İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

COMMON MEFV MUTATIONS AND HLA-B*51 FREQUENCIES IN ARMENIAN AND TURKISH POPULATIONS LIVING IN ISTANBUL

M.Sc. Thesis by

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Department : **Advanced Technologies**

Programme: **Molecular Biology - Genetics and Biotechnology**

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

İSTANBUL'DA YAŞAYAN ERMENİ VE TÜRK TOPLUMLARINDAKİ YAYGIN MEFV MUTASYONLARI VE HLA-B GEN FREKANSLARI

YÜKSEK LİSANS TEZİ
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ABBREVIATIONS

FMF	: Familial Mediterranean Fever
BD	: Behçet's Disease
MEFV	: Mediterranean Fever (gene)
MHC	: Major Histocompatibility Complex
SAA	: Serum Amyloid A
RNA	: Ribonucleic Acid
LPS	: Lipopolysaccharide
TNF-α	: Tumor Necrosis Factor - Alpha
IFN-γ	: Interferon - Gamma
TGF-β	: Transforming Growth Factor - Beta
IL	: Interleukin
ASC	: Apoptosis-Associated Speck-Like Protein
CARD	: Caspase-Recruitment Domain
mRNA	: Messenger Ribonucleic Acid
HLA	: Human Leukocyte Antigen
NK	: Natural Killer
DNA	: Deoxyribonucleic Acid
OD	: Optical Density
PCR	: Polymerase chain Reaction
TBE	: Tris – Borate – EDTA
EDTA	: Ethylenediaminetetraacedic acid
RE	: Restriction Enzyme
ETOH	: Ethanol

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COMMON MEFV MUTATIONS AND HLA-B*51 FREQUENCIES IN ARMENIAN AND TURKISH POPULATIONS LıVıNG ıN ıSTANBUL

SUMMARY

Familial Mediterranean Fever (FMF) and Behçet Disease (BD) are two autoinflammatory diseases prevalent in people from Middle-East and Mediterranean ancestory. The frequency of FMF is found to be between 1 to 2.5 in 1000, and the frequency of BD 4 in 1000 from previous studies conducted in Turkey. A recent study performed on Armenian Turkish citizens states that the frequency of FMF is higher than the Turkish population (%0.7) and that the frequency of BD was lower within the same group (<1/500). These findings led us to propose that the genetic factors have an important role in the aetiology of these two diseases.

The gene for FMF is Mediterrenean Fever Gene (MEFV) and there are more than 70 mutations which have been characterized. Among these, four mutations (M694V, M694I, M680I, V726A) and one polymorphism (E148Q) are more commonly seen in the FMF patients. These mutations are reported to be approximately 85% in patients and 10-20 % in the healthy population.

The association between HLA-B*51 and BD have been implied in many studies. The carrier rate of HLA-B*51 was reported to be between 50-80 % in patients and 20-30 % in healthy population. The occurrence of HLA-B*51 allele and the frequency of BD correlate with each other. For instance, in populations where the carrier rate of HLA-B*51 is lower, the frequency of BD is also lower.

We wanted to reveal the frequency of the MEFV mutations and HLA-B*51 frequencies in Armenian Turkish citizens and Turkish population living in the same environment, and clarify if there is any difference in mutation frequencies between these two populations with respect to disease frequencies.

For this purpose, saliva samples were collected from 115 Armenians working in the administration department of Armenian schools in Istanbul and from 98 Turkish students studying in Cerrahpaşa Medical Faculty. Mutation/polymorphism analysis revealed that there isn't any difference in the frequencies of MEFV mutations and of HLA-B*51 phenotypes between the Armenian and the Turkish groups. These results may point to other location/loci for FMF and BD.

İSTANBULDA YAŞAYAN ERMENİ VE TÜRK TOPLUMLARINDAKİ YAYGıN MEFV MUTASYONLARı VE HLA-B*51 GEN FREKANSLARI

ÖZET

Ailevi Akdeniz Ateşi ve Behçet Hastalığı, Ortadoğu ve Akdeniz toplumlarında, dünyanın diğer bölgelerine kıyasla daha sık görülen otoenflamatuvar hastalıklardır. Türkiye'de yapılan çalışmalarda Ailevi Akdeniz Ateşi sıklığının 1000'de 1 ila 2.5 arasında değiştiği, Behçet hastalığının sıklığının ise 1000'de 4 olduğu gözlenmiştir. Yakın zamanda İstanbul'da Ermeni kökenli Türkiye Cumhuriyeti vatandaşlarında yapılan bir çalışmada, bu toplumda Ailevi Akdeniz Ateşinin Türklere oranla daha sık (% 0.7), Behçet Hastalığının ise daha nadir olduğu (<1/ 500) bulunmuştur. Bu bulgular, her iki hastalığın etiyolojisinde de genetik faktörlerin önemli rolü olduğunu düşündürmektedir.

Ailevi Akdeniz Ateşi hastalığına Mediterranean Fever (MEFV) genindeki mutasyonların yol açtığı bilinmektedir. Şu ana kadar tanımlanan mutasyonların sayısı 70'den fazladır; ancak bunlardan yalnızca 4 mutasyon (M694V, M694I, M680I, V726A) ve bir polimorfizm (E148Q) daha sık görülmektedir. Yapılan çalışmalarda, bu mutasyonların hasta kişilerde % 85, sağlıklı kişilerde ise % 10-20 oranında olduğu bildirilmektedir.

Öte yandan Behçet hastalığı ile HLA-B*51 geni arasında olası bir ilişki varolduğu da çeşitli çalışmalarda belirtilmiştir. HLA-B*51 taşıyıcılığı, Behçet hastalığı olan bireylerde % 50-80, sağlıklı kişilerde ise % 20-30 oranında bildirilmektedir. Behçet hastalığının sıklığı toplumdaki HLA-B*51 taşıyıcılığına paralel seyretmektedir. Örneğin, HLA-B*51 sıklığının az olduğu toplumlarda Behçet hastalığı da nadirdir.

Bu çalışmada, T.C. Ermeni ve Türk vatandaşlarında MEFV gen mutasyonu ve HLA-B*51 gen taşıyıcılığını araştırmak ve bu iki toplumun mutasyon sıklıkları arasındaki farkları hastalık sıklıklarını göz önünde bulundurarak ortaya çıkartmak amaçlanmaktadır.

Bu doğrultuda İstanbul'daki Ermeni okullarının idari bölümünde çalışan 115 Ermeni ve Cerrahpaşa Tıp Fakültesi'nde okuyan 98 Türk vatandaştan tükürük örneği toplandı. Mutasyon/polimorfizm analizleri sonucu bu iki toplumun MEFV mutasyon ve HLA-B*51 frekansları arasında belirgin bir fark gözlenmedi. Bu sonuçlar FMF ve Behçet hastalığında diğer lokasyon veya lokasyonların rol oynayabileceğine işaret etmektedir.

1. INTRODUCTION

Autoinflammatory diseases were first defined for the hereditary periodic diseases by Kastner *et al.* in 1999. They are mainly caused by mutations on the innate immune system genes, as well as mutations in genes encoding proteins involved in apoptotic and cytokine processing pathways [1,2]. A relative lack of autoimmune pathology, like pathogenic high-titer autoantibodies or antigen-specific T cells is the characteristics of these diseases. Also environmental factors and background genetic effects may play a role in the formation of different clinical manifestations [2].

Although the immune system is regulated by many mechanisms, its occasional failures may lead to autoimmune or autoinflammatory diseases. Autoimmune diseases result from the dysfunctional activation of B and T cells, which lead to the production of receptors and specific antibodies to self-antigens. On the other hand, neither autoantigens nor autoantibodies are playing a role for the autoinflammation. Inflammation episodes may occur without any detectable causes [1].

Familial Mediterranean Fever (FMF) and Behçet Disease (BD) are both inflammatory diseases that are mainly seen in Middle Eastern and Mediterranean populations [3]. FMF is a hereditary periodic fever, which is found to be linked to MEFV gene [3,4], and BD is a polyfactorial vasculitis, linked to major histocompatibility complex (MHC) [3,5]. FMF was first observed in 1908 but was defined later in 1945 by Siegal [6,7]. The first case of FMF in Turkey was reported one year later, in 1946 [6]. On the other hand, BD is named after Dr. Hulusi Behçet, the dermatologist who demonstrated it for the first time [8].

1.1 Characteristics of FMF and BD

FMF is characterized by acute attacks of fever; abdominal, thoracic and joint pain due to peritonitis, pleuritis and synovitis; and also by erysipelas-like erythema [4,9].

The recurrent attacks of fever may cause amyloid AA protein accumulation, and the accumulation of this protein in the kidneys may lead to death [4]. The precursor of the amyloid fibrils, serum amyloid A (SAA) is an acute phase reactant, which has a role in mediating inflammation and is found to be elevated in FMF patients during and between the attacks [10]. Two phenotypes of FMF are defined according to the presence of amyloidosis as fist manifestation: in phenotype I, the attacks of fever appear first and in phenotype II, amyloidosis is initially observed [9]. Also, another phenotype was categorized as phenotype III for individuals who have the mutant genotype but does not show the FMF phenotype [8]. The existence of the phenotype III, with the results of other studies showing the dominant inheritance of FMF in some families [11], has compromised the autosomal recessive inheritance of FMF as previously described [7]. Furthermore the variation of the occurrence of amyloidosis in different ethnic groups may point to separate genetic loci other than the FMF gene [10].

AA type amyloidosis is also a rare but an important complication of BD although it is not very commonly encountered as in FMF patients [12, 13]. Diagnosis of BD is effectuated via clinical testing due to the absence of laboratory findings characterizing the disease. According to the international study group classification criteria, recurrent oral ulcerations with two of the following is sufficient for the designation of BD: recurrent genital ulcerations, ocular lesions, typical skin lesions and a positive pathergy (skin hyper reactivity) test [14].

Colchicine is generally used to treat patients who suffer from FMF and/or BD. Its action mechanism involves preventing fever attacks and amyloidosis [10,13,14]. However, some patients don’t respond to colchicine therapy. Interferon-γ and anti-TNF-α agents have been reported to be efficient for colchicine-resistant patients [14,15].

The age of onset of FMF is variable. Generally the first attacks are observed before the age of 20; moreover onset before 10 years is also a common case [7]. Occurrence of the attacks after 40 years is also reported; still they had a milder form of disease with abdominal attacks as the only symptom of FMF in many cases and without other types of chronic manifestations like amyloidosis or chronic arthritis [16].

A milder form of BD is also observed for late onset (after 40 years). Furthermore, both diseases show a severe course in young males [17,18]. The general age of onset for BD is the third decade of life [17].

1.2 Relationship between FMF and BD

The coexistence of FMF and BD has been observed in many countries. Birlik et *al*. reported a Turkish patient having both FMF and BD in 1998. In 2000, Schwartz et *al*. have reported 39 patients having BD among 4000 non-Ashkenazi Jewish FMF patients. 14 Turkish patients among 2716 (0.5 %) were reported to possess the two diseases at the same time by the Turkish FMF Study Group in 2005. The same year, Matsuda et *al.* reported a 15 year-old Japanese patient with the same case.

The concomitant occurrence of the two diseases may be due to the neutrophil and monocyte involvement in both of the diseases [13,19]. The serosal fluids of FMF patients have higher amounts of neutrophils during the attacks. Also, lesions of BD patients show neutrophil enhancement without any sign of bacterial presence [13]. Neutrophils participate to the regulation of the inflammatory response subsequent to an inflammatory stimulus, by producing soluble proinflammatory and antiinflammatory mediators. Monocytes play a central role in the coordination of inflammatory processes [20]. Pyrin, the product of MEFV gene, which is highly expressed in neutrophils, monocytes and dendritic cells is shown to be involved in both diseases [15,21].

1.3 MEFV gene, pyrin protein and its role in the formation of the inflammasome

MEFV gene has been found to be located on the short arm of chromosome 16 via linkage analysis [10]. In 1997, two consortia have cloned this gene (International FMF Consortium and French FMF Consortium). MEFV contains 10 exons and its RNA transcript encodes a protein composed of 781 amino acids [22]. This protein is called pyrin (fire in Greek) by the International FMF consortium, and marenostrin (coming from marenostrum in Latin, meaning Mediterranean Sea) by the French FMF consortium [15,23].

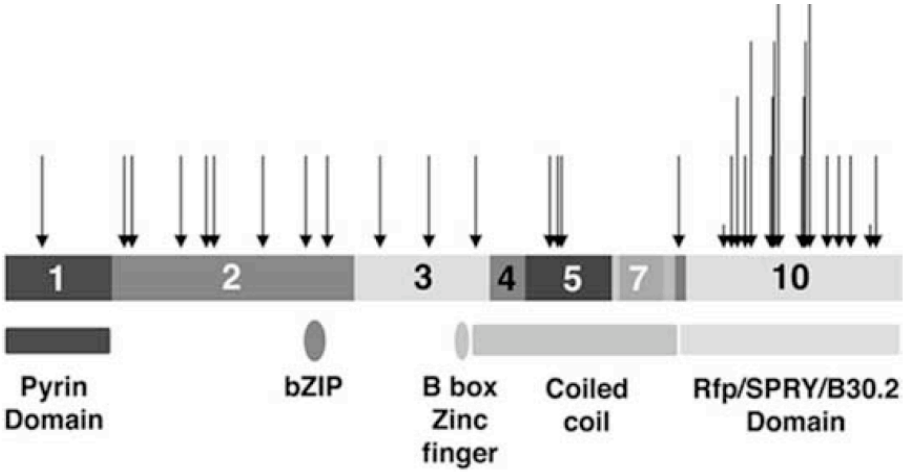

Figure 1.1. Pyrin protein and its different domains [22]

The pyrin domain on the exon 1 encodes a death domain. The exons between 2 and 10 contain other known motifs like bZIP on the second exon, a transcription factor basic domain; B box zinc finger on the exon 3; Coiled coil domain spanned on exons 3 to 8; and on the exon 10, where the majority of the mutations are found, the B30.2/SPRY/rfp domain composing the C-terminal domain [22].

Pyrin is mainly found in granulocytes, monocytes, dendritic cells; synovial, peritoneal, and skin-derived fibroblasts and has an important role in innate immunity [24]. It is up regulated by pro-inflammatory cytokines like LPS (Lipopolysaccharides), TNF-α, and IFN-γ, and down-regulated by anti-inflammatory cytokines like IL-4, IL-10, and TGF-ß [22]. This fact may lead to the conclusion that MEFV plays a role in a negative-feedback loop, specific for Th1 and proinflammatory-mediator activation of myelomonocytic cells [20].

The interaction of pyrin with two proteins, ASC (Apoptosis-associated Speck-like Protein with CARD (the caspase-recruitment domain)) and PSTPIP1 (proline serine threonine phosphatase interacting protein 1) explains its role in the cellular pathways [22,25]. ASC is a 195 amino acid protein, which plays an important role in caspase-1 activation and IL-1β secretion, NF-κB activation and apoptosis [22]. Pyrin interferes with the interaction of cryopyrin with ASC and prevents the cryopyrin-mediated inflammation through IL-1β processing [22,24,25].

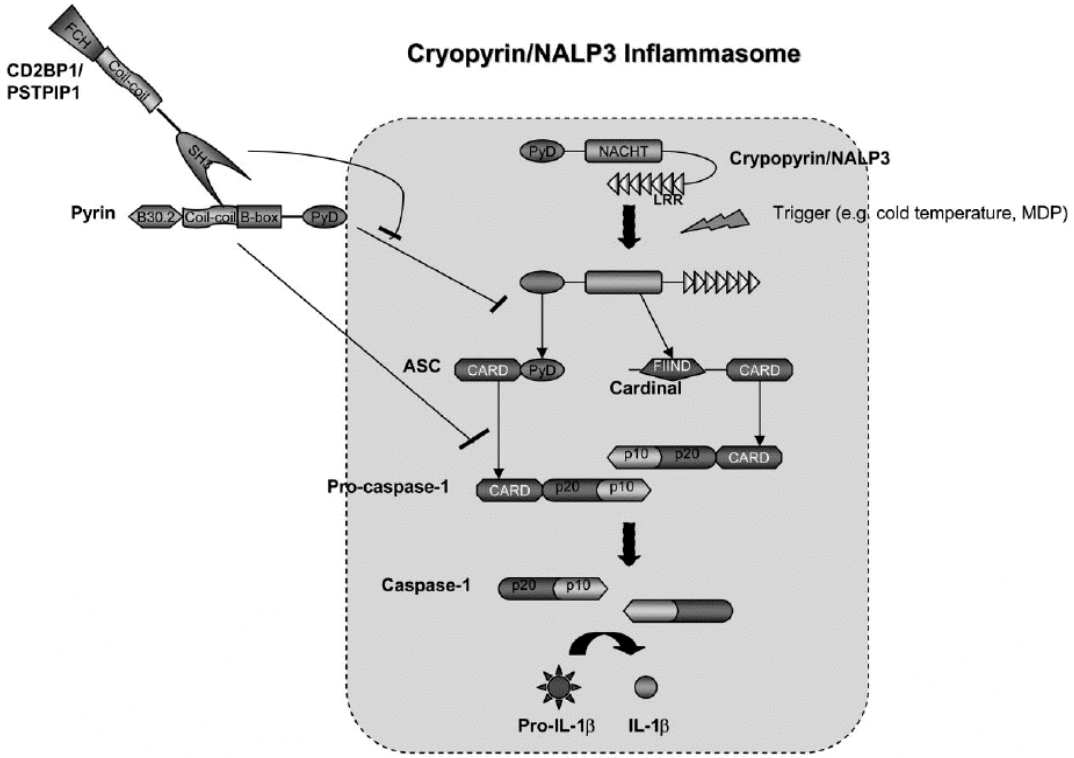

Figure 1.2. Interaction of pyrin with ASC and PSTPIP1 [25]

PSTPIP1 is an adaptor protein, which is localized in both neutrophils and monocytes as well as in lung, spleen, thymus and small intestine. It participates on the regulation of actin-based structures. The interaction of PSTPIP1 with pyrin leads to the positioning of pyrin on the signaling pathway so that it could sense and/or modulate the signals transduced by the cytoskeleton. This interaction of pyrin with actin filaments may explain the success of the colchicine treatment in FMF [22].

1.4 MEFV mutations/polymorphism, their effects and geographical distribution

Since MEFV gene has been cloned, more than 70 mutations have been reported [4,10]. Four main missense mutations and one polymorphism constitute the majority of the mutations/polymorphism encountered in patients of Mediterranean ancestry.

Table 1.1. Five main mutations/polymorphism on MEFV gene [26]

Exon	Mutation/ Polymorphism	Codon Change	Amino Acid
2	E148Q	GAG → CAG	Glu → Gln
10	M694V	ATG → GTG	Met → Val
	M694I	ATG → ATA	Met → Ile
	M680I	ATG → ATC	Met → Ile
	V726A	GTT → GCT	Val → Ala

M694V mutation is considered to cause a most severe disease phenotype since it causes renal amyloidosis in many of the reported cases, especially from Armenia, Israel and Lebanon [4]. In addition to this, patients with M694V homozygous mutation have an earlier age of onset and show a higher frequency of arthritis [6]. On the other hand, patients with compound heterozygous mutation/polymorphism like the V726A-E148Q mutation/polymorphism have severe amyloidosis in most of the cases. This might point the need to prescribe colchicine to the patients having mutations other than M694V [27].

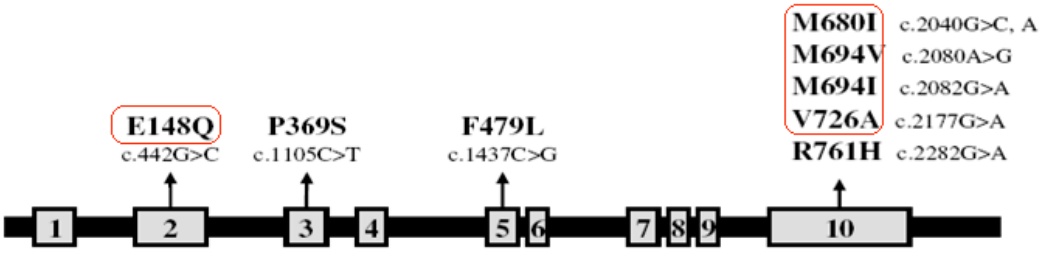

Figure 1.3. Most commonly observed /polymorphism on MEVF gene [28]

E148Q is reported to be the most frequent MEFV mutation in the general population. It is reported that patients with E148Q mutation have higher MEFV mRNA levels compared to patients with M694V mutation [29]. However, this mutation causes either a milder form or asymptomatic FMF [22]. Furthermore, this mutation shows a higher frequency in general population than observed in patients. There are some suggestions that E148Q might be a polymorphism augmenting the inflammation state [30].

The accumulation of the mutations on the 3' end of the MEFV mRNA makes it predisposed for nuclease attacks. Besides, MEFV mRNA stability might be modified by these mutations. Furthermore, the alteration in MEFV mRNA expression level due to the number and type of the mutations gives rise to the possibility that MEFV might be involved in its own expression regulation [29].

The frequency of different mutations of MEFV in different populations varies: the M694V mutation is most commonly observed among Jews, Turks and Armenians; whereas the M680I is majorly observed among Armenians and the M694I is observed among Arabs. The Turkish population has the highest carrier frequency for E148Q [9].

Despite the differences in the frequencies of the mutations, the appearance of the same mutations and the haplotypes in a wide range of populations separated geographically, points to a common ancestral genetic pool for these populations [9].

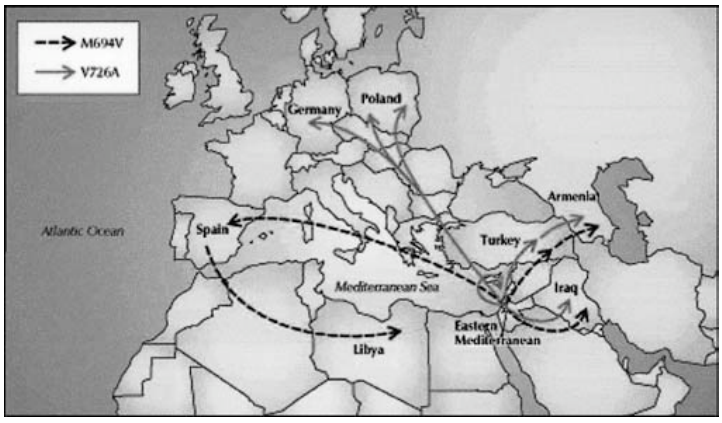

Figure 1.4. The spread of the M694 V and V726A mutations from the Middle East [9]

1.5 MEFV Mutations/polymorphism and HLA-B*51 frequencies in BD

MEFV mutations are also observed in BD patients. Patients with vascular involvement from Turkey and France, where both FMF and BD are frequent, have shown higher frequencies of MEFV mutations [19]. Nevertheless, BD is a multigenic disease [13]. HLA-B*51, a MHC class I antigen, is found in 60-70% of BD patients [19,21]. Two suballeles of HLA-B*51, HLA-B*5101 and HLA-B*5108 are mainly found in BD patients. These suballeles have the ability to bind specific peptides and natural killer (NK) cells [31]. The aetiology of BD is still unknown but the augmentation of the neutrophilic reaction is known as the main reason of the disease development [32]. An infectious agent may be involved in the pathology of BD to trigger the inflammation like in autoinflammatory disorders but an adaptive response is also supported via bacterial persistence or autoantigen-activated dendritic, T or B cells. Thus, BD may be an autoinflammatory disease with MEFV involvement, as well as an autoimmune disease with HLA-B*51 involvement [21].

1.6 The Major Histocompatibility Complex

The human major histocompatibility complex region (MHC) is located in the short arm of the 6th chromosome [33] as shown in Figure 1.5 below. It is found in all vertebrate species and has a role in the recognition of self from non-self. Thus it also has a role in the regulation of the immune function [34].

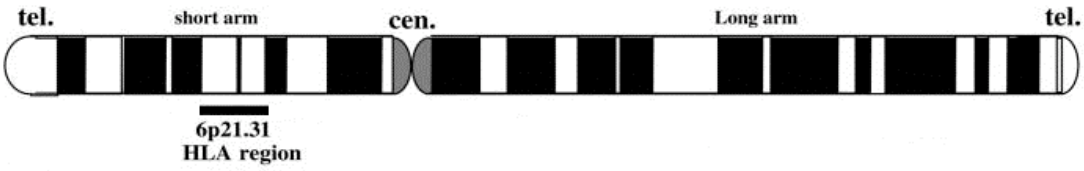

Figure 1.5 Genomic location of HLA region [34]

The MHC is clustered in three gene classes according to their structure and function. These genes are inherited in a co-dominant way, meaning that alleles on both chromosomes code for a protein product. In order to indicate the different combinations of MHC genes, "MHC haplotype" term is frequently used [34].

Class I region is composed of highly polymorphic genes (HLA-A, B and C) encoding molecules which are expressed in the membranes of many cells in different tissues. These molecules bind self and foreign peptides to present them to CDS cytotoxic T cells. The class II genes (HLA-DR, DQ, and DP) are only expressed in antigen presenting cells, for the presentation of endogenous peptides to CD4 helper T cells. The molecules of these two classes are produced in endoplasmic reticulum (ER) [34].

The third class is located between the class I and class II regions. Genes of class III region encode many humoral factors (such as complement components, heat shock proteins and tumor necrosis factor) having an immunoregulatory function [34].

Class I genes are formed by many exons coding for specific regions of the proteins; namely, the class I molecules. The first exon encompasses a signaling sequence needed for the initiation of the transcription. The second, third and fourth exons encode respectively alpha 1, alpha 2, and alpha 3 extracellular domains. Finally, the transmembrane and cytoplasmic segments are coded by exons 5, 6, 7. A beta-immunoglobulin chain, encoded by a gene on the 15^{th} chromosome, is associated with the alpha heavy chain of the class I molecules [34,36]. This motif constitutes the functional MHC molecule [36].

The second and the third domains of the class I molecules conjugate to form a peptide binding groove, "Bjorkman's groove", named after the researcher who demonstrated it by crystallography. It comprises beta pleated sheets and alpha helices as shown in the Figure 1.6 [34].

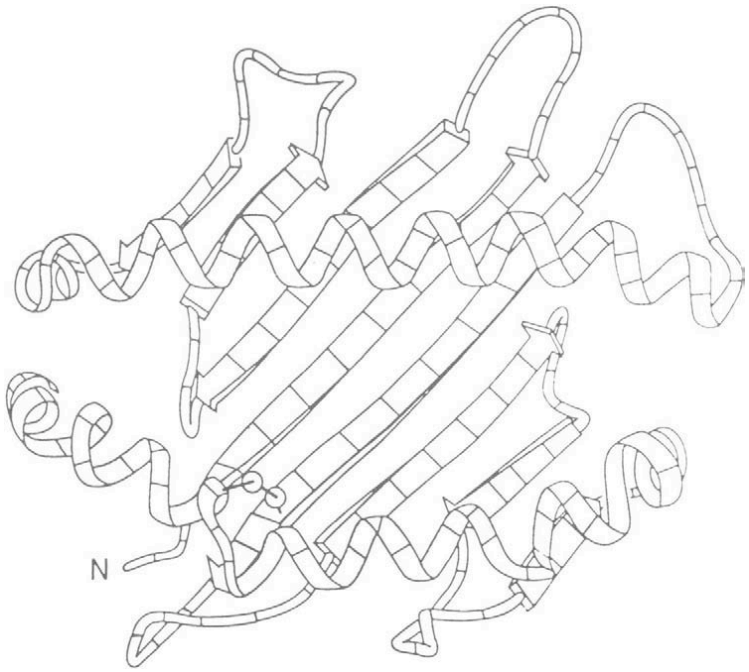

Figure 1.6 The model structure of the class I and class II molecules [34]

These grooves contain discrete pockets, which are involved in the binding of the peptide amino acid side chains: B and F binding pockets. Most of the mutations are observed in these peptide binding regions. Thus each variant bind a unique range of peptide ligands [36].

The implication mechanisms of the class I molecules in human diseases are not well known; however, molecular mimicry (shared amino acid sequence between microorganisms and MHC molecules) and preferential binding of certain viral or auto-peptides to class I and II molecules may explain the onset of these diseases [34].

1.7 Frequencies of FMF and BD

FMF and BD have also shown epidemiologic similarities [3]. Both diseases are frequent in people from Mediterranean descent [13]. The frequency of FMF in Turkey is estimated to be 1:1000 and the carrier rate is 1:5 [6]. The estimated frequency of FMF in Armenians living in Turkey is approximately 7:1000 and the carrier rate differs between 1:5 and 1:16 [37]. On the other hand, the frequency of BD in Turkish patients is approximately 4:1000 and the carrier rate is 1:4 [31,38].

For the Armenians living in Turkey, the BD frequency is estimated to be less than 2:1000 [37] and the carrier rate is still unknown.

1.9 Aim of the Study

The aim of this study was to analyze the frequencies of the five most common mutations/polymorphisms in MEFV and also HLA-B*51 allele frequencies in Armenian and Turkish populations living in the same environment, namely in Istanbul.

The results obtained from this thesis are expected to give an explanation for the difference in the frequency of FMF and BD in these two populations. Furthermore, the carrier frequency of BD in Armenians living in Turkey can be calculated.

2. MATERIALS and METHODS

2.1. Materials and Laboratory Equipment

2.1.1. Used Equipments

Appendix A displays the equipments used in this project.

2.1.2. Used Chemicals, Enzymes, Markers and Buffers

Chemicals, enzymes, markers and their related suppliers are given in Appendix B. Appendix C displays the preparation and the composition of buffers and solutions.

2.2. Collection and Storage of Saliva Samples

Saliva samples are collected from 100 Armenian working in the administration department of Armenian schools in Istanbul and from 100 Turkish students studying in Cerrahpaşa Medical Faculty. A questionnaire, shown in Appendix D, is given to the subjects before the collection of the samples. According to the selection criteria, the subjects have to be older than 18 and have to give their consent (shown in Appendix E) for the study. Saliva samples are collected in Oragene^{TM} vials [39]. The samples are kept in room temperature.

2.3. DNA Isolation from Saliva Samples

The Oragene^{TM} vials contain a DNA preserving liquid in their lids which mixes with the saliva once the subject caps the vial. Before the purification step, the sample has to be incubated in a water incubator at 50^{o}C for a minimum of 1 hour. This step is performed to ensure the release of DNA from the cells and the inactivation of nucleases.

The saliva is than divided into four 1.5 mL microcentrifuge tubes, each containing approximately 1 mL of sample. 40 mL (approximately 1/25th of the volume) Oragene•DNA Purifier is added in each tube. This step is needed for the precipitation of the impurities and inhibitors. Incubation on ice for 10 minutes is performed for effective removal of the impurities.

The four tubes are centrifugated for 3 minutes in 1500 g at room temperature. The supernatants are collected and combined in a 15 mL centrifuge tube. The pellets are discarded.

4 mL (equal volume) 95-100% ethanol at room-temperature is added for the precipitation of DNA in the form of fibers. In some cases DNA might not be visible. For the fully precipitation of the DNA, samples are kept in room temperature for 10 minutes.

Centrifugation for 10 minutes is performed at room temperature at 1100 g. The supernatants are discarded and the DNA pellet is rehydrated by 500 µL TE buffer. An incubation of the samples overnight at room temperature or 10 minutes in 50^0C is recommended for the effective dissolving.

2.4 DNA Amount, Purity and Working Solution Calculations

The absorbance values measured at 260, 280 and 320 nm are used for the calculation of the isolated DNA amount and purity. The DNA amount is calculated via the equation 2.1, which is the multiplication of the difference between absorbances at 260 and 320 nm (giving the absorbance of the DNA alone) by the dilution factor and the absorbance constant (it is assumed that 1 OD is equivalent to 50 µL of DNA).

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

DNA purity is calculated via the equation 2.2 :

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

In order to obtain comparable band patterns in genotyping, DNA concentrations are adjusted to 50ng/µL in 250 µL by dilutions from stock DNA. These dilutions serve also as aliquots preventing the contamination of the stock DNA.

2.5. MEFV Genotyping

2.5.1 Polymerase Chain Reaction (PCR)

Isolated DNAs from saliva samples are used as templates for the PCR reactions performed for the amplification of the sequences of interest containing the related MEFV SNPs. A standard mixture is prepared for all five mutations/polymorphism with relative primers.

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		

2.5.1.1 Oligonucleotide Primers

The primers used for the PCR reactions were previously chosen and prepared by my lab coworkers. They were confirmed to bind the desired sequence of MEFV gene by Amplify 3X software [40]. This software is also able to calculate the efficiency of binding and the amplicon sizes.

The hairpin, heterodimer and self dimer analysis of the primer sets were assessed with the SciTools on the IDT DNA website [41].

Table 2.2 Oligonucleotide primers

SNP	**Primer Sequence**	**Amplicon Size**	**Reference**
MEFV E148Q	F-5'-ATATTCCACACAAGAAAACGGC-3' R-5'-GCTTGCCCTGCGCG-3'	244 bp	[42]
MEFV M680I	F-5'-TGTATCATTGTTCTGGGCTCT-3' R-5'-AGGGCTGAAGATAGGTTGAA-3'	360 bp	[30]
MEFV M694V	F-5'-GCTACTGGGTGGTGAT*CAT-3' R-5'-AGGGCTGAAGATAGGTTGAA-3'	215 bp	[30]
MEFV M694I	F-5'-TGTATCATTGTTCTGGGCTCT-3' R-5'-CTGGACGCCTGGTACTCATTTTT*C-3'	195 bp	[30]
MEFV V726A	F-5'-TGTATCATTGTTCTGGGCTCT-3' R-5'-AGGGCTGAAGATAGGTTGAA-3'	360 bp	[30]

2.5.1.2 PCR Cycle Conditions

A general PCR protocol, shown in Table 2.3, was designated for all the mutations with the exception of E148Q polymorphism which has a more complicated protocol with two different loops containing two different annealing temperatures (Table 2.4). For the four mutations, only the annealing temperatures differ in protocols (shown in Tables 2.5 and 2.6).

Table 2.3 General PCR cycle conditions

Repeat	**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

Table 2.4 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

Table 2.5 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

Table 2.6 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

2.5.2 Agarose Gel Electrophoresis of PCR Products

For the observation of the PCR products of the five MEFV mutations/polymorphism analyzed in this thesis, 1% agarose gel is sufficient due to the sizes of the amplicons varying between 195 and 360. Since the right percentage of agarose gel is important for observing the PCR bands, mini and midi gels are prepared meticulously: Mini gels contain 0.5 g agarose dissolved in 50 mL of 1X TBE buffer as well as 0.5 µg/mL ethidium bromide, midi gels contain 1.5 g agarose dissolved in 150 mL of 1X TBE buffer as well as 0.5 µg/mL ethidium bromide. In order to assess the relative lengths of the PCR products, 1 kb ruler (Fermentas) is loaded into the first wells of the gels. 6 µL of the PCR products are mixed with 1 µL of 6X loading dye (Fermentas) to ease the precipitation and the observation of relative position with naked eye. The gels are run for an average of 20-25 minutes in 120V into 1X TBE buffer. The results are monitored via transilluminator under UV light, and pictures are taken via UV PhotoMW software.

2.5.3 Restriction Enzyme Digestion of PCR Products

Restriction enzyme digestions' protocols were given by the supplier of the enzymes. Nevertheless EnzymeX software was also used for the virtual performance of the enzyme digestion for its confirmation. The general protocol is given in table 2.7 and the restriction enzymes and expected fragment sizes are given in table 2.8 below

Table 2.7 Restriction Enzyme Digestion mixture

Ingredient	1X amount
10X reaction buffer	2 μL
dH_2O	5 μL
Restriction Enzyme (10 U/μL)	1 μL
Amplicon	12 μL
Final Volume	20 μL

According to the restriction enzyme digestion protocol, the PCR products are left overnight with the enzyme mix at 37 °C for efficient digestion. An additional step at80 °C for 30 minutes is needed for the inactivation of the enzyme leading to digestion arrest.

Table 2.8 Restriction Enzymes used for each mutation/polymorphism 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)

2.5.4 Agarose Gel Electrophoresis of Restriction Enzyme Digested PCR Products

Different agarose gel concentrations are needed for the genotyping of different mutations/polymorphism, varying between 1% and 4%. The digested fragments of E148Q products are run in 4%, M680I and V726A digested fragments were run in 2%, M694I and M694V digested fragments were run in 3.5% mini or midi agarose gels at 120 V for a minimum of 30 minutes in 1X TBE buffer. Low range marker (Fermentas) is used for the calculation of relative lengths of the products. 12 µL of RE digestion products are mixed with 2 µL of 6X loading dye (Fermentas) with the exception of E148Q products, from which 15 µL of PCR products are mixed with 3µL of 6X loading dye.

2.5.5 Genotyping

The analysis of the gel photos of the RE digested PCR products are performed for the genotyping. The expected bands were determined via the EnzymeX software, as shown in figures 2.1, 2.2, 2.3, 2.4, 2.5. Genotyping was done blindly by myself, my lab partners Sinem KARAMAN, and Duygu KUZUOĞLU, and by my advisor Dr Eda TAHİR TURANLI.

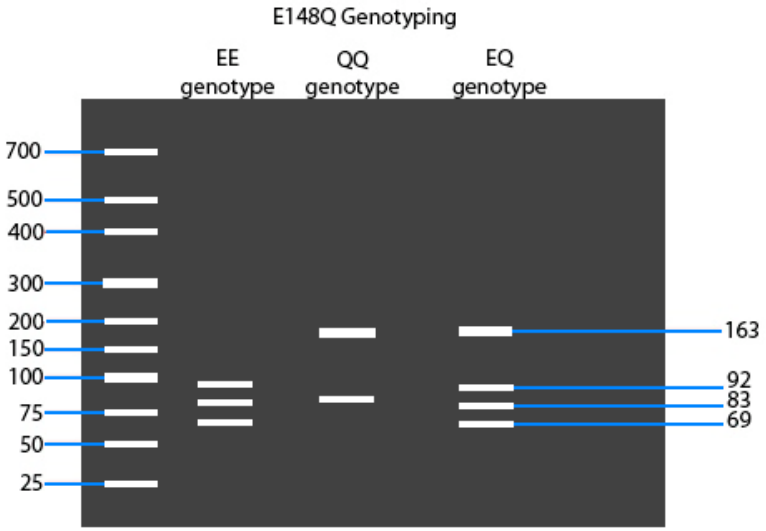

Figure 2.1 E148Q expected restriction enzyme fragment sizes

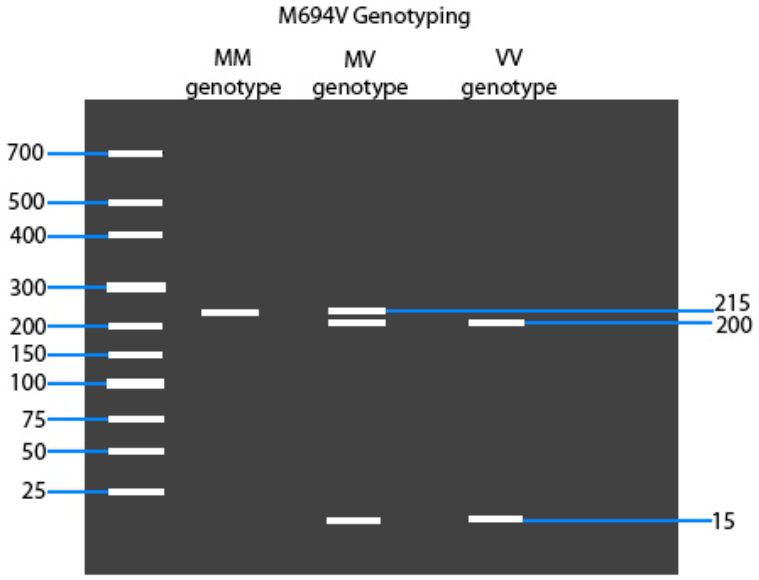

Figure 2.2 M694V expected restriction enzyme fragment sizes

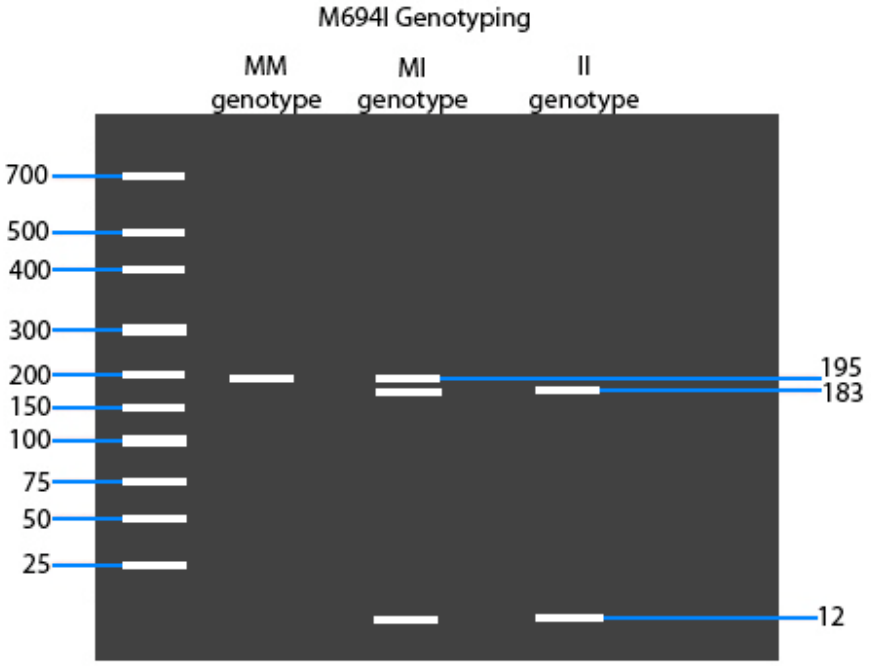

Figure 2.3 M694I expected restriction enzyme fragment sizes

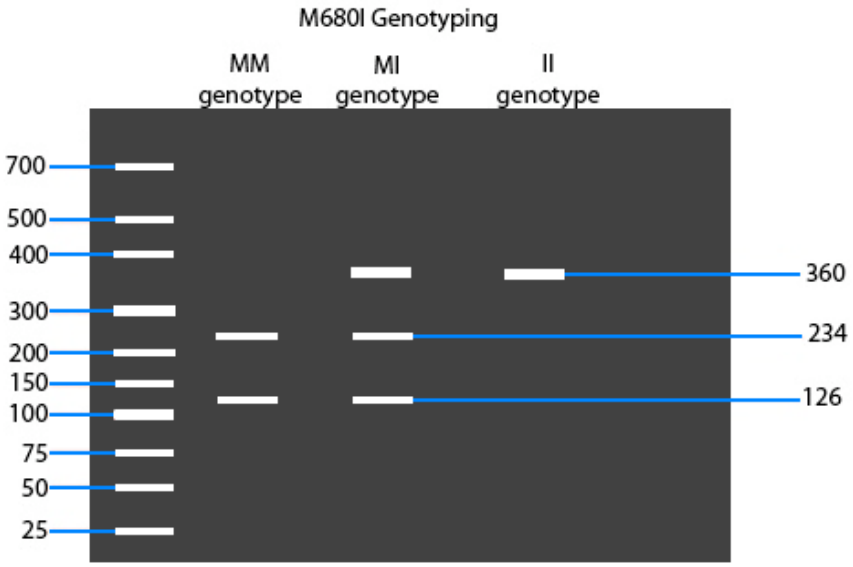

Figure 2.4 M680I expected restriction enzyme fragment sizes

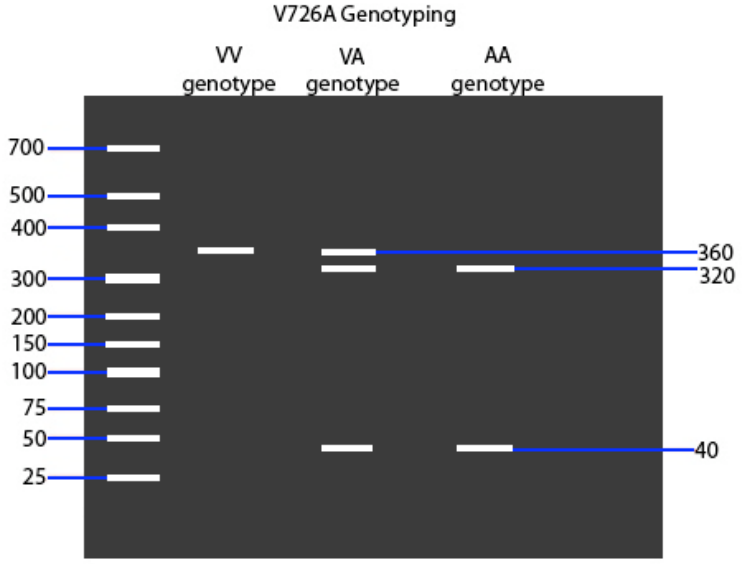

Figure 2.5 V726A expected restriction enzyme fragment sizes

2.6 HLA Genotyping

Two types of Olerup SSP HLA kits were used for the determination of the presence of the HLA-B*51 allele and its subtype: HLA-B low resolution and HLA-B*51 kits (Qiagene). The genotyping protocols for HLA are given by the suppliers of the kit.

The Micro SSP™ Seramates HLA Class I DNA Typing kit (One Lambda) was also used for one part of the study as well as for confirmation of the Olerup results.

The optimum concentration of DNA for HLA genotyping is 30 ng/µL. It is implicated in the manual that non-specific amplifications might occur with samples exceeding 50 ng/µL.

2.6.1 Olerup SSP Kit

2.6.1.1 PCR Amplification

A 48-well plate, supplied with its primers in specific positions, is used for HLA genotyping. A master mix is prepared for all 48 wells of the PCR plate, as shown in Table 2.9, 2.10 and 2.11.

Table 2.9 Standard PCR mix-1 for HLA-B low resolution

Ingredient	**Volume**
PCR Master Mix	3 µL x 53
Taq Polymerase	4.2 µL
FINAL	163,2 µL

3 µL of the standard mix-1 is added into well No. 48 - the well with the negative control primers. Then 7 µL is added to well 48.

Table 2.10 Standard PCR mix-2 for HLA-B low resolution

Ingredient	**Volume**
Standard Mix-1	160,2 µL
DNA	2 µL x 52
dH_2O	5 µL x 52 – 4,2 µL
FINAL	519,7 µL

10 µL of the Standard Mix-2 is added in each 47 wells. Than the PCR plate is closed with the provided lids.

Table 2.11 Standard PCR mix-2 for HLA-B low resolution

Ingredient	**Stock Concentration**	**Volume**	**Final Concentration**
DNA	30 ng/ µL	2 µL x 45	2,4 µg
PCR Master Mix		3 µL x 45	1.875 mM
Taq Polymerase	5 U/µL	3,6 µL	U/µL
dH_2O	–	5 µL x 45 – 3,6 µL	–
FINAL	450 µL		

10 µL of the master mix is added to each of the 40 wells. Than the PCR plate is closed with the provided lids.

A standard PCR cycle condition is used for both kits, as given in the Table 2.12.

Table 2.12 General PCR cycle condition

Repeat Number	**Degree**	**Time**	**Phase**
1	94 °C	2 minutes	Initial Denaturation
10	94 °C	10 seconds	Denaturation
	65 °C	60 seconds	Annealing and extension
20	94 °C	10 seconds	Denaturation
	61 °C	30 seconds	Annealing
	72 °C	30 seconds	Extension

2.6.1.2 Agarose Gel Electrophoresis

2% agarose gel is prepared in 0.5 X TBE buffer. The gel is stained with 5 µL per 100 mL gel solution of ethidium bromide (10 mg/mL). The gel is run in 0.5 X TBE for 15-20 minutes at 8-10 V/ cm.

2.6.1.3 Documentation and Interpretation

The gel is documented by photography with a UV transilluminator. The presence and absence of specific PCR products is recorded. The specific PCR products help in the interpretation of the results.

The relative lengths of the internal positive control bands are also recorded. These control bands help in the correct orientation of the typing. Lanes without control band or specific PCR products are repeated.

The interpretation is done via HELMBERG-SCORE® Virtual Sequencing software (Appendix F).

2.6.2 Micro SSP Seramates Kit

2.6.2.1 PCR Amplification

A 11-well plate, supplied with its primers in specific positions, is used for HLA genotyping. The first well is set for negative control by adding 1µL of Taq polymerase to the PCR mix provided by the supplier (One lambda). 1 µL of DNA sample as well as 9 µL of the PCR mix with Taq polymerase are put in the first well. Than 19 µL of DNA sample is added in the PCR mix with Taq polymerase. 10 µL of this mix was applied to the next 10 wells.

The PCR condition is given in the Table 2.13 below.

Table 2.13 PCR Condition For One Lambda Seramates SSP Kit

Repeat Number	**Degree**	**Time**	**Phase**
1	96 °C	130 seconds	Denaturation
	63 °C	60 seconds	Annealing and extension
9	96 °C	10 seconds	Denaturation
	63 °C	60 seconds	Annealing
20	96 °C	10 seconds	Denaturation
	59 °C	50 seconds	Annealing
	72 °C	30 seconds	Extension

2.6.2.2 Agarose Gel Electrophoresis

2.5 % agarose gel is prepared in 0.5 X TBE buffer. The gel is stained with 5 µL per 100 mL gel solution of ethidium bromide (10 mg/mL). The gel is run in 0.5 X TBE for 15-20 minutes at 8-10 V/ cm.

2.6.2.3 Documentation and Interpretation

The gel is documented by photography with a UV transilluminator. The presence and absence of specific PCR products is recorded. The specific PCR products help in the interpretation of the results.

The relative lengths of the internal positive control bands are also recorded. These control bands help in the correct orientation of the typing. Lanes without control band or specific PCR products are repeated.

The interpretation is done with the aid of the interpretation table given with the kit (Appendix G).

2.7 Statistical Analysis

Non-parametric statistical analyses were performed using chi-square tests. This allows to assess the significance of mutation frequencies between both Turkish and Armenian groups with their historical controls, and between each other.

The P value, which is the level of significance, is calculated to control whether the results are obtained by chance. Thus, a lower P value will allow us to reject the null hypothesis. The P value intervals are:

$P > 0.05$ pointing to the absence of significant difference

$P < 0.05$ pointing to a significant difference

$P < 0.01$ pointing to a highly significant difference

$P < 0.001$ pointing to an extremely significant difference

Calculations for P value, chi-square and Odds ratio were done with the online tool from Simple Interactive Statistical Analysis (SISA) [43].

3. RESULTS

3.1 DNA Isolation Results

DNA samples had average concentration of 182.755 ng/μL obtained by OrageneTM DNA Self-collection Kit (Genotek). The average value of A_{260}/A_{280} was 1,83 (N=213).

3.2 Demographic Data of the Armenian and Turkish Groups

The demographic data of the Armenian and Turkish groups are given in the tables 3.1 and 3.2 below.

Table 3.1 Demographic properties of the Armenian Group

Armenian Group N=113	
Gender	**Percentage / Number**
Female Male	82.3 % / 93 17.7 % / 20
FMF	4.4% / 5
BD	--
Mean Age	42 ± 12 (range 22-84)

Table 3.2 Demographic properties of the Turkish Group

Turkish Group N=100	
Gender	**Percentage / Number**
Female Male	68 % / 68 32 % / 32
FMF	--
BD	1% / 1
Mean Age	24 ± 1 (range 22-31)

3.3 Genotype Analysis

3.3.1 MEFV

The genotyping is done by counting the alleles from the obtained gel photos with the expected RE digestion patterns previously determined via EnzymeX software Sample PCR and restriction enzyme results for each mutation analysis are given in Appendix H.

The obtained genotypes are shown in the table 3.3 below.

Table 3.3 Obtained genotypes in the Armenian and Turkish groups

SNP	Genotype	Armenian Group N= 113	Turkish Group N= 98
M694V	MM	100	92
	MV	13	6
	VV	-	-
M694I	MM	106	95
	MI	7	3
	II	-	-
M680I	MM	105	97
	MI	7	1
	II	1	-
V726A	VV	105	94
	VA	8	3
	AA	-	1
E148Q	EE	101	88
	EQ	12	7
	QQ	-	3

3.3.1.1 Statistical Mutation/Polymorphism Analysis

Some subjects have been excluded from the data: 3 Turkish subjects - the genotyping was not applicable, one suggested Armenian subject for not having Armenian ethnicity and one Armenian subject for having a sibling in the study. Additionally diseased subjects are also excluded from the statistical analyses.

The mutational/polymorphism frequencies of Armenian and Turkish groups are given in the tables 3.4 and 3.5 below.

Table 3.4 Frequencies of five common MEFV mutations/polymorphisms in the Armenian group

SNP	***N=108*** ***Number of Mutations/Polymorphisms***	**Mutation/Polymorphism Frequency**
M694V	13	6 %
M694I	6	2.7 %
M680I	6	2.7 %
V726A	6	2.7 %
E148Q	12	5.5 %
TOTAL	43	19.6 %

Table 3.5 Frequencies of five common MEFV mutations/polymorphisms in the Turkish group

SNP	***N=97*** ***Number of Mutations/Polymorphisms***	**Mutation/Polymorphism Frequency**
M694V	6	3.1 %
M694I	3	1.5 %
M680I	1	0.5 %
V726A	5	2.6 %
E148Q	13	6.7 %
TOTAL	28	14.4 %

We first compared the mutation/polymorphism frequencies between our two groups which is shown in table 3.6 below.

Table 3.6 Mutation/polymorphism analysis between two groups

SNP	***N=97 the Turkish group*** (194 chromosomes)	***N=108 the Armenian group*** (216 chromosomes)
M694V	6 (3.1 %)	13 (6 %)
M694I	3 (1.5 %)	6 (2.7 %)
M680I	1 (0.5 %)	6 (2.7 %)
V726A	5 (2.6 %)	6 (2.7 %)
E148Q	13 (6.7 %)	12 (5.5 %)
TOTAL	28 (14.4 %)	43 (20 %)

The mutational/polymorphism frequencies of healthy Armenian and healthy Turkish groups are compared with historic controls, the frequency data given in the tables 3.8 and 3.9 below.

Table 3.7 Mutation/polymorphism analysis – comparison with historic controls in Turks

SNP	***N=97 the Turkish group*** (194 chromosomes)	Turkish Controls [44] (N=100) (200 chromosomes)
M694V	6 (3.1 %)	3 (1.5 %)
M694I	3 (1.5 %)	0
M680I	1 (0.5 %)	5 (2.5 %)
V726A	5 (2.6 %)	2 (1 %)
E148Q	13 (6.7 %)	12 (6 %)
TOTAL	28 (14.4 %)	22 (11 %)

Table 3.8 Mutation/polymorphism analysis – comparison with historic controls in Armenians

SNP	*N=108 the Armenian group* (216 chromosomes)	Armenian Controls [45] (N=250) (500 chromosomes)
M694V	13 (6 %)	24 (4.7%)
M694I	6 (2.7 %)	--
M680I	6 (2.7 %)	--
V726A	6 (2.7 %)	23 (4.6%)
E148Q	12 (5.5 %)	17 (3.4%)
TOTAL	43 (20 %)	64 (12.8 %)

There was no significant difference in the MEFV mutation/polymorphism frequencies between the Turkish subjects and Turkish historic controls. In addition to this, no significant difference was found in the MEFV mutation/polymorphism frequencies between the Armenian subjects and Armenian historic controls. On the other hand, when the mutation/polymorphism frequencies of MEFV between Turkish and Armenian subjects are compared there was not any significant difference either. However, in M680I mutation, a trend for significance was observed (95% CI, p=0.0847, χ^2=2.971, Table 3.8).

Table 3.9 Statistical analysis of mutations/polymorphism

	Armenian Group / Turkish Group			**Armenian Group / Historical Controls**			**Turkish Group / Historical Controls**		
	χ2	**P Val**	**OR**	**χ2**	**P Val**	**OR**	**χ2**	**P Val**	**OR**
M694V	1.807	0.18	1.5	0.41	0.52	1.25	1.069	0.30	1.35
M694I	0.691	0.41	1.42	--	--		3.1	0.08	-
M680I	3.016	0.08	3.31	--	--		2.51	0.11	2.95
V726A	0.015	0.90	1.04	1.197	0.27	1.66	1.355	0.24	1.45
E148Q	0.207	0.65	1.10	1.65	0.20	1.63	0.072	0.79	1.06

3.1.2 HLA-B*51

The genotyping was done with HELMBERG-SCORE® Virtual Sequencing software for the Olerup kit and with the table provided by the supplier for the One lambda Seramates kit.

First, we compared the HLA-B*51 frequencies of our two groups, which is shown in the table 3.10 below.

Table 3.10 HLA-B*51 phenotypes of the Armenian and the Turkish Groups

	Armenian Group **N=110** **Number of phenotypes (%)**	**Turkish Group** **N=93** **Number of phenotypes (%)**
HLA-B*51	30 (% 27.2)	19 (% 20.4)

The observed HLA-B*51 phenotypes of the two groups were than compared with historical controls. The data are given in the tables 3.11 and 3.12 below.

Table 3.11 HLA-B*51 phenotype analysis – Comparison with historical controls in Armenians

	Armenian Group **N=110** **Number of phenotypes/** **Percentage**	**Armenian Controls [46]** **N=100** **Number of phenotypes/** **Percentage**
HLA-B*51	30 (% 27.2)	10.4 (% 10.4)

Table 3.12 HLA-B*51 phenotype analysis – Comparison with historical controls in Turks

	Turkish Group **N=93** **Number of phenotypes/** **Percentage**	**Turkish Controls [47]** **N=228** **Number of phenotypes/** **Percentage**
HLA-B*51	19 (% 10.2)	72 (% 15.8)

There was no significant difference in the HLA-B*51 frequencies between the Turkish and Armenian subjects. No significant difference was found in the HLA-B*51 frequencies when Turkish subjects were compared with historic controls from the literature. On the other hand, when the HLA-B*51 frequencies between Armenian subjects and Armenian historic controls are compared there was a significant difference which can be seen in the table 3.13 below (χ^2=14.153, p=0.0001, OR=1.63).

Table 3.13 Statistical analysis of HLA-B*51 frequencies

	Armenian Group / Turkish Group			**Armenian Group / Historical Controls**			**Turkish Group / Turkish Historical Controls**		
	χ^2	**P Val**	**OR**	**χ^2**	**P Val**	**OR**	**χ^2**	**P Val**	**OR**
HLA-B*51	0.875	0.350	1.18	14.153	0.0001	1.63	2.591	0.1074	1.39

4. CONCLUSION & DISCUSSION

The results obtained from this study confirm the literature on the frequencies of the five most common MEFV mutations in general Turkish and Armenian populations who live in Turkey. Subjects showed similar frequency distributions of five most common mutations of MEFV, and there was no significant difference in the MEFV mutation frequencies between the Turkish subjects and Turkish historic controls as well as in Armenian subjects and Armenian historic controls. When the mutation frequencies of MEFV between Turkish and Armenian subjects are compared, there wasn't any significant difference either. This result might be caused by population stratification or lack of a greater sample pool. However, in M680I mutation, a slight trend was observed, which may indicate that this mutation is more common among the Armenian population (6 in 108).

Although these two populations live in the same environment, the FMF frequency is higher in Armenian Turkish citizens than in Turkish population. On the other hand, the mutation frequencies of MEFV are very high in both groups, unlike the FMF disease itself.

Recent studies have shown that MEFV mutations and polymorphisms are not specific to FMF; in fact, they are also found in other inflammatory disorders, such as Behçet disease [3,48], ulcerative colitis [49], rheumatoid arthritis [50] and polyarteritis nodosa [51]. Also, more recent studies point that there are many patients who are clinically FMF but are free of MEFV mutations [52,53].

Altogether, these facts might indicate the recessive inheritance pattern of FMF as well as the other potential genetic locus or loci that could be related with FMF.

Results from the HLA-B*51 study confirm the literature for the Turkish group, however, the Armenian group have shown a higher frequency than the historic

control group. When compared, no significant difference between the Armenian and the Turkish group was observed. These results may point to the genetic differences between the Armenians living in Turkey and the Armenians living in other countries. Also, it may point to another locus or loci, which have a role in the aetiology of Behcet disease.

In further studies, the compound heterozygous samples would be clarified via DNA sequencing to calculate the true allelic frequencies of MEFV mutations and polymorphisms. Also, the HLA-B*51 frequencies observed in both groups, would be confirmed by serological analyses.

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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
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

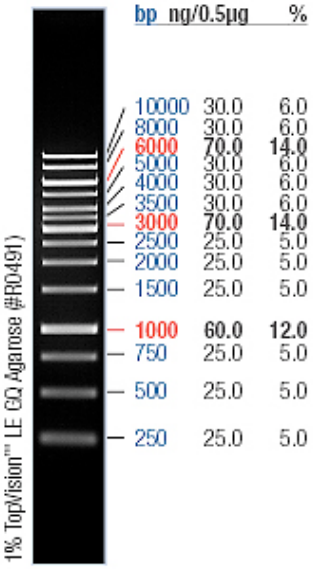
Agarose	AppliChem
Boric acid	Amresco
dNTP	Fermentas
EDTA	AppliChem
Ethanol	Riedel-de Haën
EtBr	Amresco
$MgCl_2$	Fermentas
NaOH	Riedel-de Haën
Primers	IDT DNA
NaCl	Carlo Erba
Tris Base	Amresco
10X PCR Buffer	Fermentas
	Qiagen
	Roche

ENZYMES

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

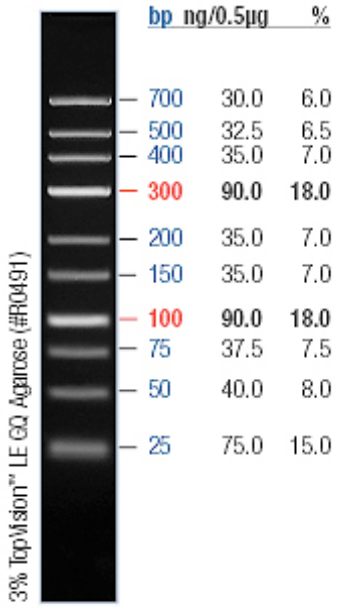
MARKERS

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.

APPENDIX D

Gönüllü Bilgilendirme ve Olur Formu

Çalışma Başlığı: Ermeni ve Türk toplumunda MEFV gen mutasyon ve HLA B51 sıklığı

Çalışmayı yürüten merkez: İstanbul Üniversitesi, Cerrahpaşa Tıp Fakültesi, Romatoloji Bilim Dalı

Ailevi Akdeniz Ateşi ve Behçet Hastalığı, Ortadoğu ve Anadolu halklarında, dünyanın diğer yörelerine oranla daha sık görülmektedir. Türkiye'de yapılan çalışmalarda Ailevi Akdeniz Ateşi sıklığı 1000'de 1-2.5 arasında değişirken, Behçet hastalığının sıklığı 1000'de 4 olarak saptanmıştır. Yakın zamanda İstanbul'da T.C. Ermeni kökenli vatandaşlarında yaptığımız öncü bir çalışmada, bu toplumda Ailevi Akdeniz Ateşinin Türklere oranla daha sık (% 0.7), Behçet hastalığının ise daha nadir olduğunu (< 1/ 500) gözledik. Bu bulgular bize, her iki hastalığın etyolojisinde de genetik sebeplerin daha ağır bastığını düşündürdü.

Son yıllarda Ailevi Akdeniz Ateşi hastalığına MEFV genindeki mutasyonların yol açtığı ortaya çıkmıştır.Tanımlanan mutasyonların sayısı 50'den fazladır ancak 4 tanesi daha sık görülmektedir; ve bu mutasyonlar hasta kişilerde % 85, sağlıklı kişilerde ise % 10-20 oranında bildirilmektedir.

Öte yandan Behçet hastalığı ile HLA B51 geni arasında bir ilişki varolduğu da bilinmektedir. HLA B51 taşıyıcılığı, Behçet hastalığı olan bireylerde % 50-80, sağlıklı kişilerde ise % 20-30 oranında bildirilmektedir. Behçet hastalığının sıklığı toplumdaki HLA B51 taşıyıcılığına paralel seyretmektedir. Örneğin, HLA B51 sıklığının az olduğu toplumlarda Behçet hastalığı da nadirdir.

Yukarıda belirttiğimiz nedenlerden ötürü, biz bu çalışmada, T.C. Ermeni ve Türk vatandaşlarında MEFV gen mutasyonunu ve HLA B51 gen taşıyıcılığını araştırmak istedik. **Bu araştırma için tükürük örneği vermeniz yeterli olacaktır.** Verdiğiniz tükürük örneği, içerdiği kan hücrelerinden DNA ve RNA elde edilmek

üzere İstanbul Teknik Üniversitesi, Moleküler Biyoloji ve Genetik laboratuarlarında saklanacak ve daha önce bahsettiğimiz genler için çalışılacaktır. Bu araştırmaya yaklaşık 200 kişinin (100 Ermeni, 100 Türk) katılması beklenmektedir.

Sizden alınmış olan tükürük örneği, sizden alındığını gösteririr herhangi bir kişisel bilgi içermeyecektir. Yapılacak bilimsel çalışmalar genellikle çok sayıda örnekle yürütülecek ve bu çalışmalardan toplu sonuçlar çıkacaktır. Bu nedenle, çalışmaların kısa vadedeki sonuçlarından kişisel olarak sizin veya ailenizin diğer bireylerinin yararlanması sözkonusu olmayabilir.

Bağışladığınız bu tükürük örneğiyle, çalışmaların sonuçlarından herhangi bir ticari kazanç beklememeyi taahhüt ediyorsunuz. Bu çalışmaya katılmak için herhangi bir ücret ödemeniz de gerekmemektedir. Tükürük vermeden önce aç olmanız ya da herzaman almakta olduğunuz ilaçları kesmeniz de gerekmemektedir.

Bu araştırmaya katılmamakta veya imzaladıktan sonra herhangi bir zamanda sebep belirtmeksizin ayrılmakta özgürsünüz.

Bu çalışmada yer aldığınız takdirde elde edilecek tıbbi bilgiler, her zaman kesinlikle gizli kalacaktır. Bununla birlikte kayıtlarınız İstanbul Üniversitesi Cerrahpaşa Tıp Fakültesi Romatoloji Bilim Dalı ve fakültenin Etik Komitesinin incelemesine açık olacaktır. Araştırmada elde edilecek bilgiler yurt içi ve yurt dışında bilimsel toplantılarda ve dergilerde açıklanabilir. Ancak herhangi bir rapor ya da yayında hiçbir şekilde kimliğinizi belirtecek bilgi bulunmayacak ve veriler izlenerek size ulaşılmayacaktır. Yürümekte olan çalışmaların genel sonuçları hakkında Dr. Emire Seyahi'den bilgi alabilir ve herhangi bir sorun olduğu takdirde bu doktora 0533 818 42 34 telefon numarasından ulaşabilirsiniz.

Yukarıda gönüllüye araştırmadan önce verilmesi gereken bilgileri içeren metni okudum (veya bu metin bana okundu). Bunlar hakkında bana yazılı veya sözlü açıklamalar yapıldı. Bu koşullarla söz konusu klinik araştırmaya katılmayı kendi rızamla, hiçbir baskı ve zorlama olmaksızın katılmayı kabul ediyorum.

Gönüllünün adı-soyadı: İmzası

Tarih:.....

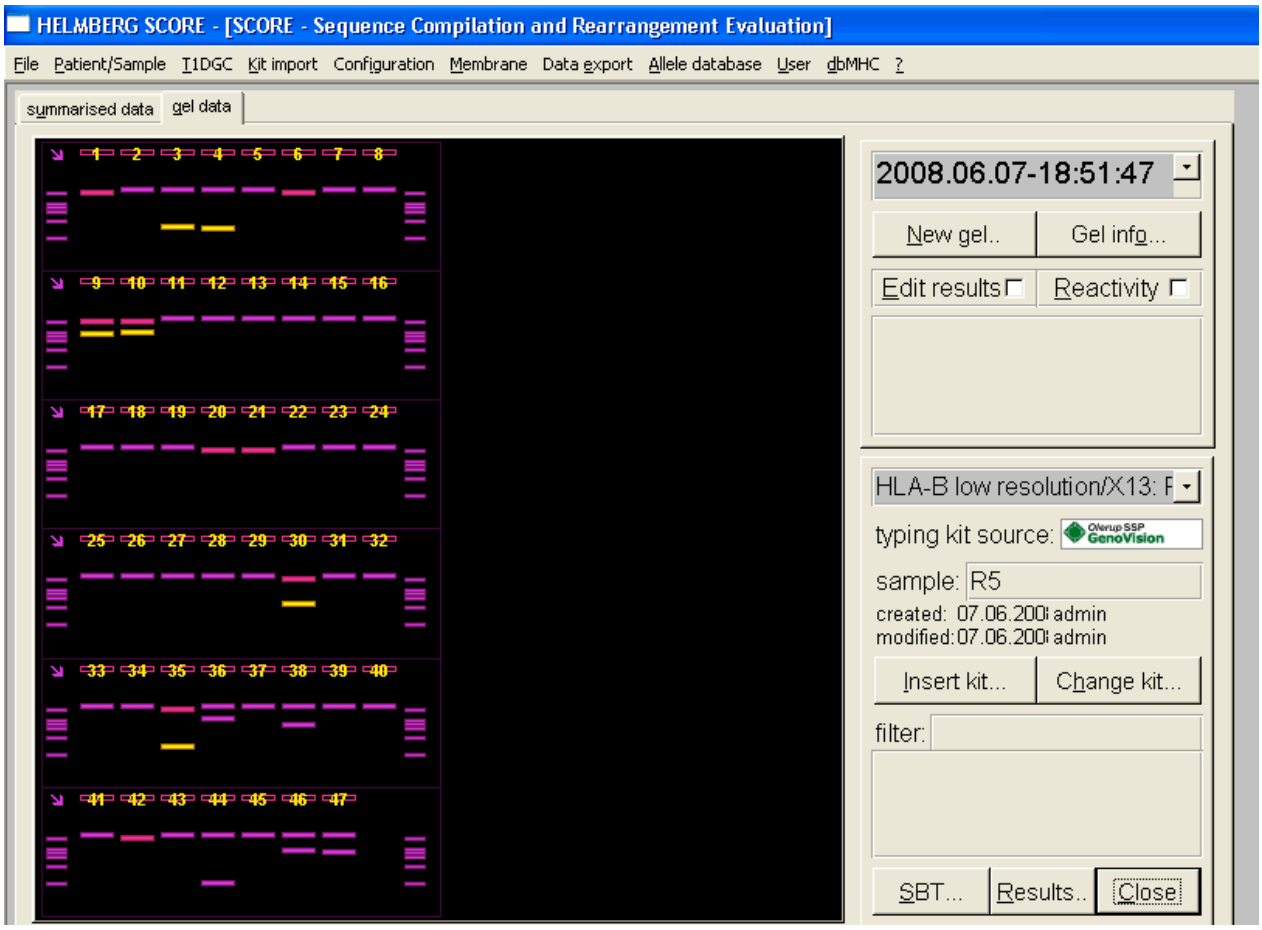
Açıklamaları yapan /örneği alan araştırmacının:

Adı-Soyadı İmzası:

Rıza alma işlemine başından sonuna kadar tanıklık eden kuruluş görevlisinin:

Adı-Soyadı İmzası:

APPENDİX E


HELMBERG SCORE - [SCORE - Sequence Compilation and Rearrangement Evaluation]
File Patient/Sample T1DGC Kit import Configuration Membrane Data export Allele database User dbMHC ?
summarised data
gel data
2008.06.07-18:51:47
New gel..
Gel info...
Edit results
Reactivity
HLA-B low resolution/X13: F
typing kit source:
sample: R5
created: 07.06.200 admin
modified: 07.06.200 admin
Insert kit...
Change kit...
filter:
SBT...
Results..
Close

APPENDİX F

WORKSHEET

Micro SSP™ Seramates HLA Class I DNA Typing Tray - B5, Lot #002

Name _____ Sample ID _____

B Locus

Reaction Results (mark positive locations)

B5 Alleles	Other Alleles	1/3/5/7/9/11 H	G	F	E	D	C	B	A	2/4/6/8/10/12 H	G	F	E	D	C	B	A
		1	2	3	4	5	6	7	8	9	10	11	-	-	-	-	-
N.C.	Negative Control (betaglobin)	1															
B*5001~04;B*5504/08/14/22;B*5601~04/08~14/16~20	B*070201~04/07~12/14~17/1801~26/28~31/33/35/37/39/41~52/54~62;B*080101~0105/04~05/07~08N/10~19N/21~28/30N~31/33~35;B*1309;B*1401/0201~05/0601~07N;B*15010101~0107/02~08/10/1101~12/14~15/18~21/25~26N/2701~29/31~35/37~40/44~57/60~66/68~72/74~82/84~86/88/90~94N/96~99;B*2708/12/18/26/33;B*350802/19/25~27/46~47/49/56/63;B*39010101~0104/0201~05/07~12/1301~17/20~31/37~43;B*400101~0106/0201~04/07~12/1401~1403/18/20~22N/24~25/27/29~43/45~46/48~50/52/54~69/71~74/77~80;B*4101~02/0301~04/06~08;B*4201~02/0501~09;B*4409/46;B*4601~07;B*460101~12;B*4702;B*480101~02/0301~04/06~14/16~17/19;B*670101~0102;B*7301;B*8101~03;B*8201~02;B*8301;B*9501~14/16~39											11					
B*510101~0107/03/11N/14/16/18/21/24~30/32~33/38~39/41N/43/48~49/51~52			2	3	4	5	6				10						
B*510108/50;B*5621			2			5	6				10						
B*510201~0202/23/34			2	3	4	5					10						
B*510203/10/17/36/40			2	3	4						10						
B*5104/06/1301~1302/35/46				3	4	5	6				10						
B*5105/08/0901~0902/19~20/31			2	3	4		6				10						
B*5107/22;B*5808			2		4	5	6				10						
B*5112			2	3	4	5	6										
B*5115/37/42/45;B*530101~02/04~08/ 14	B*3806~07;B*4406			3	4						10						
B*5144N			2		4		6				10						
B*520101~0104/05/0601~09			2		4	5	6	7	8		10						
B*5202			2			5	6	7			10						
B*5203			2		4			7	8		10						
B*5204			2		4	5	6	7	8								
B*5210			2		4		6	7	8		10						
B*5211			2		4	5		7	8		10						
B*5303/09/11~13;B*5607;B*570101~0103/0301~07/09~11/13~16	B*0727/36;B*0802;B*1301/0201~07N/08Q/10~14/17~19/21;B*1513/23/36/43/89/95;B*1809;B*2701/03/0401~07/09~11/13~17/19/21~23/25/27~29/31~32/34~39;B*3706/08;B*380101~0102/0201~05/08~09/11~17;B*4047;B*44020101~0204/0301~05/07~08/10~17/19N~24/26~45/47~49/51~59;B*47010101~010102/03~05;B*4902;B*9515										10						
B*5310				3													
B*5401~02/04~06N/07~08N/10~11/13			2														
B*5412			2								10						
B*550101~0104/03/05/15/17/21/25/28~29	B*1542		2			5						11					
B*550201~0202/07/10/12~13/16/18~20/23/26~27;B*5615	B*0809;B*1583;B*390601~0602/34;B*40060101~0602/44/63/70/75; B*4204		2									11					
B*5509/11/24	B*070501~06/32/34/40/53;B*0806/20;B*1509;B*3918/35~36;B*4005/15~16/23/26;B*4805/15					5						11					

WORKSHEET

Micro SSP™ Seramates HLA Class I DNA Typing Tray - B5, Lot #002

Name _____ Sample ID _____

B Locus

		1/3/5/7/9/11								2/4/6/8/10/12							
		H	G	F	E	D	C	B	A	H	G	F	E	D	C	B	A
Reaction Results (mark positive locations)																	
B5 Alleles	**Other Alleles**	1	2	3	4	5	6	7	8	9	10	11	-	-	-	-	-
B*560501~0502			2			5	6					11					
B*5606;B*7801/0201~ 03			2			5	6			9		11					
B*5702	B*1315						6				10						
B*5712	B*3932~33						6					11					
B*5801~07/09~16					4						10						
B*5901~02			2	3							10						
B*7804	B*3560		2							9		11					
B*7805			2			5	6		8	9		11					
	B*0738;B*0803			3							10						
	B*0832;B*180101~08/10~11/13~25/27~28;B*350101~0106/0201~03/0401~07/0801/0803/0901~0902/11~12/1401~15/17/2001~24/29~43/45/48/50~55/57~59/61~62/64~68/70~79/82~86;B*3919									9		11					
	B*1316/20;B*2720/24;B*3810					5					10						
	B*1516/170101~1702/24/67/87;B*2702/30;B*4013/19;B*4418/25/50;B*4818;B*4901/04~05							7			10						
	B*1530/58/73;B*4028/51					5	6					11					
	B*1812;B*3510/13/16/28/69/80; B*3711								8	9		11					
	B*1826;B*3518/81						6			9		11					
	B*3544					5	6			9		11					
	B*370101~04/07/09~10/12~13								8		10						
	B*3705								8			11					
	B*4903				4			7	8		10						

APPENDIX G

Sample Questionnaire

Tarih:.....

Ad Soyad:.....

Kod:.....

Doğum Tarihi:.....

Cinsiyet:.....

Doğum yeri:.....

1) Şimdiye kadar size Behçet hastalığı tanısı kondu mu?

a) Evet b) Hayır c) Emin Değilim

2) Şimdiye kadar size Ailevi Akdeniz Ateşi hastalığı tanısı kondu mu?

a) Evet b) Hayır c) Emin Değilim

3) Tekrarlayan ağız yaraları oluyor mu? (Senede 3'ten fazla)

a) Evet b) Hayır c) Emin Değilim

4) Cinsel bölgenizde tekrarlayan yaralar çıkıyor mu?

a) Evet b) Hayır c) Emin Değilim

5) Tekrarlayan ateşli veya ateşsis karın ağrısı nöbetleri olur mu? (2-3 gün süren, genellikle yatak istirahati gerektiren)

a) Evet b) Hayır c) Emin Değilim

6) Tekrarlayan göğüs ağrısı nöbetleri olur mu? (2-3 gün süren, nefes almayı zorlaştıran)

a) Evet b) Hayır c) Emin Değilim

7) Eklemlerinizde 2-3 güren süren kırmızı ağrılı şişlikler olur mu?

a) Evet b) Hayır c) Emin Değilim

APPENDIX H

SAMPLE PCR AND RESTRICTION ENZYME RESULTS

Figure H.1 E148Q PCR results

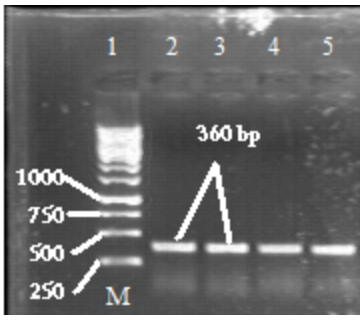


Figure H.2 E148Q Restriction Enzyme Digestion Results

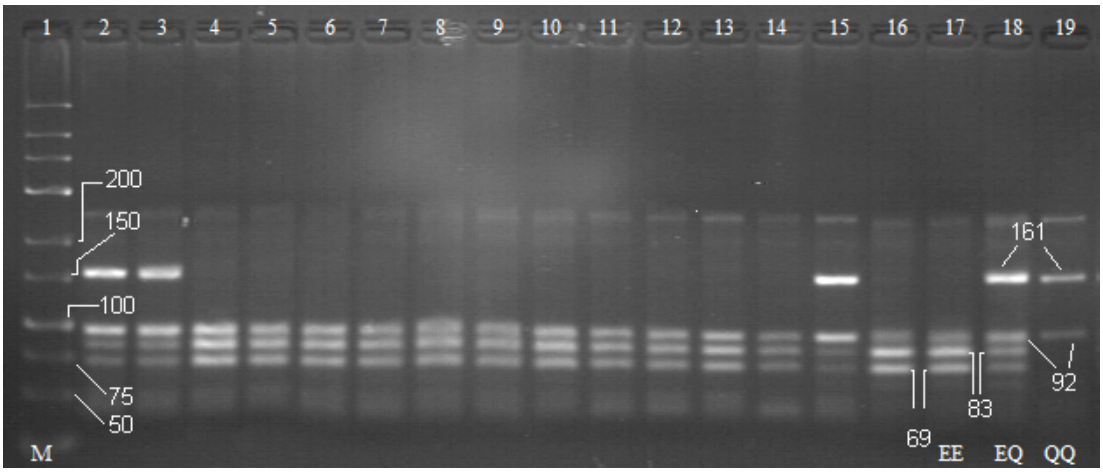


Figure H.3 M680I PCR results

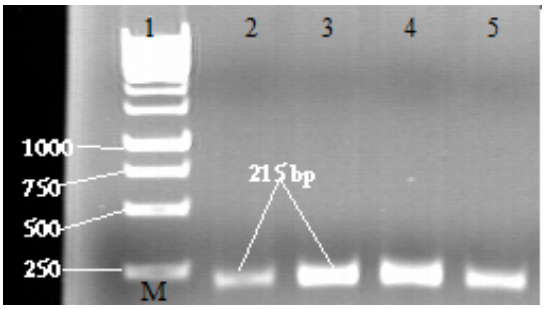


Figure H.4 M680I Restriction Enzyme Digestion Results

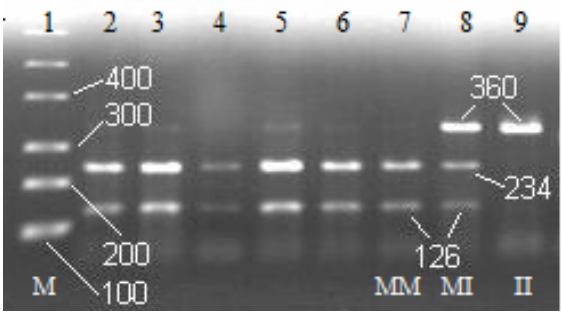


Figure H.5 M694V PCR results

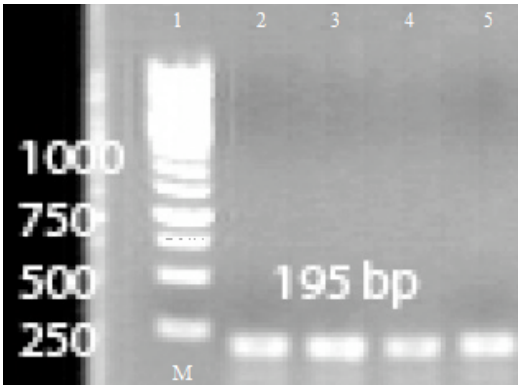


Figure H.6 M694V Restriction Enzyme Digestion Results

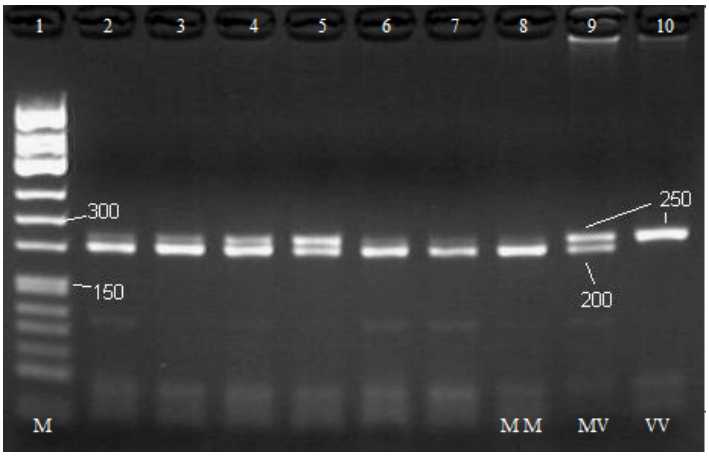


Figure H.7 M694I PCR results

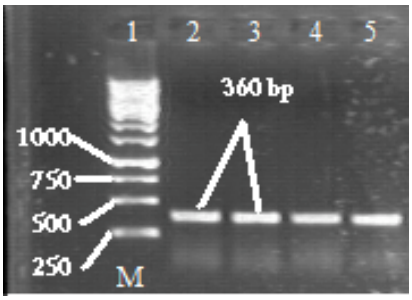


Figure H.8 M694I Restriction Enzyme Digestion Results

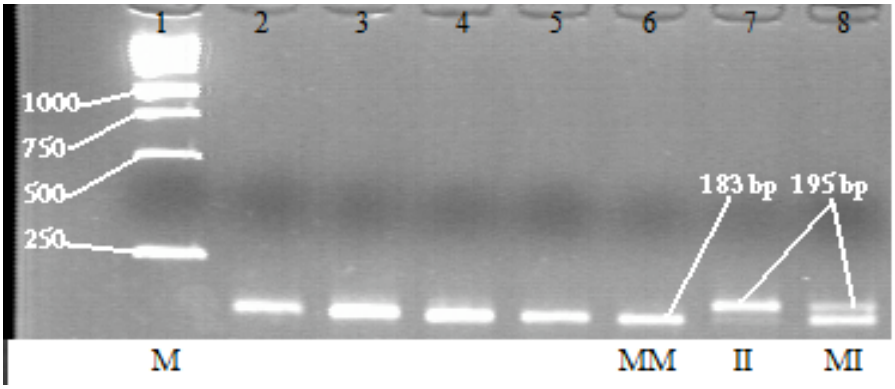


Figure H.9 V726A PCR results

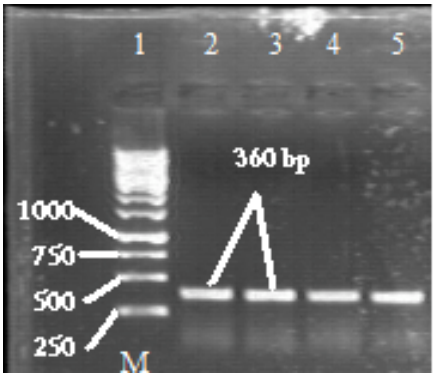
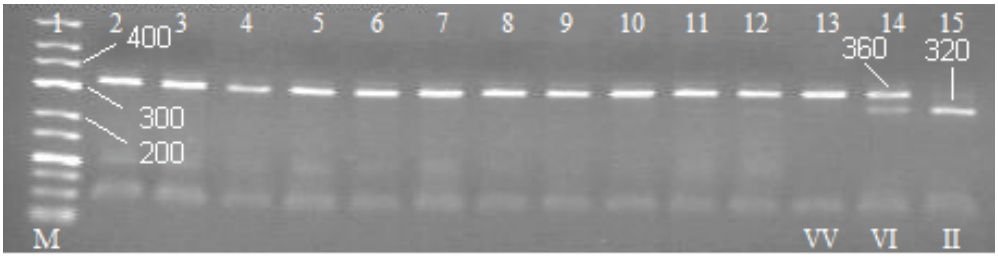


Figure H.10 V726A Restriction Enzyme Digestion Results



CURRICULUM VITAE

Gökçe Çelikyapı was born in Bursa, in 1980. She graduated from Notre Dame de Sion and enrolled to the Montpellier II University, in France for DEUG programme in Life Sciences, in 1999. Then she enrolled to Biology Programme of Istanbul University in 2003. She graduated from Biology Programme of Istanbul University in 2006 and started to M.Sc. degree education in Molecular Biology - Genetic and Biotechnology Program of the Istanbul Technical University.