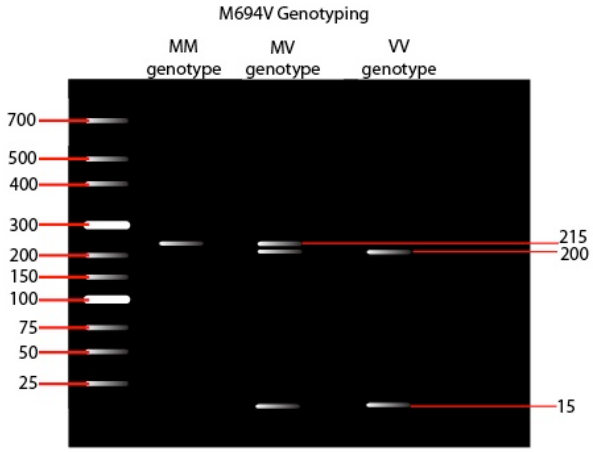

Figure 2.3 M694V expected restriction enzyme fragments sizes and genotyping

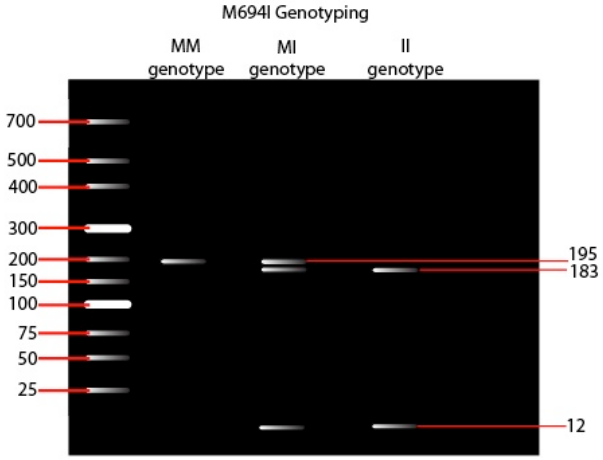

Figure 2.4 M694I expected restriction enzyme fragments sizes and genotyping

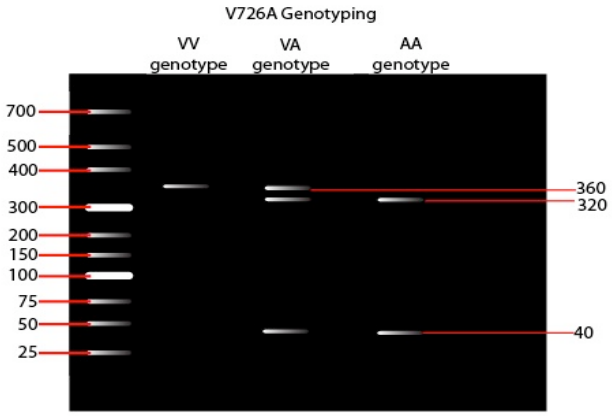

Figure 2.5 V726A expected restriction enzyme fragments sizes and genotyping

2.14 Expression Analysis

2.14.1 Collection and Storage of Blood Samples

The peripheral blood samples for expression analysis are collected both from the selected patients who came to the Geriatrics Polyclinic, and selected FMF patients carrying various mutations of MEFV from Rheumatology Polyclinic of Cerrahpaşa Medical Faculty. The blood required for the RNA isolation was collected in PAXgene^{TM} Blood RNA tubes in order to stabilize the RNA. 2.5 mL of blood was added into each PAXgene^{TM} tube and kept at −20 °C until isolation (at maximum six months).

2.14.2 RNA Isolation from Whole Human Blood

Total RNA of the case group was required for gene expression analysis with real-time PCR, for this purpose the total RNA was firstly tried to be isolated from the blood collected in vacuum EDTA tubes, which were kept at −80 °C. But the RNA isolation yield was too low for further investigation, and we concluded that the RNA was possibly degraded. In order to stabilize the RNA, and to prevent degradation; PAXgene^{TM} Blood RNA system was used. The total RNA was isolated from the samples, with High Pure RNA Isolation kit of Roche. Normally, the PAXgene^{TM} tubes were recommended to be used with Qiagen RNA isolation kit, so the protocol

of High Pure RNA Isolation kit from Roche was modified for usage with PAXgene™ Blood RNA system. Modified protocol is as follows:

- The PAXgene tubes containing 2.5 mL of peripheral blood were centrifuged at 3500 rpm for 10 minutes.
- The supernatant was removed and the pellet was resuspended in 5 mL of RNAse free dH_2O by vortexing.
- The tubes were centrifuged at 3500 rpm for 10 minutes.
- The supernatant was removed and the pellet was resuspended in 400 µL sterile PBS and transferred to a 2 mL microfuge tube.
- 800 µL Lysis/Binding buffer was added to the microfuge tube and mixed by turning the tube up and down.
- A High Pure filter tube was inserted into a collection tube and the mixture was added on the upper reservoir of the column.
- The tubes were centrifuged at 10,000 rpm for 15 seconds.
- The liquid collected in the collection tube was discarded.
- 150 µL DNase solution per sample was mixed in a separate tube (135 µL DNase incubation buffer and 15 µL DnaseI)
- 100 µL of DNase was added onto each tube, and the tubes were incubated at 15 − 25 °C for 15 minutes.
- 750 µL Wash Buffer I was added on the filter tubes and centrifuged at 10,000 rpm for 15 seconds, and the fluid was discarded.
- 750 µL Wash Buffer II was added on the filter tubes and centrifuged at 10,000 rpm for 15 seconds, and the fluid was discarded.

- 300 µL Wash Buffer II was added on the filter tubes and centrifuged at maximum speed for 2 minutes, and the fluid was discarded with the collection tube.
- The filter tube was inserted into a 1.5 mL microfuge tube.
- Approximately 80 µL of elution buffer was added to the filter tube and centrifuged at 10,000 rpm for 1 minute.
- The isolated RNAs were used as templates for cDNA synthesis reaction, used RNAs were stored in −80 °C for later usage.

2.14.3 cDNA Synthesis

cDNA was synthesized from isolated total RNA via two different kits (Transcriptor First Strand cDNA synthesis Kit from Roche, and RevertAid^{TM} First Strand cDNA synthesis Kit from Fermentas) with oligodT primers. The protocol used for Transcriptor First Strand cDNA synthesis Kit from Roche is as follows:

- 1 µL of 50 pmol/µL anchored oligo $(dT)_{18}$ primer and 5 µL of PCR grade dH_2O were added on 7 µL total RNA isolated previously.
- The template-primer mixture was denatured by keeping at 65 °C for 10 minutes, and then the mixture is immediately taken on ice.
- Then 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 10U/µL Transcriptor Reverse Transcriptase is added as in the given order.
- The mixture in the tube was mixed carefully (vortex usage was avoided).
- The samples were spinned down with a microcentrifuge.
- The samples were placed in a thermal cycler and the following conditions were programmed:
    - 30 minutes at 55 °C

- 5 minutes at 85 °C (for the inactivation of Reverse Transcriptase)

- The reaction was then stopped by taking the tubes on ice.
- The synthesized cDNAs were stored at -20 °C for later usage.

The protocol used for and RevertAid™ First Strand cDNA synthesis Kit from Fermentas is as follows:

- The reaction mixture containing 8 µL of total RNA, 1 µL of 0.5 µg/µL oligo $(dT)_{18}$ primer and 3 µL DEPC treated was prepared by mixing on ice.
- The mixture was mixed gently and spinned down for 3-5 seconds with a microcentrifuge.
- The mixture was incubated at 70 °C for 5 minutes and cooled on ice.
- 4 µL of 5X reaction buffer, 1 µL of Ribolock, 2 µL of 10 mM dNTP mix were added onto the mixture, which was still on ice.
- The mixture was incubated at 37 °C for 5 minutes.
- 1 µL of RevertAid M-MuLV Reverse Transcriptase (200 U/µL) was added to each tube, completing the final volume to 20 µL.
- The mixture was incubated at 42 °C for 60 minutes and then at 70 °C for 10 minutes to end the reaction.
- The newly synthesized cDNAs are immediately placed on ice and stored at -20 °C for later usage.

2.14.4. Real-Time PCR

Real Time PCR was performed to obtain the relative change in mRNA amounts of target gene (MEFV) between defined groups such as, mutation carriers, wild type allele carriers and FMF patients. The Real-Time PCR reaction was set with LightCycler® TaqMan Master Kit from Roche and the Reaction was performed in Roche LightCycler® 2.0 instrument. Calibration standards were prepared from a

cDNA sample with relatively higher concentration, as undiluted, 1/10 dilution and 1/100 dilutions. For each sample two reactions were set including negative controls, one for the target gene (MEFV) and one for the reference gene (internal control−β2M-Beta 2 Microglobulin). Each reaction is set with its specific primers and probes. The internal control reaction was set in order to normalize the errors result from differences in total RNA amount. The optimized reaction ingredients for MEFV are given in table 2.11 and the optimized reaction ingredients for β2M is given in table 2.12. The Real-Time PCR, cycle conditions are given in the table 2.13 below.

Table 2.11 MEFV Real-Time PCR mixture

Ingredient	**1X volume**	**Final Concentration**
Water	9.85 µL	–
UPL Probe #8 (10 µM)	0.15 µL	75 nM
Forward Primer (10 µM)	0.5 µL	250 nM
Reverse Primer (10 µM)	0.5 µL	250 nM
TaqMan Master (5X)	4 µL	1X
cDNA template	5 µL	–

Table 2.12 β2M Real-Time PCR mixture

Ingredient	**1X volume**	**Final Concentration**
Water	9.4 µL	–
UPL Probe #64 (10 µM)	0.2 µL	100 nM
Forward Primer (10 µM)	0.7 µL	350 nM
Reverse Primer (10 µM)	0.7 µL	350 nM
TaqMan Master (5X)	4 µL	1X
cDNA template	5 µL	–

Table 2.13 Real-Time PCR Cycle conditions

Repeat Number	Temperature	Time	Phase
1	95	10 minutes	Pre Incubation
45	95	10 seconds	Denaturation
	60	30 seconds	Annealing
	72	1 second	Extension

2.14.5. Relative Quantification of the Samples

The relative quantification of the samples is done by $2^{-\Delta\Delta C_T}$ method. [29]. In this thesis the relative expression levels of MEFV gene were obtained by first calculating the average C_T values from different experiments for internal control (β2M) and MEFV of each control (free of mutations, N=16), then the average of these average values is used as the normalizing C_T for the cases with the mutations. The average C_T values from different experiments for internal control (β2M) and MEFV of each case (mutants) are also calculated. Then, the ΔC_T values for MEFV and β2M are calculated via subtracting the average C_T values of cases from the previously obtained average control C_T values ($C_{TControl} - C_{TCase}$). These obtained ΔC_T values are then placed as the power of 2 ($2^{\Delta CT}$) and the expression levels are then calculated via dividing the $2^{\Delta CT}$ of "Target gene" (MEFV) with the $2^{\Delta CT}$ of "Reference gene" value, The resulting values give the relative expression levels of each sample, normalized with the values obtained from the control samples.

2.15. Statistical Analysis

P value (the level of significance) accounts for the probability of any difference observed between two data sets is only by chance. As a result, the lower the p value, the more evidence for rejecting the null hypothesis. In this thesis, the obtained data was designated into levels of significance according to the p value intervals:

If P>0.05, there is no significant difference

If $P<0.05$, the difference is significant

If $P<0.01$, the difference is highly significant

If $P<0.001$, the difference is extremely significant

In addition to this, chi square (χ^2) test is used to test the significance of the distribution of mutations between the healthy elderly and the historical controls. These tests are done with SPSS software (version 15.0). Lastly, odds ratio calculations were done with the online tool from Simple Interactive Statistical Analysis (SISA) [39]. Odds ratio is generally used to compare the probability of a certain event in two groups. An odds ratio of 1 implies that the condition (or event) is equally possible in both of the case and the control groups [40].

3. RESULTS

3.1 DNA Isolation Results

In this thesis, Magtration System 8Lx DNA isolation procedure was followed for all the samples. The average DNA concentration obtained from Magtration System 8Lx instrument and kit is 252.9 ng/μL and average A_{260}/A_{280} value is 1.8 (N=164).

3.2 Demographic Data of the Healthy Elderly Group

The healthy elderly group in this study was made up of mostly women (75 %) and had an average age of 74±0.5 (range 61-92). The percentages of having hypertension, osteoporosis, osteoarthrosis; and hsCRP and RF negativity are shown in table 3.1 below.

Table 3.1 Demographic properties of the Healthy Elderly Group

Healthy Elderly N=170	
Gender	**Percentage**
Female Male	75% 25%
Hypertension	69%
Osteoporosis	52%
Osteoarthrosis	29%
hsCRP (IU/mL)	hsCRP < 3 IU/mL: 72 % mean=2.8±0.3 (range 0.16-43.5)
RF (mg/dL)	RF < 15mg/dL: 92 % (range <9.8 – 154)
BMI	Mean= 27.34 ±0.3 (range 14.67-40.89)
Mean Age	Mean=74±0.5 (range 61-92)

3.3 Genotype Analysis

The genotyping is done according to the comparison of agarose gel results with the expected restriction enzyme digestion patterns. Sample PCR and restriction enzyme results for each mutation analysis are given in Appendix E.

3.3.1 Statistical Mutation Analysis

The obtained mutation data was used to compare the distribution of mutations between the healthy elderly and historical controls. No significant difference was found between the mutation frequencies among healthy elderly group and any of the historical controls, instead the data confirmed the previous studies. χ^2 analysis was not significant for all but one mutation in which an excess of M694I alleles were found significantly higher in healthy elderly population than in the historical controls (χ^2 =3.6, P=0.05). There was no difference between mutation frequencies between females or males in this population. The observed allele frequencies are given in table 3.2 below.

Table 3.2 . Frequency of five common MEFV mutations in the elderly population

SNP	***N=164*** ***Number of Alleles***	**Carrier Frequency (mutant genotypes)**	**Allele Frequency**
M694V	6	3.7 %	1.8 %
M694I	6	3.7 %	1.8 %
M680I	2	1.2 %	0.6 %
V726A	7	4.3 %	2.1%
E148Q	17	10 %	5 %
TOTAL	38	23 % (1/4)	11.3 %

Table 3.3 Mutation analysis – comparison with historical controls

SNP	***N=164 the healthy elderly group of genetic analysis*** (328 chromosomes)	Turkish Controls (N=100) (200 chromosomes) [16]	Turkish Controls (N=49) (98 chromosomes) [17]
M694V	6 / 1.8 %	3 / 1.5 %	3 / 3.06 %
M694I	6 / 1.8 %	0	0
M680I	2 / 0.6 %	5 / 2.5 %	0
V726A	7 / 2.1 %	2 / 1 %	2 / 2.04 %
E148Q	17 / 5.0 %	12 / 6 %	2 / 2.04 %
TOTAL	38 / 11.3 %	22 / 11 %	7 / 7.14 %

3.3.2 Subgroup Analysis

Subgroup analyses of hsCRP and RF positivity among common MEFV mutations were done and hsCRP values were analyzed for two levels of positivity. In the first group, hsCRP values higher than 5 IU/mL were accepted as hsCRP positive (hsCRP > 5 IU/mL), and in the second group hsCRP values higher than 3 IU/mL were accepted as hsCRP positive (hsCRP > 3 IU/mL). In both of these analyses, hsCRP positivity was not found to be significantly different between M694V mutation carriers and non-carriers (χ^2=2.834, p=0.092, OR=4.12 for hsCRP > 5 IU/mL, and χ^2=1.275, p=0.258, OR=2.09 for hsCRP > 3 IU/mL). On the other hand, RF positivity was found highly significant (χ^2=7.358, p=0.007, OR=5.41) in E148Q allele carriers compared to the non-carriers. The data tables of hsCRP and RF statistical-significance subgroup analyses are given in the tables 3.4, 3.5 and 3.6 below.

Table 3.4 hsCRP positivity for hsCRP > 5 IU/mL-subgroup analysis

		hsCRP value	
MEFV M694V	**Alleles**	(Low hsCRP) hsCRP ≤ 5 IU/mL	(High hsCRP) hsCRP > 5 IU/mL
	MM	132	16
	MV	4	2
	TOTAL	136	18
	Pearson χ^2**=2.834, DF =1, p=0.092**		**OR=4.12, 95% CI 0.69-24.33**

Table 3.5 hsCRP positivity for hsCRP > 3 IU/mL-subgroup analysis

		hsCRP value	
MEFV M694V	**Alleles**	(Low hsCRP) hsCRP ≤ 3 IU/mL	(High hsCRP) hsCRP > 3 IU/mL
	MM	105	42
	MV	3	3
	TOTAL	108	45
	Pearson χ^2**=1.275, DF =1, p=0.258**		**OR=2.09, 95% CI 0.48-12.88**

Table 3.6 RF value subgroup analysis

		RF value	
MEFV E148Q	**Alleles**	(Low RF) RF ≤ 15 mg/UL	(High RF) RF > 15 mg/UL
	EE	130	8
	EQ	12	4
	TOTAL	142	12
	Pearson χ^2**=7.358, DF =1, p=0.007**		**OR=5.41, 95% CI 1.41-20.64**

3.4 Expression Analysis

The error rates of the Real-Time PCR reactions are about 10% and the efficiencies of these reactions are approximately 2 (given in table 3.7). The standard curves for MEFV and β2M are given in the figures 3.1 and 3.2 below.

Table 3.7 Error rates and efficiencies of MEFV and β2M Real-Time PCRs.

	MEFV	**β2M**
Error Rate	0.105	0.116
Efficiency	2.061	1.983

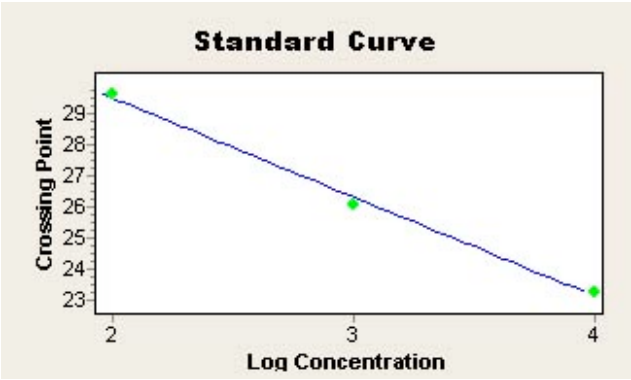

Figure 3.1 Standard curve for MEFV

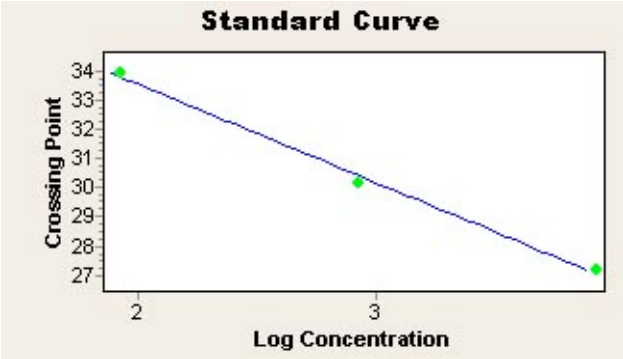

Figure 3.2 Standard curve for β2M.

When the mRNA levels are normalized and calibrated according to the endogenous control and the standard dilutions, relative expression levels were obtained. In the overall data, the lowest mRNA levels were observed in M694V homozygous mutants (N=7, 0.0055 Relative Expression Units), and the highest MEFV mRNA levels were observed in E148Q heterozygous mutants (N=7, 1 patient with FMF, 6 healthy control, 0.032 Relative Expression Units). M694V heterozygous mutants had 0.027 relative expression units of mRNA (N=2, 1 patient with FMF, 1 healthy elderly). On the other hand, in the healthy elderly group without mutations, the relative

expression of MEFV mRNA was higher than M694V homozygous mutants, but lower than E148Q and M694V heterozygous mutants (N=16, 0.017 relative expression units). The graph showing relative expression of MEFV mRNA among described groups is shown in figure 3.3 below.

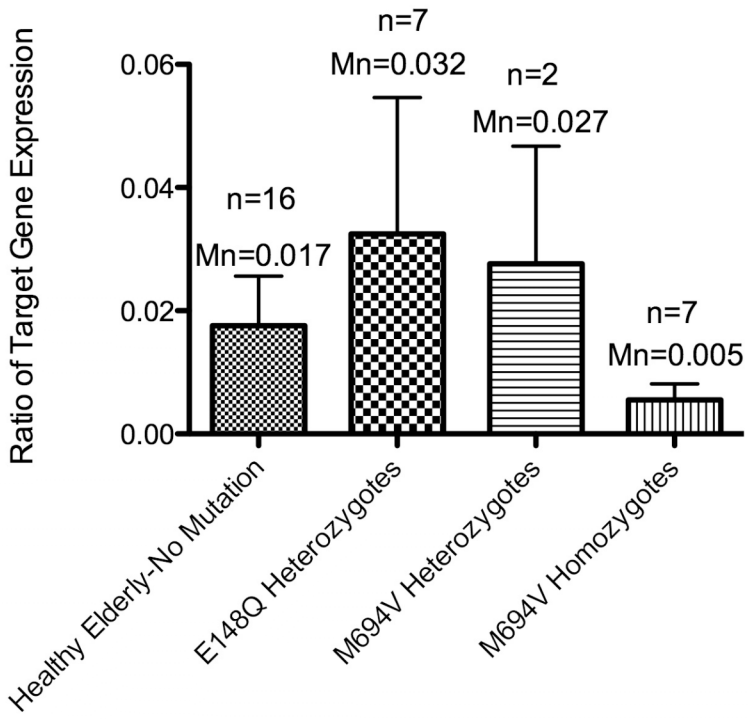

Figure 3.3 Comparison of average MEFV mRNA levels between the M694V and E148Q mutations of MEFV, and Healthy Elderly group without specific mutations.

The relative expression of MEFV was also compared between FMF patients with mutations (N=9, 0.0045 relative expression units) and healthy elderly with no mutations (N=16, 0.0175 relative expression units), and approximately four-fold decrease in MEFV mRNA level is observed in FMF patients compared to healthy elderlies without mutations. (Figure 3.4). Moreover, E148Q heterozygousity seemed to increase the MEFV mRNA level more than two-fold within the healthy elderly group (Figure 3.5).

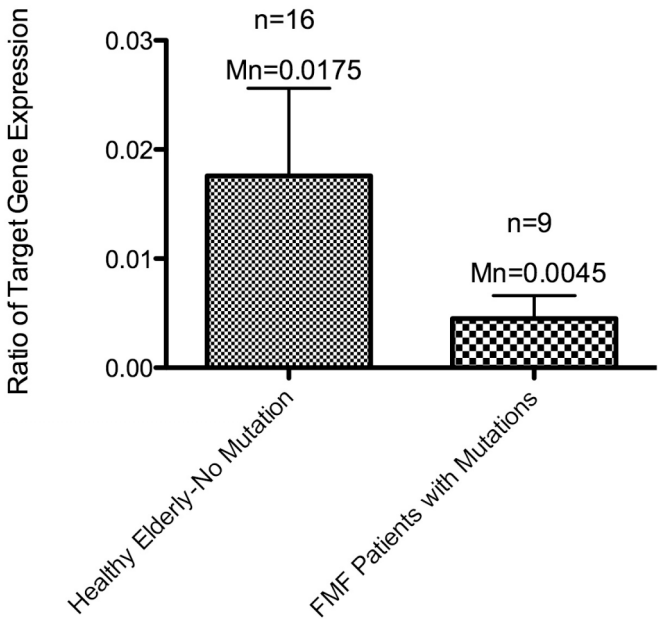

Figure 3.4 Comparison of MEFV mRNA levels between FMF patients with mutations and Healthy Elderly group without specific mutations.

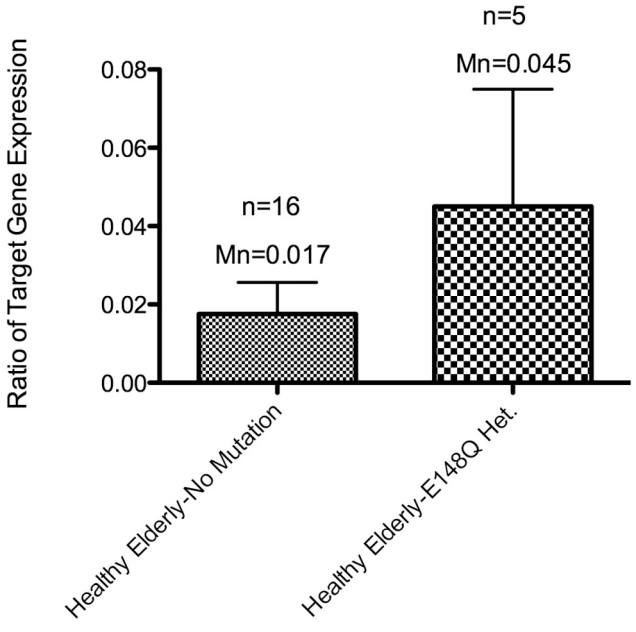

Figure 3.5 Comparison of MEFV mRNA levels within Healthy Elderly group, healthy elderlies without mutations and E148Q heterozygous mutants.

4. CONCLUSION & DISCUSSION

A negative correlation between healthy ageing and MEFV mutation frequencies was expected. But the mutation analysis, which is done in 164 cases, showed similar distributions of five most common mutations of MEFV in the healthy elderly group compared to the historical controls. As a result, no significant difference was found between the mutation frequencies among healthy elderly group and any of the historical controls, instead the data confirmed the previous studies. On the contrary to the expected frequencies, the M694I mutation was observed in a higher frequency in our group compared to the historical controls. It is known that the M694I mutation is more prevalent in populations with Arabic origin, so this observation might be caused by population stratification due to the fact that the historical control groups were studied in Aegean area.

In addition to this, subgroup analyses were done for hsCRP and RF values with M694V and E148Q mutations in order. In the subgroup analysis for hsCRP and M694V mutation, the hsCRP positivity was not significantly different between M694V carriers and non-carriers for hsCRP values higher than 3 and 5 IU/mL. On the other hand, RF positivity (RF > 15 mg/UL) was found highly significant in E148Q allele carriers compared to the non-carrier healthy elderlies. This result supports the findings of other studies, which imply higher occurrence of E148Q mutations among RF related diseases (such as Rheumatoid Arthritis). Furthermore, increase in RF positivity with age is a common observation. Our findings suggest that MEFV mutations may contribute to the causation of this observation.

The results obtained from the preliminary data from gene expression analysis which is done with quantitative real-time PCR, indicated that MEFV mRNA levels differ with mutation occurrence. Also, MEFV mRNA levels are found to be lower in FMF patients with certain mutations, which confirms previous studies. It is also found that different mutations in MEFV gene altered the mRNA levels differently. To be more

specific, it is observed that E148Q heterozygous mutation carriers have an increased MEFV mRNA level (more than two fold) compared to non-carriers within the healthy elderly group, whereas M694V homozygous mutants have decreased MEFV mRNA levels. Interestingly, M694V heterozygous mutants have a higher MEFV mRNA level compared to non-carriers, but this may be due to the fact that there are 2 cases, which are heterozygous for M694V, and the data fluctuates within a large range. In summary, MEFV mRNA expression level changes with the presence of mutations. However further analysis should be done covering broader sample pool including, healthy carriers and people with FMF but free of mutations.

When these results are combined with the preliminary data obtained from gene expression analysis, one might suggest that M694V mutation decreases MEFV mRNA level and may result in an inflammatory phenotype. Another suggestion might be that this mutation could cause mRNA instability which in order results in a lower level of MEFV mRNA. Moreover, the inflammatory effect of M694V mutation might be due to aberrant protein interactions, since this mutation is shown to be localized at caspase-1 interaction site. On the other hand, E148Q mutation of MEFV increases the MEFV mRNA level dramatically and causes the higher prevalence of rheumatoid factor, which is a sign of chronic inflammation. The E148Q mutation might change MEFV expression regulation that results in such a dramatic increase in MEFV mRNA production. This mutation is localized in the second exon of MEFV, which has a CpG island; and the E to Q mutation actually causes the disruption of a CpG motif in the DNA level, which may cause a difference in local DNA methylation pattern. It is known that the CpG islands in the mammalian genomes are methylated except from the promoter regions. Since the CpG island is in the second exon of the gene it might be related with the expression regulation of MEFV. If E148Q mutation, somehow inactivates the methylation in this region, it might lead to such overexpression of MEFV mRNA. The overexpressed MEFV mRNA, may then result in an increase in the RF level causing inflammation. Alternatively, observed decrease in MEFV mRNA in M694V carriers who suffer from FMF or other acute inflammation, may result in a decrease in the activity of Pyrin because of the site of the mutation or by directly decreased MEFV mRNA, which in turn leads to inflammation.

Findings from previous studies, our studies and our gene expression studies showing a change in MEFV gene product in general inflammation, led us to hypothesize that MEFV gene may not be only specific to FMF but may generally be involved in the inflammatory pathway.

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APPENDIX A

LABORATORY EQUIPMENT

Balances	Precisa 620C SCS
	Precisa BJ 610C
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
	Thermo Finnpipette 10 µL,
pH meter	Mettler Toledo MP220
Real time PCR	Roche Lightcycler 2.0
Spectrophotometer	PerkinElmer Lambda25 UV/VIS 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

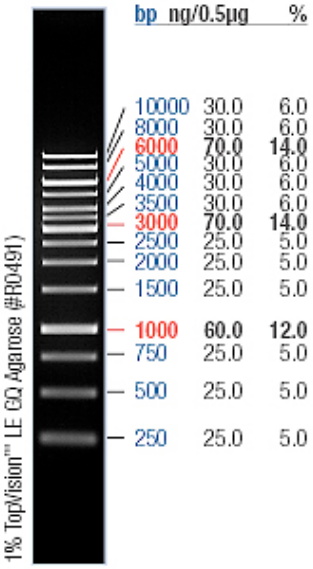
Agarose	AppliChem
Boric acid	Amresco
dNTP	Fermentas
EDTA	AppliChem
Ethanol	Riedel-de Haën
EtBr	Amresco
$MgCl_2$	Fermentas
$Na_2HPO_4.7H_2O$	Riedel-de Haën
NaH_2PO_4	Riedel-de Haën
NaOH	Riedel-de Haën
Primers	IDT DNA
NaCl	Carlo Erba
Tris Base	Amresco
10X PCR Buffer	Fermentas
	Qiagen
	Roche

ENZYMES

Alu*I*	Roche
Ava*I*	Fermantas
Hinf*I*	Roche
Mbo*II*	Fermantas
Pag*I*	Fermantas
Taq DNA Polymerase	Fermentas
	Qiagen
	Roche

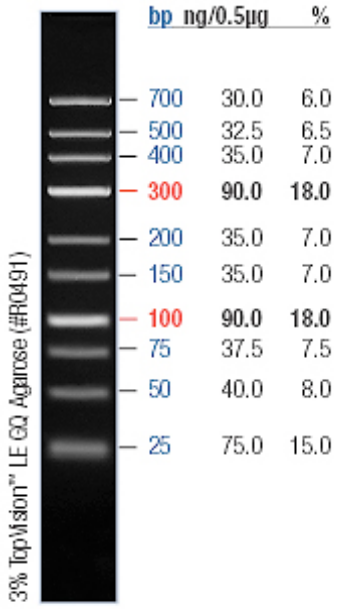
MARKERS

Gene Ruler™ 1 kb DNA Ladder Fermentas



0.5µg/lane, 8cm length gel,
1X TAE, 7V/cm, 45min

Gene Ruler™ DNA Ladder Low Range Fermentas



0.5µg/lane, 8cm length gel,
1X TBE, 5V/cm, 1h

APPENDIX C

BUFFERS

TE Buffer

Tris base	10 mM
EDTA	1 mM

Add ddH_2O to 1 liter and adjust the pH to 8.0

TBE (Tris-Borate-EDTA) Buffer (10X)

Tris base	108 g
Boric Acid	55 g
EDTA	40 ml (0.5 M, pH 8.0)

Add ddH_2O to 1 liter and adjust the pH to 8.0

Mini Agarose Gel (1%)

Agarose	0.5 g
TBE 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
TBE buffer (1X)	150 mL

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

Mini Agarose Gel (3%)

Agarose	1.5 g
TBE buffer (1X)	50 mL

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

Midi Agarose Gel (3%)

Agarose	4.5 g
TBE buffer (1X)	150 mL

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

Mini Agarose Gel (4%)

Agarose	2 g
TBE buffer (1X)	50 mL

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

Midi Agarose Gel (4%)

Agarose	6 g
TBE buffer (1X)	150 mL

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

10X Phosphate Buffered Saline (PBS)

NaCl	90 g
$Na_2HPO_4.7H_2O$	26.8 g
NaH_2PO_4	3.2 g

Add up to 1 L by ddH_2O, and adjust the pH to 7.2 after every 1/10 dilution.

APPENDIX D

USED KITS

DNA Isolation	8 Lx Magtration® Genomic DNA kit
RNA Isolation	Roche – High Pure RNA isolation kit
cDNA synthesis kit	Fermantas − RevertAid™ First Strand cDNA synthesis kit
	Roche – Transcriptor First Strand cDNA synthesis kit
Real-Time PCR	Roche – LightCycler® TaqMan Master Kit

APPENDIX E

SAMPLE PCR AND RESTRICTION ENZYME RESULTS

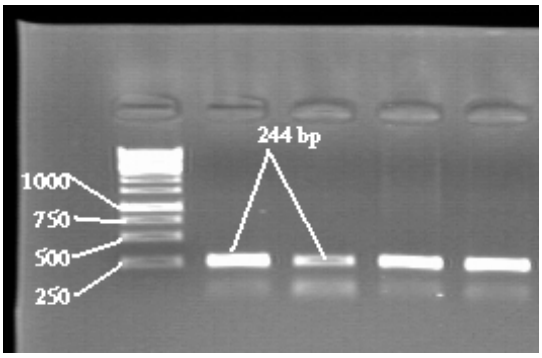


Figure E.1 E148Q PCR results

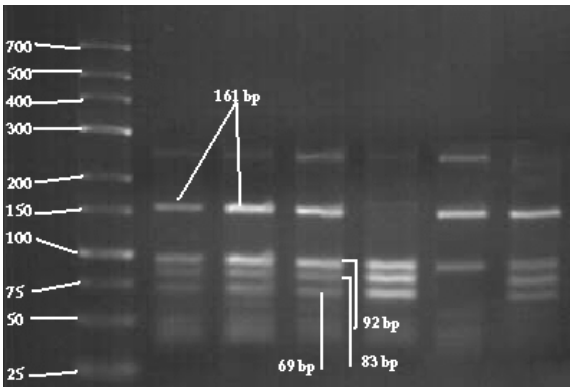


Figure E.2 E148Q Restriction Enzyme Digestion Results

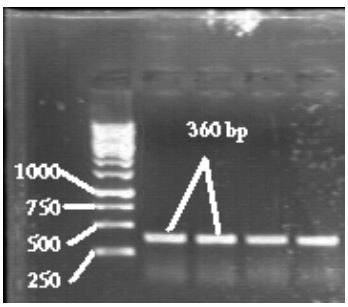


Figure E.3 M680I PCR results

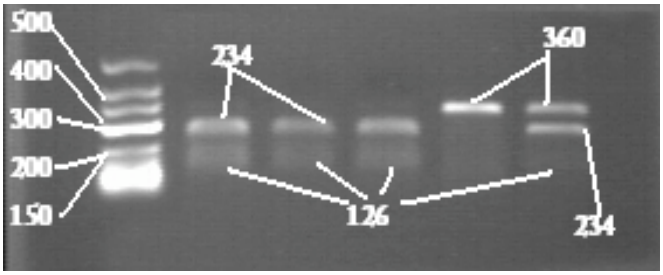


Figure E.4 M680I Restriction Enzyme Digestion Results

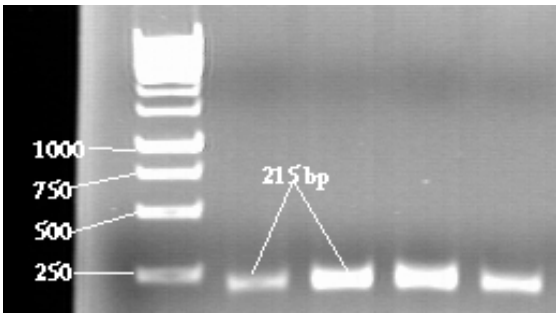


Figure E.5 M694V PCR results

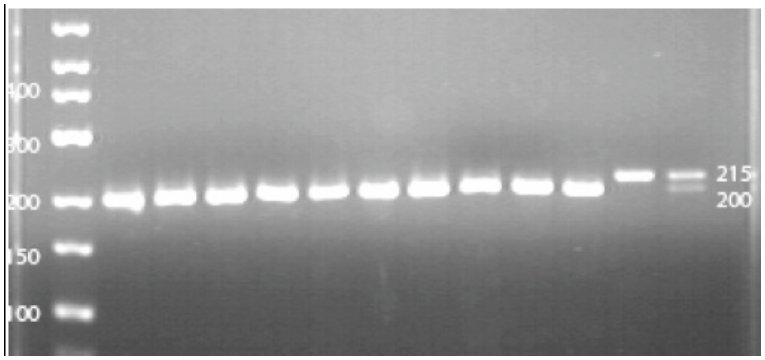


Figure E.6 M694V Restriction Enzyme Digestion Results

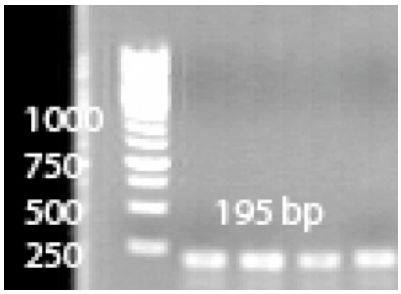


Figure E.7 M694I PCR results

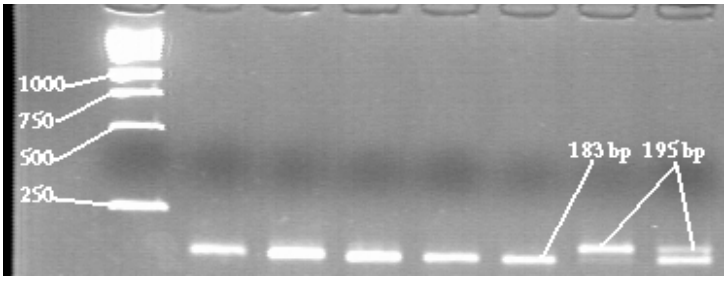


Figure E.8 M694I Restriction Enzyme Digestion Results

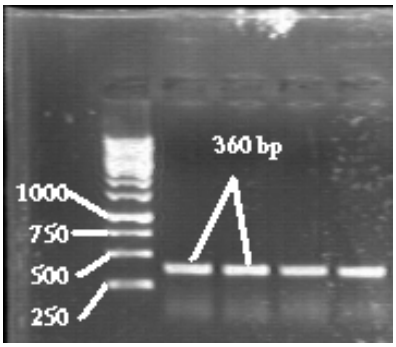


Figure E.9 V726A PCR results

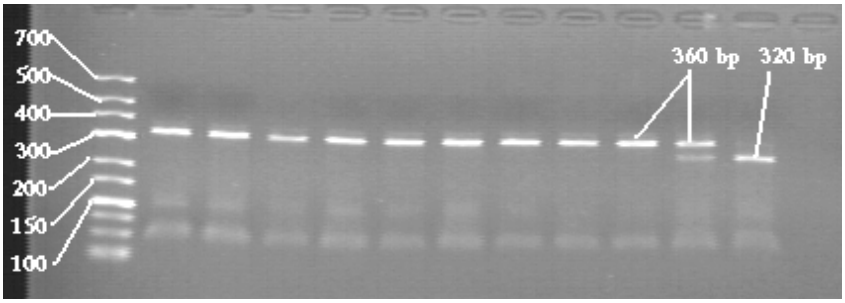


Figure E.10 V726A Restriction Enzyme Digestion Results

CURRICULUM VITAE

Sinem Karaman was born in Istanbul, in 1984. She graduated from Besiktas Ataturk Anatolian High School and enrolled to the Istanbul Technical University, Molecular Biology and Genetic Department in 2002. She graduated from the Molecular Biology and Genetic Department in 2006 and started to M.Sc. degree education in Molecular Biology - Genetic and Biotechnology Program of the same university.