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GENETIC AND EPIGENETIC ANALYSES OF HLA-B5 GENE IN FAMILIAL BEHÇET SYNDROME PEDIGREES

M.Sc. THESIS

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AİLESEL BEHÇET SENDROMU AİLELERİNDE HLA-B5 GENİNİN GENETİK VE EPİGENETİK ANALİZLERİ

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To my dear parents and friends,

FOREWORD

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Mayıs 2015

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ABBREVIATIONS

ADP : Adenosine Diphosphate
ASM : Allele Specific Methylation
ATP : Adenosine Triphosphate
BS : Behçet's Syndrome
CD : Classification Determinant
DBP : DNA Binding Proteins
DNA : Deoxyribonucleic Acid
DNMT : DNA Methyltransferases
DNTP : Deoxynucleoside Triphosphate
DZ : Dizygotic
EDTA : Ethylenediaminetetraacetic Acid
GWAS : Genome-wide Association Study
HAT : Histone Acetyltranferases
HDAC : Histone Deacetylases
HIV : Human Immunodeficiency Virus
HLA : Human Leucocyte Antigen
ICR : Imprinting Control Genes
LINE : Long Interspersed Nuclear Elements
MHC : Human Histocompatibility Complex
MiRNA : Microribonucleic Acid
MRNA : Messenger Ribonucleic Acid
MSRE : Methylation Specific Restriction Enzyme
MZ : Monozygotic
OD : Odds Ratio
PCR : Polymerase Chain Reaction
RISC : RNA Induced Silencing Complex
RNA : Ribonucleic Acid
SNP : Single Nucleotide Polymorphism
TGF : Tumor Growth Factor
TNF : Tumor Necrosis Factor
XCI : X-chromosome Inactivation

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GENETIC AND EPIGENETIC ANALYSES OF HLA-B5 GENE IN FAMILIAL BEHÇET SYNDROME PEDIGREES

SUMMARY

Behçet's Syndrome (BS) is a chronic multisystemic inflammatory disorder, primary symptoms of which are oral and genital ulceration and uveitis. The disease is also chracterized by inflammation in tissues and organs throughout the body such as vessels, lungs, kidneys, joints, gastrointestinal and central nervous system. BS usually occurs in second and third decades of the life with a severe disease course. It exists most commonly in Mediteranean and Middle East populations. In Turkey, frequency of the disease changes between 20 and 420 of 100.000.

BS risk factors include genetic predisposition which is mainly based on familial aggregation and increased HLA-B51 carrier rate as well as infectious agents, environmental causes and yet undefined immunological mechanisms. Even though HLA-B51 allele has shown to be the most associated marker for BS, the presence of healthy individuals who carry HLA-B51 allele and similarly BS patients without HLA-B51 allele have led us question other mechanisms for BS causation.

Epigenetic mechanisms have gained exceeding importance for their effects on DNA and chromatin structures without altering DNA sequence. DNA methylation is one of the mostly studied epigenetic mechanisms. HLA-B gene has shown to comprise a CpG island (CpGi) which span 1346 nucletides where CG ratio is 66.6 %, therefore methylation of cytosine residues may lead to a change in gene expression levels.

We previously investigated global methylation and HLA-B locus specific methylation in a group of 4 MZ BS twins and 4 DZ BS twins; which suggested an involvement of an epigenetic regulation in HLA-B exon 1-2 region. BS twins had a higher methylation levels for HLA-B gene (p=0.0024). In this thesis we wanted to further our preliminary findings in familial BS cases and more directly examine the influence of HLA-B51 carrier rate and its methylation upon the presence of the clinical phenotype. Therefore, involvement of genetic and epigenetic roles of HLA-B51 region in BS would be better analysed and the sample size would be increased by including familial BS cases with their affected and unaffected relatives. 850 BS patients were contacted from Cerrahpaşa Medical Faculty, Rheumatology Polyclinic between 2013 and 2015 and asked if they have an affected relative. Within those 850 index patients, 150 patients had a family member with BS. Among those, 15 families accepted to enroll in the study. So the study consists of 15 index patients; 17 affected relatives and 26 unaffected relatives. Peripheral blood samples were collected and genomic DNA was isolated from leukocytes. Patients and relatives were genotyped for HLA-B51 alleles using sequence specific PCR method. HLA-B51 positivity ratio was found to be 12/15 for index patients, 13/17 for affected relatives , 22/26 for unaffected relatives. Among BS and healthy family members, HLA-B51 frequency did not show a statistically significant diffrence. For healthy controls, who are not related with families, HLA-B51 positivity was 8/25. However, HLA-B51 positivity

for BS patients was statistically higher when compared to healthy controls *(p*=0.0005*).* After determining HLA-B51 positivity, methylation profiles on HLA-B gene were analyzed and compared between groups using Real-time PCR based OneStep qMethyl Kit. For exon-1 and exon-2 region of HLA-B gene, BS patients had statistically higher methylation levels compared with healthy individuals in the family (*p*=0.0065). Methylation levels were not significantly different among HLA-B51(+) and HLA-B51(-) individuals for index patients, relatives with BS and healthy relatives. Observed results were also correlated with the data from our previous studies. These findings suggested us a room for epigenetic modifications which might be independent from HLA-B51 positivity.

AİLESEL BEHÇET SENDROMU AİLELERİNDE HLA-B5 GENİNİN GENETİK VE EPİGENETİK ANALİZLERİ

ÖZET

Behçet Sendromu, vücuttaki birçok doku ve sistemin iltihaplı tutulumuyla öne çıkan, kronik romatolojik bir hastalıktır. Başlıca belirtileri ağızda ve cinsel organda sıklıkla tekrar eden yaralar, vücutta sivilceler ve kızarıklıklardır. Bununla beraber, eklemlerde, dolaşım sisteminde, merkezi sinir sisteminde, gözün görme tabakasında, sindirim sisteminde ve akciğer, böbrek gibi organlarda iltihaplanma görülmektedir. Hastalık genellikle daha ağır bir seyirle 20'li ve 30'lu yaşlarda başlar. Behçet Sendromu'na, İpek Yolu olarak tanımlanan, Akdeniz ve Orta Doğu popülasyonlarının da üzerinde bulunduğu güzergahta sık rastlanmaktadır. Türkiye'de ise hastalığın sıklığının, 100.000' de 20 ile 420 arasında değiştiği gösterilmiştir.

Hastalık etkenleri, ailesel birikim ve Behçet Sendromlu ailelerde artan HLA-B51 sıklığı gibi indikasyonlar ile birlikte enfeksiyonel ajanlar, çevresel faktörler ve henüz tanımlanmamış immünolojik mekanizmalar olarak öne sürülmüştür. Bunların içinde HLA-B geninin bir alt aleli olan HLA-B51 aleli, bugüne kadar Behçet Sendromu ile ilişkilendirilmiş en önemli işaretlerden biridir.

HLA-B geni 6. Kromozomun p(kısa) kolunda 21.3 ile tanımlanan bölgesinde bulunmaktadır.HLA-B, insan lökosit antijeni (HLA) denilen kompleks grubun başlıca genlerindendir. Görevi bağışıklık siteminde T hücrelerine antijen tanıtımını sağlamaktır. HLA-B geninin birden çok aleli bulunmaktadır. Bu alellerden Behçet Sendromu ile bağlantısı bilinen aleller HLA-B51, HLA-B15, HLA-B27 ve HLA-B57 olarak gösterilmiştir. Fakat bunların içinden HLA-B51 aleli günümüze kadar yapılan çalışmalarda, hastalık ile en çok bağlantılı bulunan alel olmuştur. HLA-B51 alelinin 89 dan fazla alt tip aleli bulunmaktadır. Günümüzde, HLA-B 51 alelinin HLA-B5101 ve HLA-B5108 gibi bazı alt alellerinin Behçet Sendromlu hastalarla bağlantısı olduğu gözlemlenmiştir. Genom genelinde yapılan bağlantı çalışmalarında, IL-10, IL-23R, IL-12A, MICA, STAT4 gibi farklı genlerinde Behçet Senromu ile ilişkili olduğu bulunmuştur.

Tüm bu bulgulara rağmen, HLA-B51 alelini taşıyan sağlıklı bireylerin varlığı, aynı şekilde HLA-B51 alelini taşımayan Behçet hastalarının varlığı, hastalığın oluşumunda başka mekanizmaların olabileceğini düşündürmüştür.

DNA metilasyonu ve kromatin modifikasyonları gibi DNA dizisinde herhangi bir değişime sebep olmadan gen aktivitesini değiştiren epigenetik modifikasyonlar, tanımlanamayan kompleks hastalıkların nedenlerinin aydınlatılmasında önemli rol kazanmaktadır. Epigenetik modifikasyonlar içinde ise DNA metilasyonu en sık çalışılan regülasyon mekanizmalarından biridir. HLA-B geninde, 1346 nükleotit

uzunluğundaki CpG adacığının varlığı ve bu bölgede CG dinükleotitlerinin %66.6 oranında yoğun olarak kümelendiği gözlemlenmiştir. CpG adacığında bulunan sitozin ve guanin bazlarının metillenmesi ile gen aktivitesinin dolayısıyla ekspresyonun değişmesi beklenmektedir.

Yaptığımız öncül araştırmalarda, 4 monozigotik ve 4 dizigotik Behçet Sendrom'lu ikizlerin oluşturduğu çalışmamızda HLA-B geninin 1. ve 2. ekzonlarının epigenetik regülasyonunu düşündüren bulgulara rastladık. Bu çalışmada ise ailesel Behçet Sendromu vakaları ile birlikte ailelerinde bulunan diğer Behçet Sendrom'lu bireyler ve sağlıklı aile bireyleri de incelenmek istenmiştir. Bu sayede, HLA-B51 alelinin genetik ve epigenetik etkilerinin daha iyi anlaşılması planlanmıştır. 2013-2015 yılları arasında Cerrahpaşa Tıp Fakültesi Romatoloji Polikliniği'ne tedavi olmaya gelen hastalar içerisinden irtibat kurulan yaklaşık 850 Behçet Sendrom'lu bireyden, ailesinde en az bir BS tanısı konulmuş akrabası bulunan 150 Behçet Sendrom'lu bireye ulaşılmıştır. İletişime geçilen 150 Behçet Sendrom'lu aileden 15 aile çalışmamıza katılmayı kabul etmiştir. Araştırma grubumuz toplamda 15 Behçet Sendrom'lu birey, ailelerindeki Behçet Sendrom'lu 17 akraba ve yine ailelerindeki 26 sağlıklı akrabalardan oluşmaktadır. Hastalardan ve ailelerinden alınan periferal kan örneklerindeki lökositlerden genomik DNA izolasyonu yapılmıştır. Çalışma grubunda bulunan tüm bireyler, bölge spesifik Polimeraz Zincir Reaksiyonu yöntemiyle HLA-B51 aleli için genotiplenmiştir. Yapılan çalışmalar sonucunda, HLA-B51 pozitifliği, indeks hasta grubu için 12/15, hasta akrabalar için 13/17 ve sağlıklı akrabalar için 22/26 olarak hesaplanmıştır. 25 kişiden oluşan aile dışı sağlıklı kontrollerde ise HLA-B51 pozitifliği 8/25 oranında bulunmuştur. HLA-B51 genotiplendirmesi sonrasında, HLA-B geninin 1.ekzon, 1.intron ve 2.ekzon bölgelerinin metilasyon seviyeleri Real-time PCR temelli OneStep qMethyl kiti kullanılarak analiz edilmiştir. HLA-B genine ait ekzon-1, intron-1 ve ekzon-2 bölgelerini içeren metilasyon analizlerinde, indeks hasta grubu ve ailelerindeki diğer Behçet Sendrom'lu ve sağlıklı bireyler arasında yapılan karşılaştırmada, tüm Behçet Sendrom'lu bireylerdeki metilasyon oranlarının sağlıklı akrabalarına göre anlamlı olarak yüksek olduğu görülmüştür (p=0.0065). Fakat tüm gruplar ayrı ayrı incelendiğinde, HLA-B51 taşıyıcılığının metilasyon seviyeleri üzerinde anlamlı bi fark yaratmadığı ortaya çıkmıştır. Elde ettiğimiz sonuçlar, Behçet ikizleriyle yaptığımız önceki çalışmamızın sonuçlarıyla tutarlı olarak bulunmuştur. Bu veriler ışığında, HLA-B51 alelinden bağımsız olarak muhtemel epigenetik mekanizmaların, hastalığın oluşumunda etkili olabileceği düşünülmüştür.

Bu çalışmanın devamı niteliğinde, epigenetik mekanizmaların değişken olabildiği göz önünde bulundurularak, Behçet Sendrom'lu ailelelerdeki sağlıklı akrabalar, ortaya çıkabilecek çeşitli Behçet Sendromu benzeri bulgular açısından takip altında tutulmalıdır. Aynı şekilde HLA-B bölgesinde saptanmış DNA metilasyon farklılıklarının gen ekspresyonuna etkisinin görülmesi amacıyla, aynı bölge için gen ekpresyonu analizleri yapılmalıdır.

Bu çalışmalara ek olarak, münferit Behçet vakalarında da aynı şekilde, HLA-B51 genotiplemesi ve HLA-B bölgesi için DNA metilasyon analizleri tekrarlanmalıdır. Bu sayede ailesel ve münferit vakalarda HLA-B51 sıklığı ve HLA-B genindeki DNA

metilasyon farklılıkları gözlemlenebilir.

Gelecekte yapılacak çalışmalar için, HLA-B51 aleline spesifik metilasyonun araştırılması, gen içerisinde ya da genler arasında etkili olabilecek faktörlerin anlaşılmasında yararlı olacaktır. Buna ek olarak, HLA-B5101 ve HLA-B5108 gibi alt alel farklılıkları, Behçet Sendrom'lu bireyler arasında saptanarak, alt alel değişimlerinin hastalık üzerindeki etkisi gösterilebilir.

1.INTRODUCTION

1.1 Behçet's Syndrome

From ancient Greece, descriptions of an illness which has similarities to BS was found in Hippocrates' writings in 450 B.C. However in 1937, Behçet's Syndrome (BS) was firstly described by Hulusi Behçet as a chronic multisystemic inflammatory disorder, the hallmarks of which are oral and genital ulceration and uveitis (Yurdakul and Yazıcı, 2008). Since the disease is well known through its wide spectrum of clinical features and its recurrent acute inflammation, it is also characterized with the systemic vasculitis of small and large vessels including thromboplebitis, gastrointestinal and central nervous system involvement and epididymitis (Yurdakul et al., 1988a ; Mizuki et al., 1997). Inflammation in tissues and organs throughout the body such as lungs, kidney and joints are other commonly observed BS symptoms (Misushima et al., 1988).

BS occurs most commonly in the second and third decades of life. Young patients and male patients have a severe disease course with regard to the ocular, vascular and neurological effects with increased mortality (Yazici et al., 1984). BS usually follows a remission phase in many patients (Kural-Seyahi et al., 2003).

The diagnosis of BS is based on the recognition of a group of clinical features. Furthermore, pathergy test is still a reliable method for diagnosis together with clinical examination. Even though pathergy positivity is specific to Behcet's patients; its sensitivity depens on different ethnicities (Tüzün et al., 1979). Despite 60–70% of patients in Turkey and Japan have a positive pathergy test, it is rarely observed in patients with BS from Northern Europe and North America (Yazıcı et al., 1984).

Exact causes of BS are still not known for sure. Genetic factors, infectious agents, environmental causes, immunological mechanisms have postulated as risk factors. It is clearly evident that both genetic and environmental factors play role in the causation of the disease with a complex mode of inheritance. Among those, HLA-B*51 allele has shown to be major susceptibility gene. Nonetheless, it is not precisely known if HLA-B*51 is pathogenic gene itself or genetic predisposition

causes the disease when environmental factors trigger. Also HLA-B*51 allele has shown to be in linkage disequilibrium with other genes (Muzuki et al., 1997; Azizlerli et al., 2003).

1.2 Epidemiology of the disease

Behçet's disease has a worldwide distribution but epidemiological reports show that it occurs most frequently in Asian, Mediteranean and Middle East populations; geographically this suggests a possible relationship to the Silk Road, extending from Japan to the Mediterranean and Middle Eastern countries including Turkey and Iran (Michelson and Friedlaender, 1990). Due to this geographical association, it was named after 'Silk Road Disease' (James et al., 1986). The relation of BS with the Silk Road, and the distribution of HLA-B51 along this route provides important clues to the cause of this disease (Table 1.1) (Yurdakul et al., 1988; Piga and Mathieu, 2011).

Table 1.1: Prevalence of BS and its association with HLA-B51 in patients of different world populations NR: Not reported. (adapted from Piga and Mathieu, 2011).

Population(Region)	Prevalence (per 100.000)	HLA-B frequency in BS patients (%)
TURKEY		
İstanbul	420.0	NR
North-Eastern Area	370.0	26
North-Western Area	20.0	NR
ISRAEL		
Druze-Northern Region	146.4	100
Arab-Taibe City	120.0	83
Arab-Northern Region	26.2	81
Nationwide	15.2	NR
Jewish-Northern Region	8.6	72
JAPAN		
Hokkaido Region	30.5	NR
Nationwide	13.5	59
SAUDI ARABIA		
Al Quassim Region	20.0	72
Al Quassim Region	20.0	72
GERMANY		
German(West Berlin)	0.55	36
Turkish(West Berlin)	20.75	75
IRAQ		
Nationwide	17.0	62
CHINA		
Nationwide	14.0	56
EYGPT		
Alexandra Region	7.6	58
SPAIN		
Nationwide	7.5	37
KOREA		
Nationwide	7.3	53
ITALY		
Reggio Emilia Area	3.8	57
GREAT BRITAIN		
Yorkshire	0.63	13
Scotland	0.27	18

Numerous studies have shown that Turkey has high prevalence rates of BS. With the numbers of 420/100,000 in İstanbul (Azizlerli et al., 2003). In one of these studies the prevalence of BS in northern Turkey was found to be 380/100,000 (Yurdakul et al., 1988). In another regional study, the prevalence of BS was found to be 41/100,000 in the population of southern Turkey (Acar et al., 1989). A study in Saudi Arabia revealed a prevalence of Behçet's disease of 20/100,000 (Al-Dalaan et

al., 1997). Prevalence of Behçet's disease in the Japanese population diminishes as 7–8.5/100,000 (Shimizu et al., 1979). Lower rates of occurrences are seen in Australia, Africa, Germany and in the United States (Figure 1.1) (Michelson and Friedlaender, 1990).

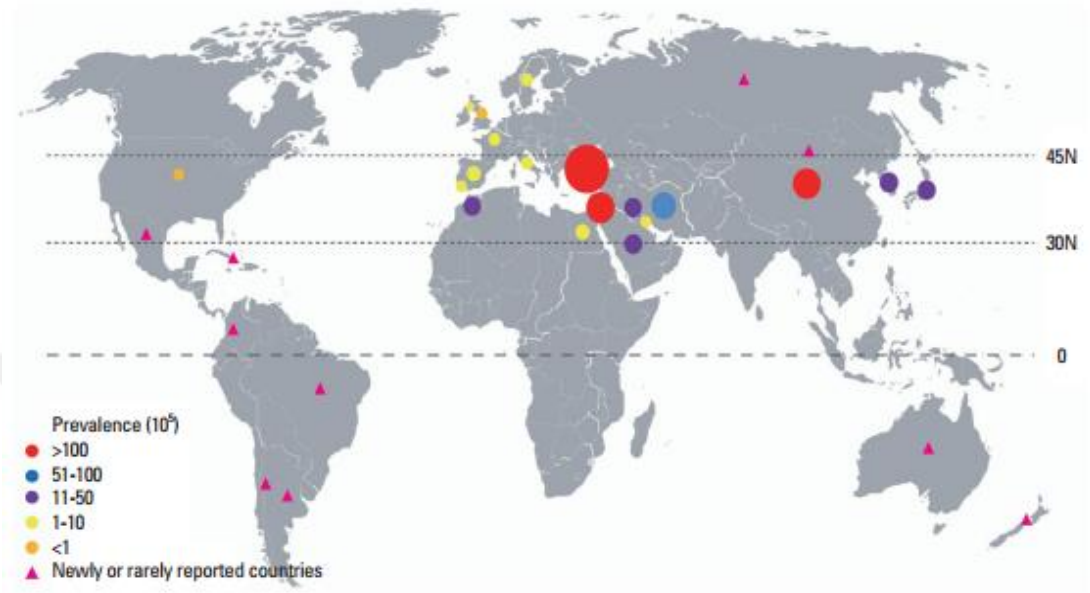

Figure 1.1: Global Distribution of Behçet's Syndrome.Dot size reflects prevalence (Cho et al., 2012).

1.3 Classical Genetic Studies

Classic genetic studies are perfomed to find out relative contributon of genetic and environmental factors on the disease phenotype using heritability estimates in familial cases and twins (Verity et al., 2003).

1.3.1 Familial Aggregation

Most cases of BS are sporadic and the relatives of patients are unaffected, whereas familial aggregation of BS has been reported less frequently (Figure 1.2) (Kone-Paut et al., 1999). Familial aggregation has been considered as evidence supporting genetic influence on the pathogenesis of BS. The familial recurrence of BS does not display a Mendelian pattern (Fietta et al., 2005).

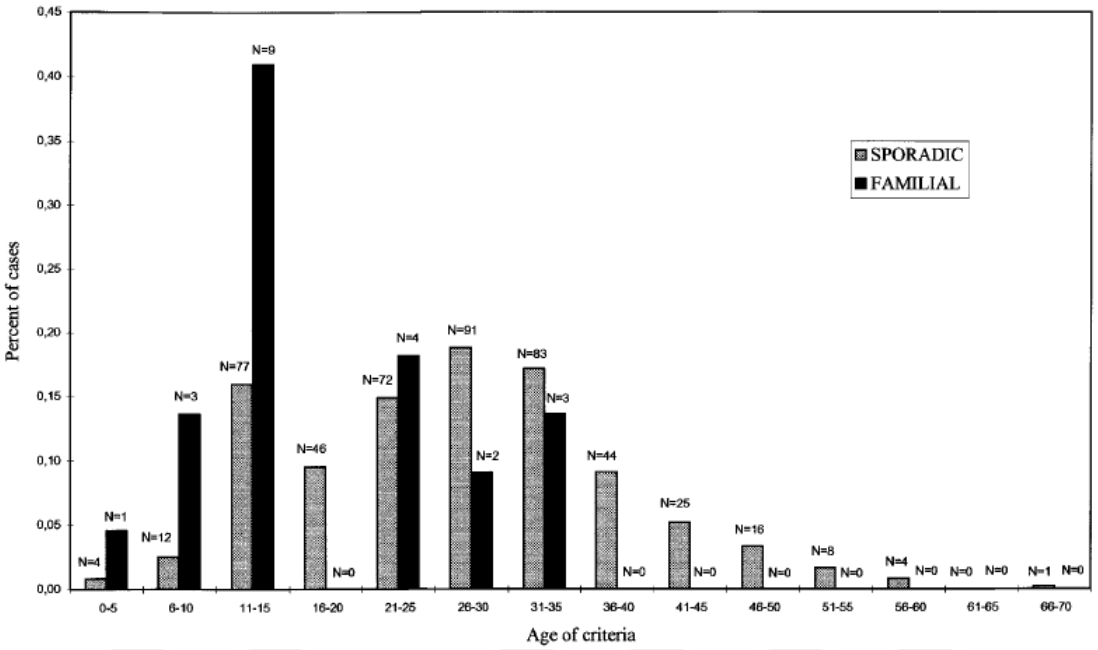

Figure 1.2: Distribution of familial and sporadic cases as a function of age of attending diagnostic criteria (Kone-Paut et al., 1999).

The familial aggregation of BS has variable frequency in different ethnic groups. It is higher in Turks (18.2%), Koreans (15.4%) and Jews (13.2%)than in Chinese Japanese and Europeans(which are approximately %1-2) (Zouboulis, 1999). In another study it was observed that familial clustering is significantly more common in juvenile than adult patients (Kone-Paut et al., 1999; Gül et al., 2000). Recurrence mostly occurs between siblings, but also mother/son, mother/daughter, father/son, father/daughter, cousin/cousin and uncle/nephew cases, and even a four generation BS family has been reported (Berman et al., 1979; Nıshıyama et al., 2002; Bang et al., 1997). From Turkey, a study which included seven BS families also proven that HLA-B5 frequency is not coincidental among BS families (Dündar et al., 1985). In familial BS, the HLA-B*51 positivity is much more common than in sporadic cases, being 68-83.3 % in Turkish studies. In Japanese reports, it ranges from 60.9 % to 92% (Nıshıyama et al., 2002; Nıshıura et al., 1996; Gül et al., 2001). According to that, in Turkish studies, the sibling recurrence risk ratio was determined to be 11.4-52.5 which support a hereditary base for the disease (Gül et al., 2000).

1.3.2 Twin Studies

Comparison between monozygotic(MZ) and dizygotic(DZ) twin groups enables how the phenotypic variability is influenced by the contribution of genetic and both shared and nonshared environmental factors. Monozygotic twins show different

degrees of concordance which range between 5%-75% for complex diseases (Nance and Alan, 1981). Higher concordance rates for MZ twins indicate higher genetic predisposition whereas persistance of discordance for MZ twins point out non-genetic factors. Variable concordance rates in genetically identical individuals could be explained by the influence of both external and internal factors (Jaenisch and Bird, 2003). In the literature, there are instances of both monozygotic twins concordant for BS and monozygotic twins discordant for BS (Hamuryudan et al., 1991; Gul et al., 1997).

Our previous twin research by Masatlıoğlu et al. (2010) also indicates that concordance rates differ among twins. In the study, which consisted of 14 BS twins (6 monozygotic and 8 dizygotic), pairwise concordance rate for BS was 2/6 in monozygotic twins whilst in dizygotic twins it was 1/8. Amongst twins, heritibility of BS was estimated to be 41%.

1.4 Molecular Genetic Studies

By the studies have been designed with BS patients of many ethnic groups, specifically those from the Middle to Far East including Turks, Greeks, Italians, French, English, Tunisians, Israelis, Jordanians, Iranians, Saudi Arabians, Kuwaitis, Han Chinese, Koreans, Taiwanese and Japanese, the association between BS and HLA-B51 has been approved. One of the very early examples of HLA-B5 association to BS is a study by Yazıcı H. et al. in which HLA-B51 positivity was found 84% in BS patients and 27 % in healthy controls (Yazıcı et al., 1977). Thus, HLA-B51 is accepted as the most associated marker with BS due to 40-80 % frequency. However it is shown to be decreased as 13% for BS patients with different ethnicities (Mizuki et al., 2007).

A meta-analysis by Menthon et al. (2009) which consisted of 4,800 BS cases and 16,289 controls revealed that the pooled prevalence for HLA–B51/B5 in BS cases were 57.2% and for control populations were 18.1%. In addition to that the odds ratio of HLA–B51/B5 carriers developing BS was increased by a factor of 5.78. (95% CI=5.00–6.67). To determine the contribution of HLA–B51 to BS development in the overall population, population-attributable risks(PAR) were calculated using pooled odds ratios for geographic grade. The results were 44.4% for Eastern Asia, 49.4% for Middle East/North Africa, 52.2% for Southern Europe, and 31.7% for

Northern/Eastern Europe (Menthon et al., 2009). Akpolat et al. (1992), evaluated 27 patients with familial BS in 12 families. Among the 137 patients they studied, they observed a high rate of (68%) HLAB51 positivity in the familial form.

A study from Greece in which 38 patients with BS and 40 control subjects were examined, stated MICA is another susceptibility gene and HLA-B*5101 allele was found in nine controls (22.5%) and in 30 BS cases (81.1%), however MICA-A6 allele was found in 20 controls (50.0%) and in 33 BS cases (86.8%). Statistical analysis of these results displayed that the association of MICA-A6 with BS imputed an OR of 6.60 (p=0.0012; 95% CI=1.92–24.24), whereas B*5101 imputed an OR of 14.76 (p=0.00000028; 95% CI=54.33 to 53.28) (Yabuki et al., 1999).

In several reports, it is indicated that TNF has a critical role on BS pathogenesis such as increased secretion of TNF from monocytes and high TNF production from T cells in BS patients (Mege et al., 1993; Yamashita et al., 1997). In a meta-analysis consisted of Turkish, South Korean, Tunisian, Lebanese and German population samples sustained that polymorphisms in promoter of TNF-α gene at positions -1031C, -857T and -238A are consequent with BS (Touma et al., 2010). A study by Ateş et al. (2010) suggested that TNF-a and IL10 may be regulated by several SNPs of both genes and at the transcriptional level so was associated with changes in expression levels.

In an other study, it was found that BS patients had lower incidence of TGFBR1*6A compared with control subjects (11.3% ,13.3%, respectively). Also, the incidence of TGFB1*CC was lower in cases than control subjects (24.6% , 27.0%, respectively) Results suggested lower incidence of TGFBR1*6A in BS patients might play a protective role against development of disease. Therefore, differences of the results were not statistically significant (Kaklamani et al., 2008).

1.4.1 HLA-B gene

HLA-B gene belongs to MHC(Major Histocompatibility Complex) Class genes which ubiquitiously express highly polymorphic, membrane-bound short peptides (Figure 1.3) (Song and Kang, 2012). These short peptides are presented to T cells to be discriminated whether extraneous or not (Björkman and Parham, 1990). Hence, this gene family influence numerous inflammatory and autoimmune conditions. Also, variants of these MHC genes raise susceptibility to many infectious diseases, such as malaria and HIV (Traherne, 2008).

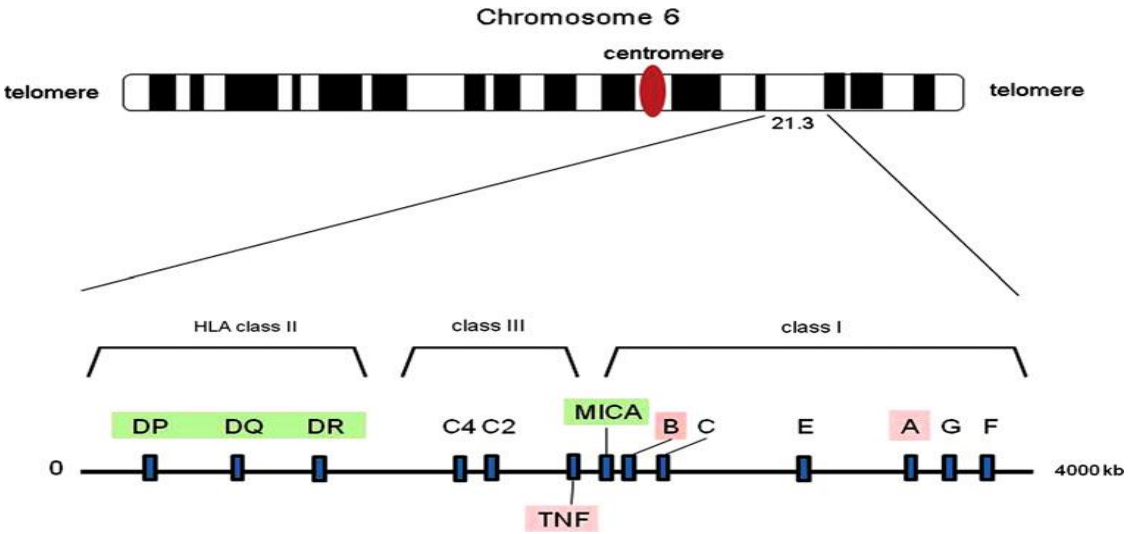

Figure 1.3: Chromosomal mapping of 6p21.3, which contains the major histocompatibility complex (MHC) region (Song and Kang, 2012).

HLA-B gene is 3,341 bps which located on the short (p) arm of chromosome 6 at position 21.3 with the genomic coordinates of 31,321,649 bp and 31,324,989 bp. (ncbi, nih) There are known 3,760 alleles of HLA-B gene which codes for 2,789 proteins and 122 alleles of 3,760 are nulls. Also, HLA-B51 is one of HLA-B alleles. HLA-B51 has approximately 89 suballeles. Among them, HLA-B5101 is the most correlated one with BS (Kaya, 2012).

The primary role of HLA class I antigens such as Bw51 which expressed from HLA-B51 gene is to present endogenous peptides to CD8+ T cells. These antigens which are expressed from HLA class genes have an important role on self-recognition of cells. When a cell becomes infected by a pathogen, proteins presented will be resulting from the breakdown of foreign proteins so recognized as 'non-self'. However, in normal status of the cell, the peptides presented are the breakdown products of normal proteins and they will be recognized as 'self' and will not trigger any immune response. Hence, immune recognition system is in need of different kinds of peptides presented for to be specific for each person (Hurley et al., 2003).

The pathogenetic role of HLA-B51 in BS is yet to be elucidated. However, Bw51, itself may be responsible, at least for neutrophil hyperfunction in BS, since HLA-B51-transgenic mice show enhanced neutrophil function as seen in BS patients, although these mice did not develop the symptoms of BS. Because the lack of the disease phenotype in this mouse model can be explained by the absence of an triggering microbial or injury-related peptide that would activate the disease-relevant

CD8+ T cells (Takeno et al., 1995). Through high percentage of polymorphisms on HLA region, genetic diversity is provided. Fleischhauer et al. (1990) demonstrated that a single amino acid difference in the HLA-B molecule is sufficient for the development of alloreactivity in vivo. Therefore, each variation of this region might result in large scale of genetic diversity which may lead to unknown inflammatory reactions.

1.5 Genome-Wide Association Studies

Disease causations can be learned and new insights can be gained by genome-wide association studies (GWAS) when performed in different populations. The purpose of GWAS is to identify the susceptibility genes in complex diseases by searching more frequent single nucleotide polymorphisms (SNPs) in a group of people with the particular disease compared to healthy group. Several GWAS and genetic polymorphism studies have found new genetic associations with BS, which may have a complementary role in disease susceptibility. A cooperative GWAS identified new genes as associated with BS which are STAT4, CCR1, KLRC4 and ERAP1. ERAP1 was also found to be in epistasis with HLA-B51 (Kirino et al., 2013). In a Class I HLA analysis, HLA-B51 was found to be the strongest susceptibility allele for the development of BS. Interestingly, among the HLA-B51-negative patients, HLA-A26 was found to be the most strongly associated allele with BS (OR =2.96) (Meguro et al., 2010). Fei et al., recognized novel single nucleotide polymorphisms (SNPs) in five candidate genes (KIAA1529, CPVL, LOC100129342, UBASH3B, UBAC2) among 152 BS patients and 172 control subjects with the odds ratios (95% CI) of 2.04, 2.26, 1.84, 1.71 and 1.61 and p values of 4.2×10^{-5}, 1.0×10^{-4}, 3.0×10^{-4}, 1.5×10^{-3}, and 5.8×10^{-3}, respectively (Fei et al., 2009). In a recent GWAS study from Turkey within the MHC Class I region, including 311, 459 SNPs in 1215 BS patients and 1278 healthy controls, the association between HLA-B51 and BS was verfied, besides IL10 (OR:1.45) and IL23R-IL12RB2 locus (OR:1.28) marked to be in association with BS (Remmers et al., 2010). In a supporting GWAS study which includes a Japanese cohort consisting 612 BS patients and 740 unaffected individuals. In that study, a meta-analysis embracing both Turkish and Korean studies showed genome-wide associations for IL23R-IL12RB2 and IL10 with the odds ratios of 1.35 and 1.45, respectively (Mizuki et al., 2010).

1.6 Epigenetics

Irreversible changes in the DNA sequence, including chromosomal deletions and gene mutations, have been liable for initiation and progression of the human diseases until now. All the same, increasing attention is targeted towards epigenetic regulations. Epigenetics involve heritable and reversible changes in the pattern of gene expression without altering the nucleotide sequence. Also, epigenetic information is transferable from one generation to the next both at the cellular level or at the level of the whole organism (Haluskova et al., 2010). Epigenetic mechanisms can be divided into four groups such as DNA methylation, histone modifications, nucleosome positioning and RNA interference (RNAi). Furthermore, Reversible RNA methylation adds a new dimension to the post-transcriptional regulation of gene expression (Fu et al., 2014). Such mechanisms as; X chromosome inactivation, genomic imprinting, cell differentiation, embryogenesis, DNA-protein interactions, regulation of gene and miRNA expression are also regulated by epigenetic modifications (Dupont et al., 2009). As a result of these modifications, genes are repressed or activated.

Epigenetic changes may also occur in response to environmental exposure including embryonic development and adult life. These effects can vary from maternal diet, hormonal changes, nutrients, pharmacologic treatments to toxics (Aguilera et al., 2010). A twin study also revealed that the epigenome varies from tissue to tissue, generating specific identity to each cell type. It is proven that even though monozygotic twins have the same genotype, they do not share the same epigenome (Fraga et al., 2005). Due to the discrepancy of their environmental exposure or other unknown cellular interactions, twins' concordance rates usually differ. The profiles of epigenetic modifications in MZ twin pairs diverge as they become older (Fraga et al., 2005; Jaenisch and Bird, 2003).

In accordance with that, our previous research in which HLA-B51 genotyping and methylation analyses performed among 8 BS twin groups (4 monozygotic and 4 dizygotic) and 8 healthy control twin groups, phenotypic discordance between BS twins were 4/8. Within the pairwise methylation analyses on exon resulted that MZ twins with BS or healthy MZ twins did not correlate with each other. What is more, DZ twins with BS and healthy DZ twins did not correlate with each other, either. BS twins had a higher methylation level, compared to control twins

(p=0.0024) (Özkılınç, MSc. Thesis). Differences in concordances between monozygotic and dizygotic twins represented asset of genetic predisposition. Regardless of that, discordance for monozygotic twins points out non-genetic factors which might be effective**.**

1.6.1 DNA Methylation

DNA methylation is so far the most studied epigenetic modification which has a crucial role for controlling gene expression. In mammals, cytosine methylation is mainly restricted to the CpG context. CpG islands (CpGi) can be described as regions greater than 200 bps where cytosine nucleotides takes place next to guanine nucleotides repetitively, with a minimum of %50 GC content (Aissani and Bernardi, 1991).

DNA methyltransferases (DNMTs) direct methylation process by adding a methyl group to a 5' carbon of the cytosine ring located within CpG dinucleotides. Consequently, gene expresion is repressed in those regions. There are two types of methylation process in eukaryotic cells. First one is de novo methylation which is involved in the rearrangement of methylation pattern during embryogenesis or differentiation in adult cells and controlled by DNMT3a, DNMT3b and DNMT3L enzymes. Whereas, second one as maintenance methylation which is responsible for maintaining the methylation pattern during DNA replication and controlled by DNMT1 enzyme (Okano et al., 1999). In both conditions, methyltransferases have critical role. Especially, in developmental stage, DNMTs are fatally important (Li et al., 1992).

When CpG islands are found at promoters of many housekeeping or developmentally regulated genes, they are essentially hypomethylated. Even CpG islands, most of which are unmethylated at all times in normal cells, can acquire methylation under special developmental circumstances in abnormal cells. In accordance with that, CpG islands that occur at intragenic regions are more frequently methylated. Some studies revealed that in normal cells, most repetitive CpGs within the coding region of genes are methylated, whereas most CpG island (CpGi) regions are remained in an unmethylated state (Illingworth et al., 2010). Besides, methylation of the CpG sites in gene exons is a major cause of C to T transition mutations, leading to disease-causing mutations. Most gene bodies are not CGIs, and when CGIs are situated in intragenic regions, with a few exceptions, thought to remain unmethylated. However,

recent experiments, have changed this perception. It has shown that, even though gene body CGIs can become extensively methylated, this does not block transcription elongation. Most transcription start site associated CpG islands are protected from DNA methylation due to active transcription and formation of nascent RNA helices. In order to silence promoter regions stably, repressive transcription factors are needed. Specifically, at germline gene promoters, DNA methyltransferase 3B (DNMT3B) is more directly involved in gene silencing than at other genes. Pericentromeric repeats which are transcriptionally silent are extensively targeted by DNMT3B, thus DNA in those regions are methylated. Moreover, repetitive sequences such as long terminal repeats, long interspersed nuclear elements (LINEs) and endoparasitic sequences are also silenced by DNA methylation to maintain chromosomal integrity (Smith et al., 2013).

DNA methylation patterns differ between cell lines. Moreover, DNA methylation may display differences among two alleles of the same gene which is called allele specific methylation (ASM). Throughout these discrepancies, allele specific gene expression takes place (Shoemaker et al., 2010). Genomic imprinting is the first example of ASM. Genomic imprinting is where parent-of-origin specific genes' expression controlled by imprinting control regions (ICRs) which have parental specific epigenetic modifications such as DNA methylation. Thus, only single parental allele is expressed. An other example for allele specific regulation mechanisms is X-chromosome inactivation (XCI). By this mechanism, in females, DNA methylation coordinates the random silencing of either maternally or paternally derived X chromosome, thereby dosage compensation with males is ensured. As a third model for ASM is whereby DNA metylation determined by DNA sequence in cis conformation and inherited in Mendelian patterns. There are additional factors to be identified such as environmental effects, sporadic abnormalities for understanding the persistence of ASM. These epigenetic pathways may lead to improper expression or silencing of the genes and induce new pathologies or be the cause of diseases which remain unidentified (Santos-Rebouças et al., 2007).

1.6.2 Histone Modifications

DNA chromatin is packaged into chromatin formation with a set of histone proteins. And transcriptional regulation is influenced by the post translational modifications of histones. The nucleosome is the fundamental unit of chromatin and it is composed of

an octamer of the four core histones (H3, H4, H2A, H2B) around which 147 base pairs of DNA are wrapped (Roth et al., 2001). Addition to four core histone proteins, there are two linker histone proteins so-called H1 and H5 which bind the nucleosome at the entry and exit sites of the DNA, thus locking the DNA into place (Kuo et al., 1998). Histones are modified at many sites. Post-translational histone modifications can be listed as; acetylation, lysine methylation, arginine methylation, phosphorylation, ubiquitilation, sumoylation, ADP ribosylation, deimination and proline isomerization (Kouzarides et al., 2007). Among them, the most studied one is lysine acetylation which occurs on lysine residues of histones. Levels of acetylation of the core histones result from the balance between the opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs). HDACs remove acetyl groups from an ε-N-acetyllysine amino acid on a histone, allowing the histones to wrap the DNA more tightly so that gene expression through that region is repressed. HATs transfer the acetyl group from acetyl coenzyme A (acetyl-CoA) on to ε-amino group of lysine residues therefore chromatin turns into euchromatin state and gene expression gets activated (Ohtani et al., 2011). Regulating chromatin structure can in turn affect many DNA related processes such as recombination, DNA repair and replication, transcription, and chromosomal organization. This vast array of modifications gives potential for functional responses.

1.6.3 Chromatin Remodelling

As an addition to highly progressive epigenetic systems such as DNA methylation and histone modifications, chromatin remodelling is an other system to balance gene expression. Nucleosome is the key element of chromatin formation which consist of an octamer of core histone proteins and DNA wrapped around them, as mentioned before. This dynamic DNA packaging or accessibility is controlled by histone modifying enzymes with ATP-dependent nucleosome remodeling factors. (Bao and Shen, 2007). Members of this enzyme family promote DNA-histone interactions within the nucleosome by utilizing the energy of ATP-hydrolysis. Thereby mobilizing histone octamers, regulatory factors are inhibited or arranged to their specific sites (Becker and Hirz, 2002).

Already deposited histone octamers assembled by chromatin remodelers. The remodeling activity on a nucleosome can be classified in two categories. First one as

site exposure where DNA becomes accessible by "nucleosomal sliding" (repositioning) or "nucleosomal eviction" (ejection) or local unwrapping so that DNA-binding protein (DBP) can attach to DNA. Secondly, altered histone composition, in which the nucleosome content is modified by dimer replacement (exchange of H2A-H2B dimer with a histone variant) or through dimer ejection (Schrader, 2009).

1.6.4 RNA Interference

MicroRNAs (miRNAs)are regulatory small RNAs which operate the translation of messenger RNA (mRNA) by binding to it and effecting target's cleavage or act as translational block depending on the extent of sequence complementarity with the target. MiRNAs also influence gene expression through posttranslational modifications of histone methylation through silencing complexes, thus represses trancription (Bartel et al., 2004). According to recent studies, miRNAs which are 18-23 nucleotides in length can be transcribed from both coding and non-coding regions (Bentwich et al., 2005). MicroRNAs are transcribed by RNA Polymerase II as primary microRNAs (pri-miRNAs) which are poly-adenylated and capped (Bartel et al., 2004). Pri-miRNAs are then processed by nuclear localized enzymes Drosha or Pasha to produce hairpin structures known as pre-miRNAs which are approximately 70 bases. These pre-microRNAs are then exported to the cytoplasm by Exportin-5 and form duplexes of 18–23 bases by the RNAase III enzyme Dicer. After that, miRNA gets associated with RNA Induced Silencing Complex (RISC). After this complex formation, there are two mechanisms; translational repression and target degradation (Scaria et al., 2006). It was shown that microRNA bound transcripts are moved into P bodies where they are associating with proteins that prevent translation or their cap structure is removed, thereby retained in a silent state (Liu et al., 2005). Interestingly, one target can be repressed by multiple microRNAs, moreover, one microRNA can have binding sites in multiple targets (John et al., 2004). Additionly, recent studies suggested that target repression is likely to be dosage dependent (Grimm et al., 2006).

1.6.5 RNA Methylation

As a last epigenetic modification, methylation of N6-methyladenine (m^6A) is the most prevalent reversible alteration on mRNA and long non-coding RNA (Fu et al.,

2014). Though it is not precisely known yet, methyltransferases are shown to be responsible for RNA methylation. Particularly, there has been several researches on evidencing tRNA methylation by DNMT2 enzymes (Goll et al., 2006). DNA and histone modifications affect mostly transcriptional events, whereas reversible RNA methylation is primarily effective on regulation of post-transcriptional gene expression. In the cell nucleus, m^6A may affect RNA export, nuclear retention and splicing, possibly through interactions with other proteins of RNA export, retention and splicing mechanisms. Fustin et al. show that m^6A methylation puts mRNA production onto a fast-track for facilitating nuclear processing and so provides an early control point of the feedback. Whereby, recent researches indicate that cellular protein levels are not necessarily correlated with the mRNA levels, these findings make post-translational modifications even more important (Fustin et al., 2013).

1.7 Aim of the study

Our previous studies on global methylation and HLA-B51 locus specific methylation in a group consisting of 4 MZ and 4 DZ BS twins, suggested an involvement of an epigenetic regulation in HLA-B51 exon 1-2 region. In this thesis we wanted to further our preliminary findings in familial BS cases and more directly examine the influence of HLA-B51 frequency and its methylation upon the presence of the clinical phenotype. Therefore, involvement of genetic and epigenetic roles of HLA-B51 region in BS would be better analysed and the sample size would be increased by including familial BS cases with their affected and unaffected relatives. In total 15 familial BS patients with their 17 BS diagnosed and 26 healthy relatives were included in the study. Also 25 age-gender matched individuals are compared for HLA-B51 frequency using sequence specific PCR and HLA-B methylation using Realtime based OneStep qMethyl Kit. Our ultimate goal is to be able to understand the relative contribution of HLA-B51 allele frequency and its methylation status on BS development in familial cases.

2. MATERIALS AND METHOD

Used chemicals, enzymes, buffers and methods for this study are explained in this section.

2.1 Materials

2.1.1 Equipment

Equipments, used in this study, are shown in Appendix .

2.1.2 Chemicals and buffers

Chemicals, enzymes and buffers, used in study, are shown in Appendix.

2.1.3 Preparation of buffers

Preparation of buffers, which are used, is explained in Appendix.

2.2 Methods

Main methods used in this study are Sequence-Specific PCR and Real-Time PCR methods.

2.2.1 Sample collection

Blood samples are collected from 15 individuals with Behçet Syndrome (BS) who have at least one relative diagnosed with BS in their family and their relatives which consist of 17 relatives with BS and 26 healthy relatives. In addition to that, age and gender matched healthy individuals were also collected in Cerrahpaşa Medical Faculty Rheumatology Polyclinic. 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 give their consent which is approved by ethic committee for the study. A 10-milliliter tube was obtained from each person. Blood samples are collected in EDTA tubes and kept in 4°C.

2.2.2 DNA isolation

The EDTA tubes contain a substance which mixes with the blood once have an anti-coagulating effect. Before the purification step, the sample has to be incubated in room temperature.

DNA isolation was performed with Roche DNA Isolation Kit for Mammalian Blood according to its given procedure;

- 30 ml Red Blood Cell Lysis Buffer is added to a sterile 50 ml centrifuge tube for each of the samples.
- 10 ml of mammalian blood is added to each tube and mixed gently by just inversion, not vortex.
- Centrifuge tube is placed on a rocking platform for 10 min.
- Tube is put into centrifuge machine for 10 min at 875x g.
- Red supernatant part is carefully poured off and white cell pellet is obtained after centrifuge process. A residual liquid remain with the white cell pellet.
- Remaining white cell pellet is vortexed thoroughly at the bottom of the tube in residual supernatant.
- After that, 5ml White Cell Lysis Buffer is added and mixed thoroughly via vortexing.
- Sample is transferred to a sterile 17 x 100 mm tube.
- After transfer, 2.6 ml Protein Precipitation Solution is added to each sample and vortexed frequently for 30 seconds.
- Tubes are put into centrifuge machine at 12.000 x g for 10 min.
- Supernatant now contains DNA, so it is carefully poured into a new sterile 50ml centrifuge tube. Protein pellet is disposed properly.
- Two volumes of +15 to +25^{o}C ethanol is added to the supernatant from one step before and it is mixed gently by inversion until remaining liquid is no longer cloudy.
- Sample is put into centrifuge machine at 875 x g for 10 min and after it, supernatant part is discarded.
- From cold 70 % ethanol, 3 ml is added to the DNA pellet in the tube and sample is mixed carefully several times via gentle inversion. Sample is centrifuged at 875 x g for 5 minutes and supernatant is discarded again.
- After all, DNA pellet is allowed to air dry until no longer ethanol remains.

• To resuspend DNA pellet, 1ml TE Buffer (ph=8) is added and sample is vortexed thoroughly. Samples are placed at 65^{o}C for 30- 60 min to aid in resuspension.

• Samples are stored at +2 to +8^{o}C until use.

2.2.3 Pedigree designing

In order to model biological connections between BS patients and their relatives a Java programme called CeGat Pedigree Chart Designer 2.1 was used. Family connections were based on questionnaries which were filled by individuals themselves.

2.2.4 HLA-B51 genotyping with sequence specific PCR method

HLA-B51 positivity was analyzed with sequence specific PCR method after designing of primers that produce 622 bp DNA product from HLA-B51 including all base substitutions for HLA-B51. In addition to that, as a result of non-specific binding of PCR primers to HLA-H and HLA-K regions, two other bands were observed which are 619 bps and 624 bps, respectively.

Primers were selected to amplify that 622 bp sequence on HLA-B51 region such as showed in above Table 2.1.

Table 2.1: Primers for amplification of 622 bp sequence region found on exon 2-3 of HLA-B51 gene.

Forward Primer: 5'-CCGGAGTATTGGGACCGGAAC-3'
Reverse Primer: 5'-GCGCGCTGCAGCGTCTCC-3'

Primers were designed for amplification of specific region on HLA-B gene where base substitutions that lead to HLA-B51 suballele differentiations were considered.

PCR mixture is shown as above in Table 2.2;

Table 2.2: Ingredients in PCR mixture for genotyping of HLA-B51 via sequence specific PCR.

Ingredients	Stock Solution	Volume
Taq Buffer	10X	2 µl
Betain	10X	3 µl
dNTP	2.5 mM	0.4 µl
Forward Primer	10 pmol/µl	0.4 µl
Reverse Primer	10 pmol/µl	0.4 µl
Taq	5U/µl	0.25 µl
Final		20 µl

PCR protocol is shown as above in table;

Table 2.3: PCR procedure used for sequence specific PCR.

Step	Temperature(°C)	Time	Number of cycles
1.1	95	3 min	1
2.1 2.2 2.3	95 68 72	30 sec 30 sec 1 min	35
3.1	72	8 min	1
4.1	4	∞	1

After PCR procedure, for discriminating HLA-B51 sequence among those non-specific sequences, a restriction enzyme which is specific to HLA-B51 was used. PCR products were treated with restriction enzyme for 4 hours at 37 ^{o}C. When restriction enzyme reaction is finished, products were determined under %1 agarose gel including TAE as buffer. Gel electrophoresis was performed at 100V for 30 minutes. Interperation of results was done according to 100bp DNA marker.

2.2.5 Determination of HLA-B methylation levels with Real-Time PCR

Methylation levels of HLA-B region between BS patients, their relatives with BS, healthy relatives and age – gender matched healthy control groups were analyzed with Zymo OneStep qMethyl™ Kit via Real-Time machine.

2.2.5.1 Overview of procedure

This kit is used for detection of region specific DNA methylation via selective amplification of methylated cytosines in CpG dinucleotide context. DNA, which will be tested, is separated in two parts as “Test Reaction” and “Reference Reaction”. DNA in “Test Reaction” is digested with Methylation Sensitive Restriction Enzymes (MSREs) while DNA in “Reference Reaction” is not. DNA from both reactions then is amplified via Real-Time PCR. Cycle threshold values for test and reference reactions vary due to different methylation status. Large Ct differences are generally characteristic of non- methylated DNA.

Figure represents a schematic view of this workflow above;

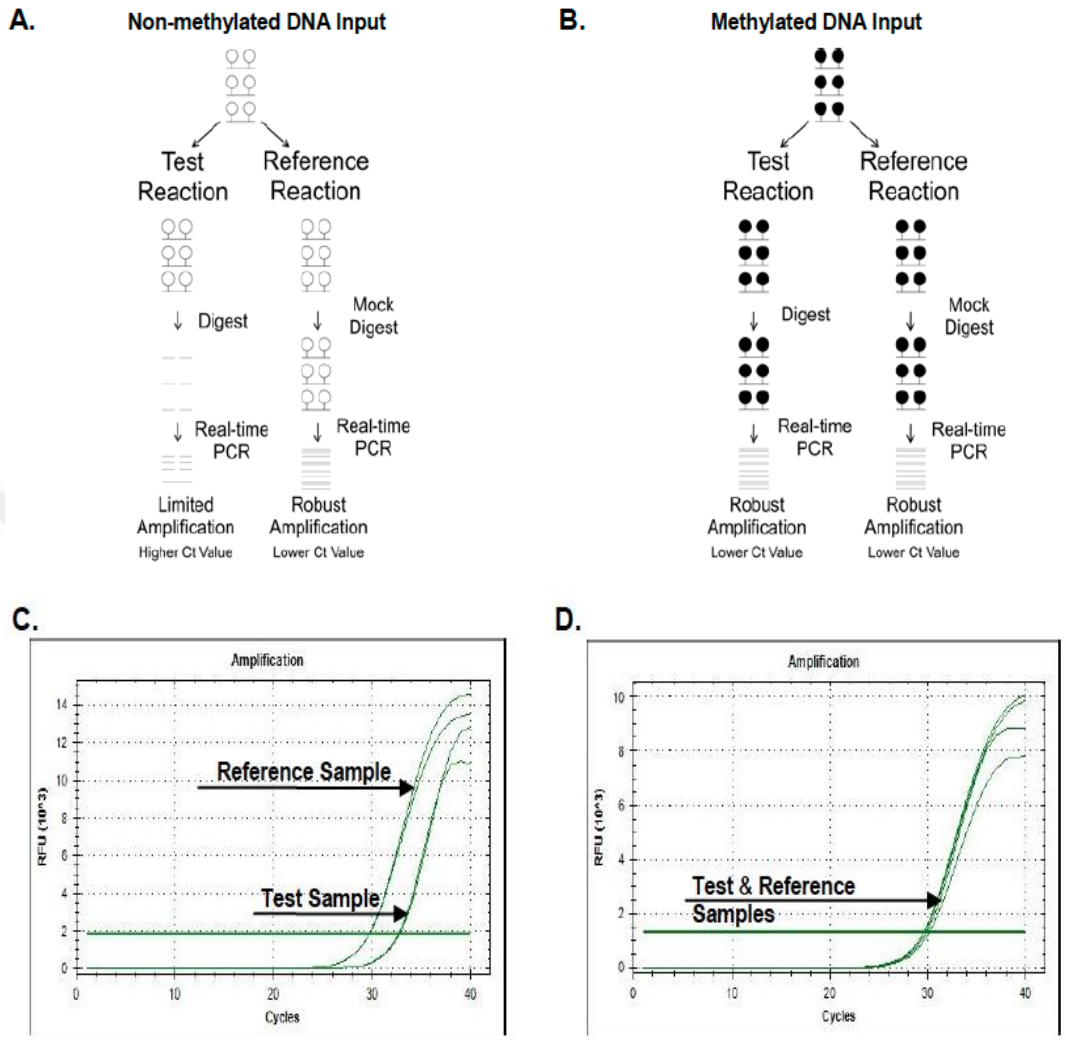

Figure 2 . 1 : A and B part illustrate sample workflow of non- methylated and methylated DNA samples. White dots represent unmethylated cytosines, whereas black dots represent methylated cytosines in CpG dinucleotide context. C and D part are amplification plots demonstrating non-methylated DNA exhibits large differences in Ct values between test and reference reactions (C) while methylated DNA exhibits slight differences in Ct values between test and reference reactions (Taken from Instruction Manual of OneStep qMethyl™- Kit).

2.2.5.2 Protocol

For each DNA sample a Test and a Reference Reaction mixtures were prepared separately**.**

Test Reaction (per well)

10 µl	2X Test Reaction-Lite Premix (contain MSREs)
1 µl	10µM Forward Primer (MGMT Primer I for standards)
1 µl	10µM Reverse Primer (MGMT Primer II for standards)
3µl	DNAase/ RNAase free water
15µl	Total volume

Reference Reaction (per well)

10 µl	2X Reference Reaction-Lite Premix
1 µl	10µM Forward Primer (MGMT Primer I for standards)
1 µl	10µM Reverse Primer (MGMT Primer II for standards)
3µl	DNAase/ RNAase free water
15µl	Total volume

After preparation of master mixes for Test and Reference Reactions, add 5 µl of DNA sample (4ng/µl) to the wells of PCR plate. Seal the plate with sealing tape.

Table 2.4: Primers sets for Real-Time PCR.

HLA-B Exon1-İntron1 Forward Primer: 5'- GTC CCA GTT CTA AAG TCC C -3'
HLA-B Exon1-İntron1 Reverse Primer: 5'- TGA GGA GGG GCT GAG AC -3'
HLA-B Exon-2 Forward Primer: 5'- AGC CCC TCC TCA CCC CCA GG -3'
HLA-B Exon-2 Reverse Primer: 5'- CCG GGG TCA CTC ACC GGC C -3'

Primer sets were designed to span exon-1, intron-1 and exon-2 region where CpG island present.

Table 2 . 5: Real-Time PCR conditions for OneStep qMethyl™ Kit.

	Temperature	Time
MSRE Digestion	37°C	2 hours
Initial Denaturation	95°C	10 minutes
Denaturation	95°C	30 seconds
Annealing	54°C/62 °C	60 seconds
Extension	72°C	60 seconds
Final Extension	72°C	7 minutes
Hold	4°C	>5 minutes

Zymo Calculator programme on Zymo Research web page was used to determine methylation levels via usage of this equation:

Percent Methylation = $100 \times 2^{-\Delta Ct}$

2.2.6 Statistical analysis

Statistical analysis were done with Graphpad Prism 6th Version programme. Fisher's and chi-square tests were performed to determine significance levels of genotyping results among groups. Two tailed t-test analysis was performed to the methylation results obtained from zymo methylation calculator. P values were calculated for methylation differences between BS patients and healthy individuals in families and healthy control groups.

The p-value is calculated to determine the significance of the results. The p-value intervals are:

$p < 0.05$: significant difference,

$p > 0.05$: no significant difference.

3. RESULTS

3.1 Demographic Data Of Study Groups

Samples were collected from 15 BS families including 15 index patients, 17 BS relatives and 26 healthy relatives and 25 age-gender matched healthy controls. The demographic data of the patients are given in the Table 3.1.

Table 3.1: Demographic Data of the Study Groups.

	Mean Age(Female)	Mean Age(Male)
Index patients(n=15)	38±7.5(n=9)	38±6.1(n=6)
Affected Relatives(n=17)	47± 11.3(n=9)	41± 4.7(n=8)
Healthy Relatives(n=26)	36± 18.6(n=11)	26± 12.8(n=15)
Healthy Controls(n=25)	43± 8.4(n=12)	43± 8.7(n=13)

3.2 HLA-B51 Genotyping With Sequence Specific PCR Method

PCR Primers were designed specifically for the amplification of HLA-B51 gene. 622 bp bands were determined in some samples which indicate the existence of HLA-B5 gene. In addition to that, as a result of non-specific binding of PCR primers to HLA-H and HLA-K regions, two other bands were observed which are 619 bps and 624 bps, respectively (Figure 3.1). Desired bands were indistinguishable from non-specific bands due to the close-by band lengths on agarose gel. Therefore, a target specific restriction enzyme was used to cleave the desired bands.

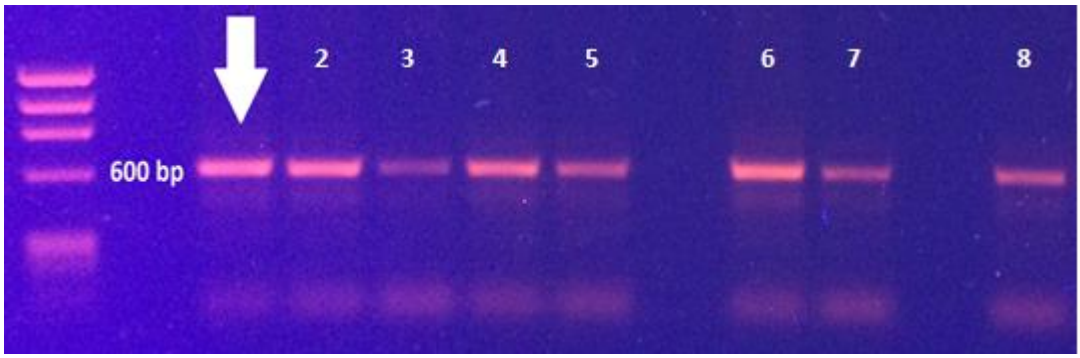

Figure 3.1: Bands which include HLA-B51, HLA-H and HLA-K sequences are seen on 1., 2., 3., 4., 5., 6. , 7. and 8. wells.

3.3 Restriction Enzyme Reaction Of HLA-B51 PCR Products

After sequence specific PCR method, observed 622 base pair PCR products were cleaved into 464 bps and 158 bps DNA fragments by a restriction enzyme which specifically recognizes HLA-B51 allele restriction site (Figure 3.2).

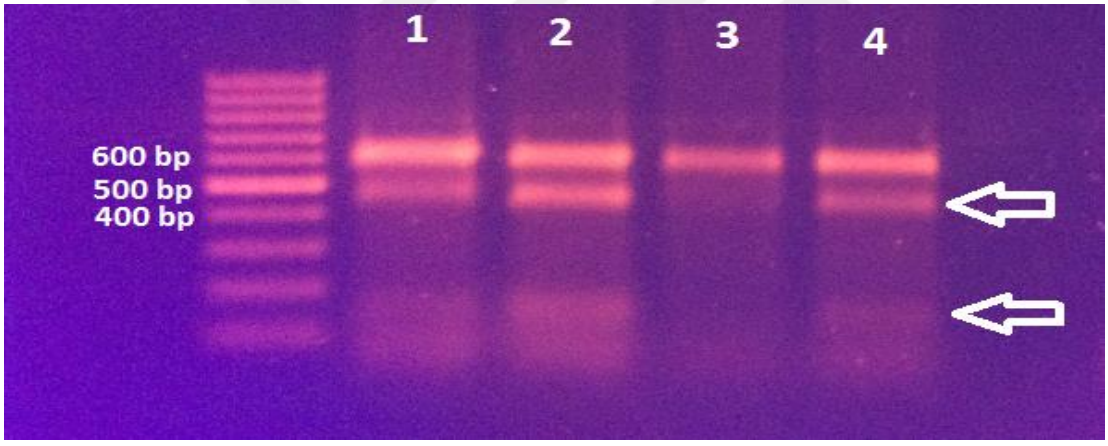

Figure 3.2: Restriction enzyme results for patients with BS. Bands seen on 1. , 2. , 4. wells indicate HLA-B51 positivity; band seen on 3. well indicates HLA-B51 negativity.

3.4 HLA-B51 Genotyping Results On Familial BS Pedigrees

Famial BS patients and their relatives are shown on the pedigrees with HLA-B51 positivity due to their degree of relation.

On pedigrees, family members only who have their genotype information below, included in the study.

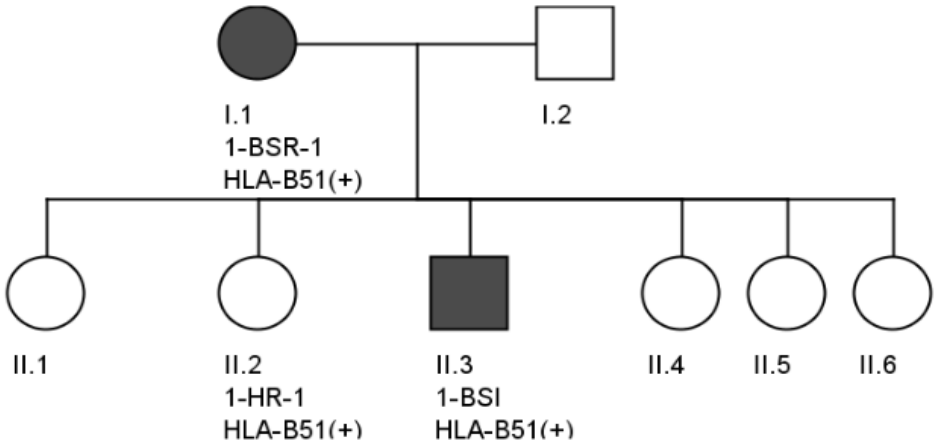

Figure3.3: Family 1: 1-BSI: index patient, HLA-B51(+); 1-BSR-1: first degree relative with BS, HLA-B51(+); 1-HR-1: first degree healthy relative, HLA-B51(+).

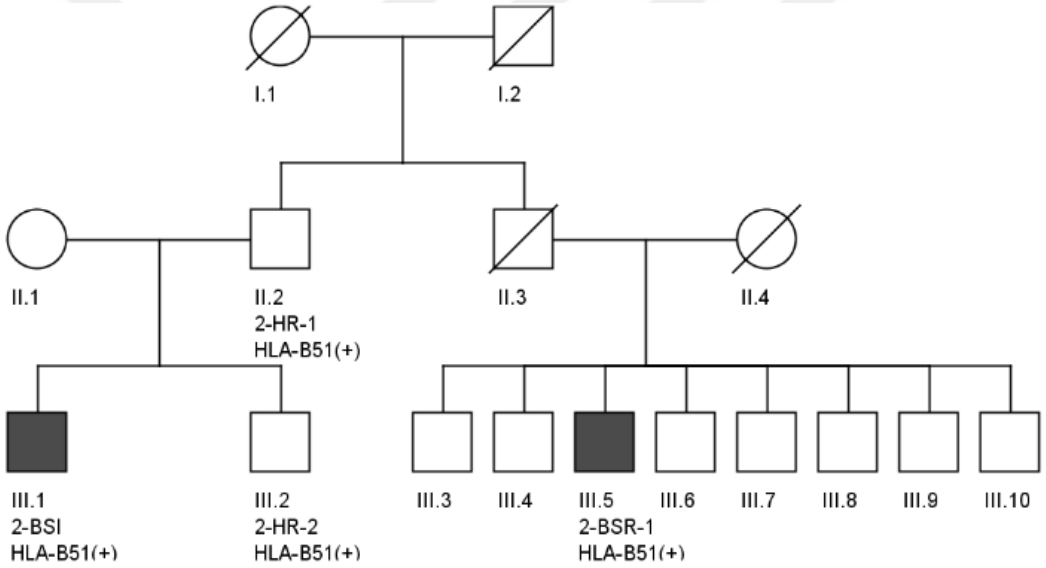

Figure 3.4: Family2: 2-BSI: index patient, HLA-B51(+); 2-BSR-1: third degree relative with BS, HLA-B51(+); 2-HR-1: first degree healthy relative; HLA-B51(+); 2-HR-2: first degree healthy relative, HLA-B51(+).

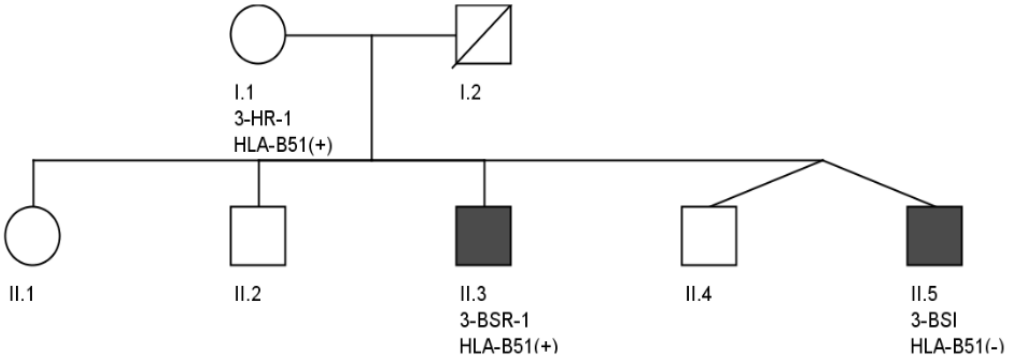

Figure 3.5 : Family 3: 3-BSI: index patient: HLA-B51(-); 3-BSR-1: first degree relative with BS, HLA-B51 (+); 3-HR-1: first degree healthy relative, HLA-B51(+).

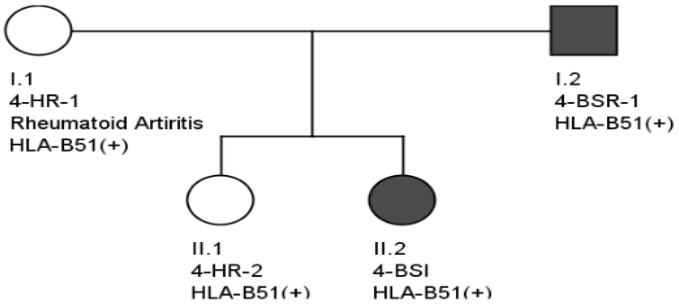

Figure 3.6: Family 4: 4-BSI: index patient, HLA-B51(+); 4-BSR-1: first degree relative with BS, HLA-B51(+); 4-HR-1: first degree relative with RA, HLA-B51(+); 4-HR-2:first degree healthy relative, HLA-B51(+).

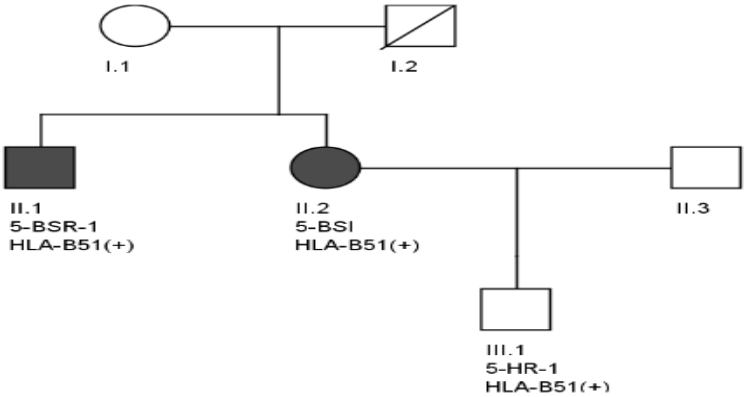

Figure 3.7: Family 5: 5-BSI: index patient, HLA-B51(+); 5-BSR-1: first degree relative with BS, HLA-B51(+); 5-HR-1: first degree relative, HLA-B51(+).

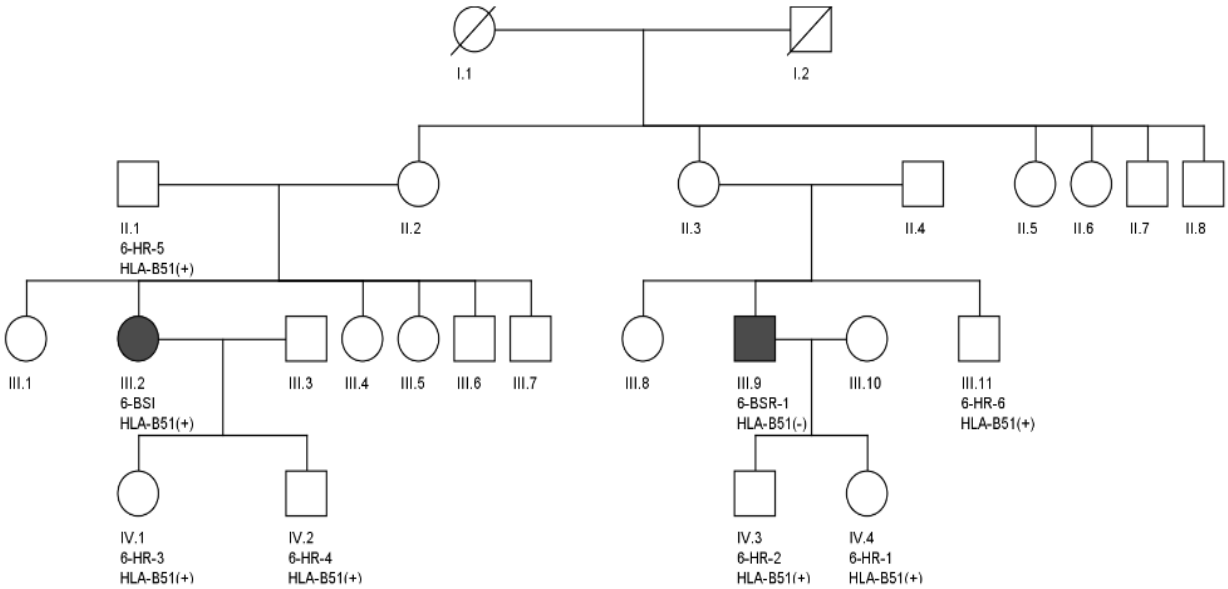

Figure 3.8: Family 6: 6-BSI:index patient, HLA-B51(+); 6-BSR-1:third degree relative with BS, HLA-B51(-); 6-HR-1: fourth degree healthy relative, HLA-B51(+); 6-HR-2: fourth degree healthy relative, HLA-B51(+); 6-HR-3:first degree healthy relative, HLA-B51(+); 6-HR-4: first degree healthy relative, HLA-B51(+); 6-HR-5: first degree healthy relative, HLA-B51(+); 6-HR-6: third degree healthy relative, HLA-B51(+).

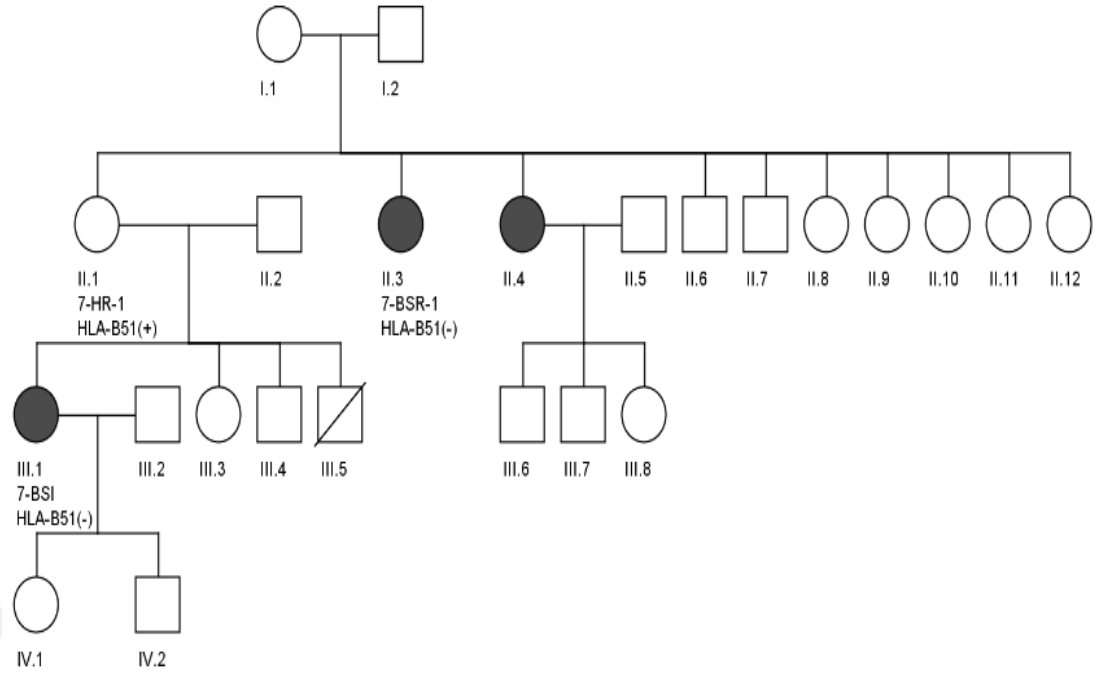

Figure 3.9: Family 7: 7-BSI:index patient, HLA-B51(-); 7-BSR-1:second degree relative with BS, HLA-B51(-); 7-HR-1: first degree healthy relative, HLA-B51(+).

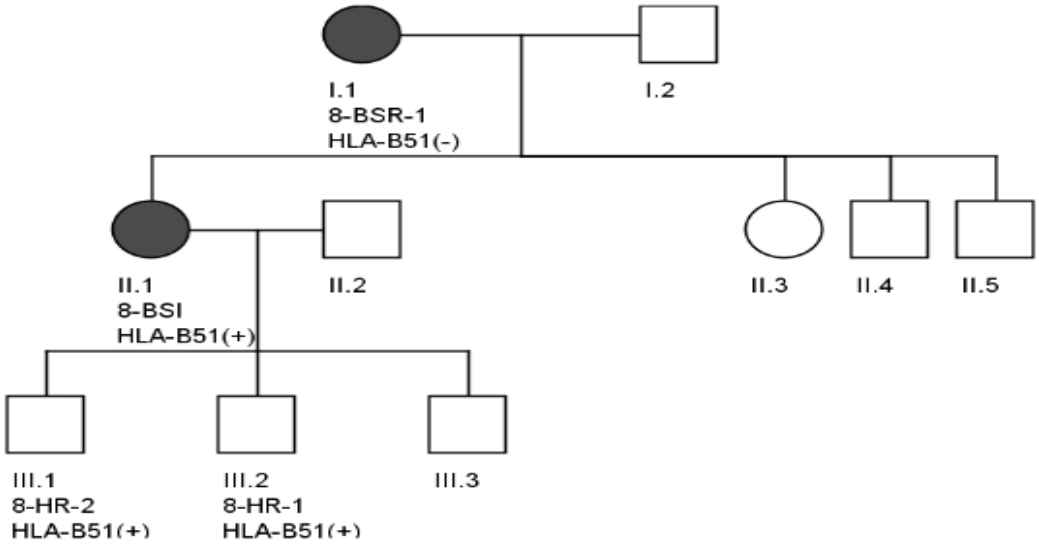

Figure 3.10: Family 8: 8-BSI: index patient, HLA-B51(+); 8-BSR-1: first degree relative with BS; HLA-B51(-); 8-HR-1: first degree healthy relative, HLA-B51(+); 8-HR-2: first degree healthy relative, HLA-B51(+).

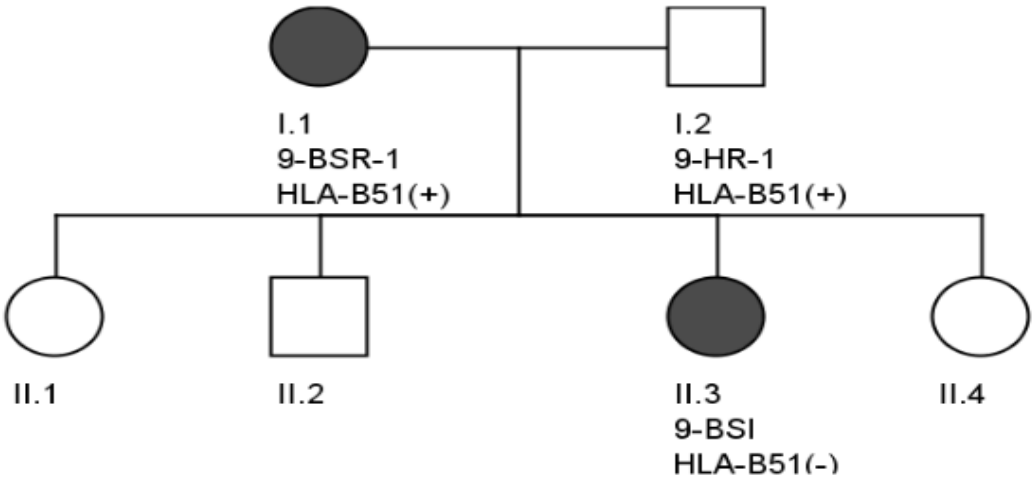

Figure 3.11: Family 9: 9-BSI:index patient, HLA-B51(-); 9-BSR-1:first degree relative with BS, HLA-B51(+); 9-HR-1: first degree healthy relative, HLA-B51(+).

Rest of the pedigress are listed in Appendix E.

3.5 HLA-B51 Genotyping Results

HLA-B51 genotyping results of 15 families including 17 relatives with BS, 26 healthy relatives and 25 healthy controls out of families (Table 3.2).

Table 3.2: HLA-B51 positivity among groups due to their degree of relation.

	HLA-B51(+)	HLA-B51(-)	
Index patients	12	3	12/15
Affected Relatives			
1.Degree	8	1	8/9
2.Degree	1	1	1/2
3.Degree	2	2	2/4
4.Degree	2	-	2/2
			13/17
Healthy Relatives			
1.Degree	18	3	18/21
2.Degree	-	1	-/1
3.Degree	2	-	2/2
4.Degree	2	-	2/2
			22/26
Healthy controls	8	17	8/25

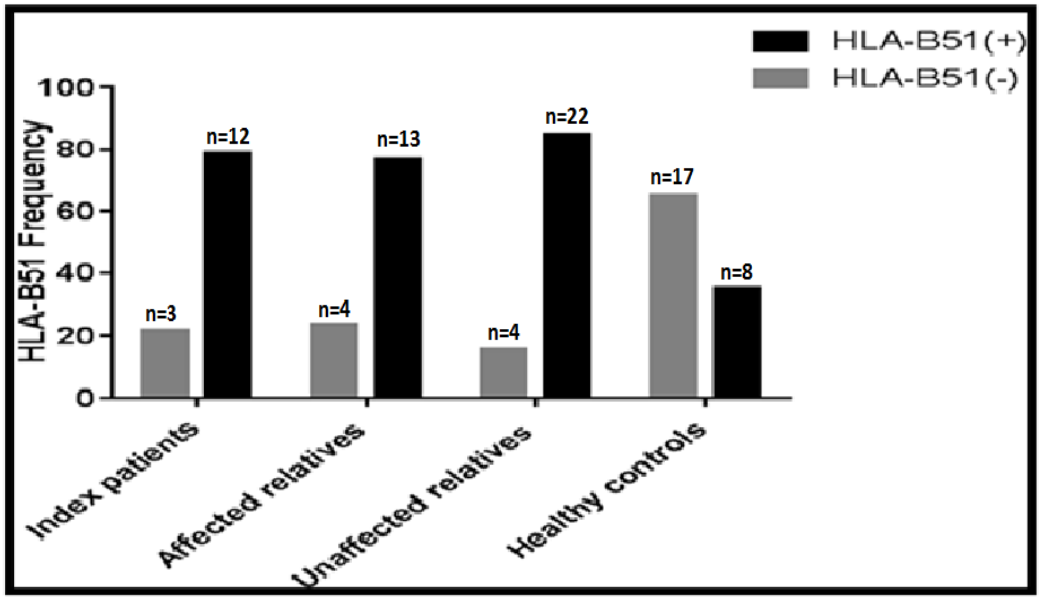

Figure 3.12:HLA-B51 frequency among groups of index patient, affected relatives, unaffected relatives and healthy controls.

Index patients , affected relatives and unaffected relatives were not significantly different when compared with each other. However, HLA-B51 frequency for healthy controls were statistically lower when compared with index patients, affected relatives and healthy individuals in families (p=0.0112, p=0.0107, p=0.0003, respectively) (Figure 3.12).

3.6 Methylation Analyses of HLA-B gene

3.6.1 Methylation levels of BS patients, their relatives and healthy controls

Methylation levels were detected with OneStep qMethyl Kit via Real-Time PCR and calculated according to the equation of Percent Methylation = 100 x 2-Δct through calculator programme of kit itself on internet. Exon1-Intron1 region and Exon 2 region were analyzed seperately due to the Real-Time procedure (Table3.3, Table 3.4, Table 3.5, Table 3.6).

Table 3.3: Exon1-Intron1 Methylation Levels for index patients and affected relatives.

Exon-1 Methylation Levels for Index Patients	
1-BSI	175 ,77%
2-BSI	202 ,39%
3-BSI	106 ,48%
4-BSI	268 ,16%
5-BSI	226 ,57%
6-BSI	163 ,26%
7-BSI	167 ,08%
8-BSI	173 ,60%
9-BSI	214 ,35%
10-BSI	207 ,99%
11-BSI	201 ,05%
12-BSI	103 ,03%
13-BSI	100 ,50%
14-BSI	127 ,16%
15-BSI	271 ,82%

Exon-1 Methylation Levels for Affected Relatives	
1-BSR-1	172,02%
2-BSR-1	266,12%
3-BSR-1	148,74%
4-BSR-1	240,70%
5-BSR-1	180,71%
6-BSR-1	174,90%
7-BSR-1	127,74%
8-BSR-1	184,13%
9-BSR-1	222,64%
10-BSR-1	236,36%
10-BSR-2	113,84%
11-BSR-1	210,95%
12-BSR-1	169,05%
13-BSR-1	103,14%
14-BSR-1	131,15%
15-BSR-1	337,67%
15-BSR-2	315,44%

Table 3.4: Exon1-Intron1 methylation levels for healthy relatives and healthy controls.

Exon-1 Methylation Levels for Healthy Relatives	
1-HR-1	131,16%
2-HR-1	135,01%
2-HR-2	152,31%
3-HR-1	105,23%
4-HR-1	104,27%
4-HR-2	124,68%
5-HR-1	165,89%
6-HR-1	113,54%
6-HR-2	50,06%
6-HR-3	59,04%
6-HR-4	96,91%
6-HR-5	100,28%
6-HR-6	130,27%
7-HR-1	105,68%
8-HR-1	110,37%
8-HR-2	114,67%
9-HR-1	139,61%
10-HR-1	122,30%
11-HR-1	165,71%
11-HR-2	140,18%
11-HR-3	152,48%
11-HR-4	116,14%
12-HR-1	139,19%
13-HR-1	90,07%
14-HR-1	93,71%
15-HR-1	280,77%

Exon-1 Methylation Levels for Healthy Controls	
C-1	164,39%
C-2	126,67%
C-3	150,08%
C-4	123,20%
C-5	149,49%
C-6	46,17%
C-7	158,54%
C-8	165,63%
C-9	98,52%
C-10	81,85%
C-11	81,60%
C-12	52,64%
C-13	63,58%
C-14	85,22%
C-15	85,28%
C-16	72,63%
C-17	48,12%
C-18	94,24%
C-19	72,01%
C-20	58,97%

Table 3.5: Exon-2 Methylation Levels for index patients and affected relatives.

Exon-2 Methylation Levels for Index Patients	
1-BSI	62,41%
2-BSI	50,44%
3-BSI	43,45%
4-BSI	44,25%
5-BSI	68,37%
6-BSI	60,64%
7-BSI	42,74%
8-BSI	35,86%
9-BSI	47,94%
10-BSI	29,26%
11-BSI	36,37%
12-BSI	25,84%
13-BSI	33,47%
14-BSI	32,40%
15-BSI	49,56%

Exon-2 Methylation Levels for Affected Relatives	
1-BSR-1	27,43%
2-BSR-1	29,42%
3-BSR-1	81,18%
4-BSR-1	61,60%
5-BSR-1	70,36%
6-BSR-1	38,87%
7-BSR-1	31,51%
8-BSR-1	37,32%
9-BSR-1	87,34%
10-BSR-1	42,33%
10-BSR-2	29,38%
11-BSR-1	57,53%
12-BSR-1	35,88%
13-BSR-1	33,47%
14-BSR-1	33,71%
15-BSR-1	58,61%
15-BSR-2	56,85%

Table 3.6: Exon-2 Methylation Levels for healthy relatives and healthy controls.

Exon-2 Methylation Levels for Healthy Relatives	
1-HR-1	62,00%
2-HR-1	36,87%
2-HR-2	36,86%
3-HR-1	28,64%
4-HR-1	27,15%
4-HR-2	36,67%
5-HR-1	35,12%
6-HR-1	43,20%
6-HR-2	29,00%
6-HR-3	30,39%
6-HR-4	32,38%
6-HR-5	34,24%
6-HR-6	36,33%
7-HR-1	33,98%
8-HR-1	38,86%
8-HR-2	37,46%
9-HR-1	19,54%
10-HR-1	25,26%
11-HR-1	37,00%
11-HR-2	13,29%
11-HR-3	50,14%
11-HR-4	20,69%
12-HR-1	37,50%
13-HR-1	37,21%
14-HR-1	30,95%
15-HR-1	63,95%

Exon-2 Methylation Levels for Healthy Controls	
C-1	60,90%
C-2	77,21%
C-3	97,35%
C-4	64,08%
C-5	73,51%
C-6	67,22%
C-7	83,33%
C-8	51,55%
C-9	10,57%
C-10	13,23%
C-11	17,91%
C-12	9,82%
C-13	17,92%
C-14	23,26%
C-15	23,50%
C-16	19,22%
C-17	13,09%
C-18	22,20%
C-19	22,89%
C-20	24,62%

3.6.2 Statistical analyses of methylation level differences between groups

For Exon-1-Intron-1 region, methylation levels were significantly higher in index patients when compared with unaffected relatives ($p<0.0001$). Methylation levels were compared between index patients and affected relatives, the difference was not statistically significant (Figure 3.13).

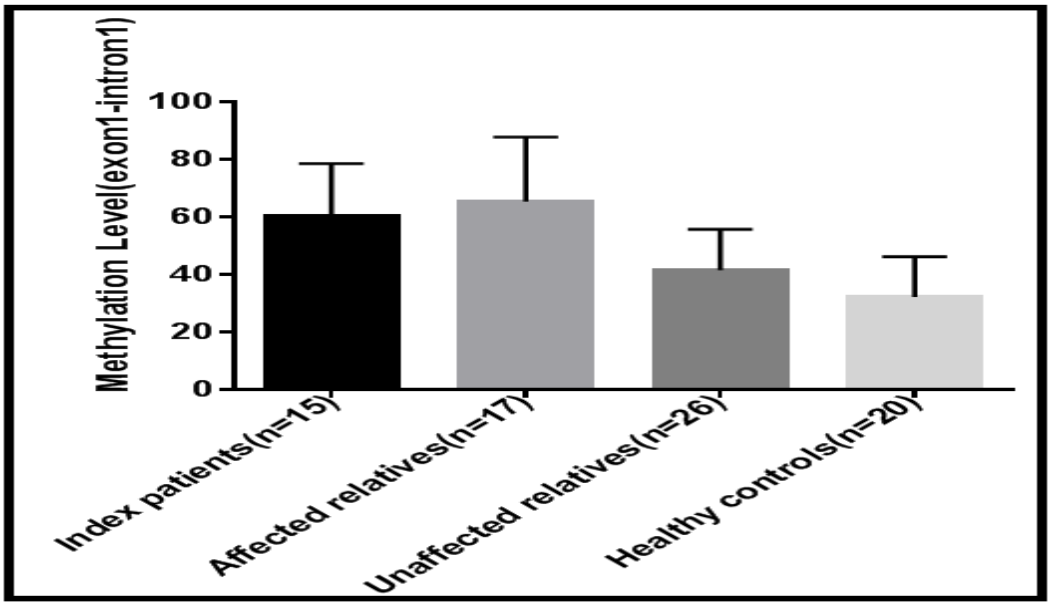

Figure 3.13: Methylation level comparison for region spanning exon1 and intron1 of HLA-B among groups.

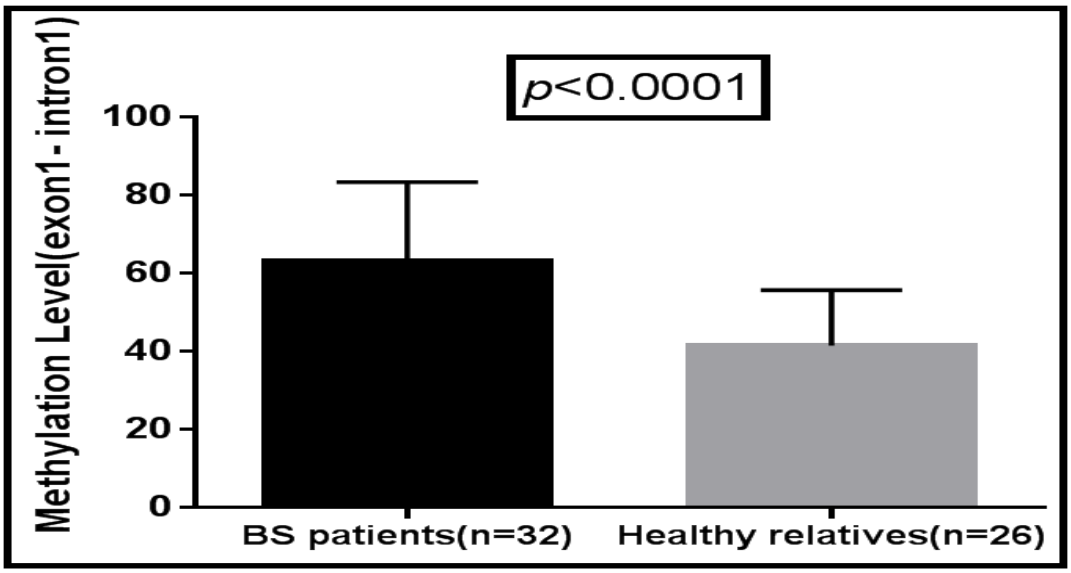

Figure 3.14: Exon1- intron1 region methylation level comparison between all BS patients and their healthy relatives.

BS patients' methylations levels were significantly higher than their healthy relatives ($p<0.0001$). This finding point out methylation differences between BS patients and healthy relatives. As well as that, through the region spanning exon-1 and intron-1 of HLA-B gene, methylation levels were significantly different between BS patients and healthy controls. BS patients had higher methylation levels ($p<0.0001$) (Figure 3.14).

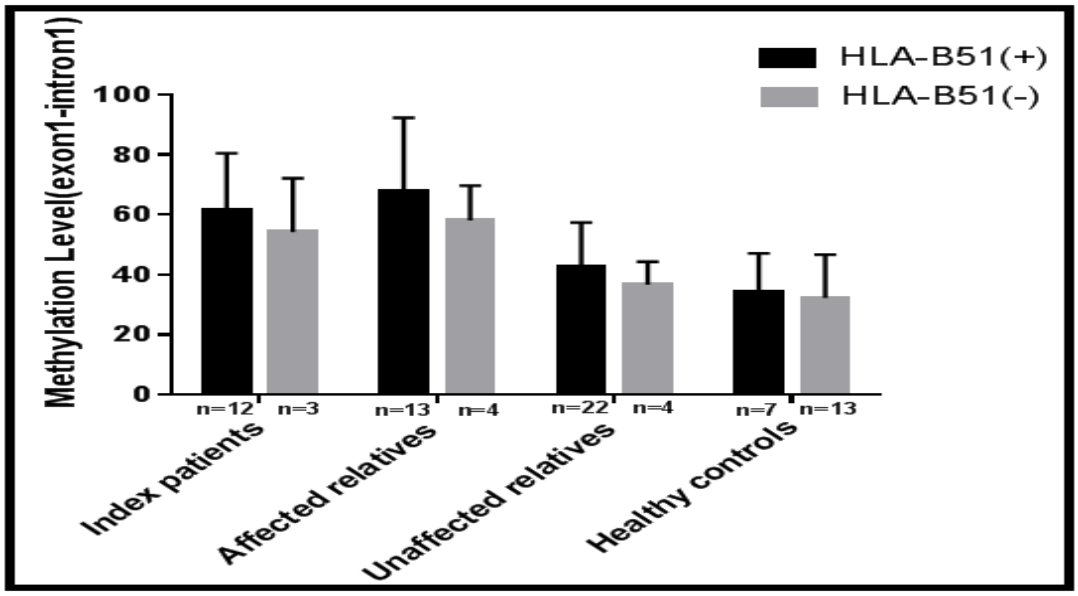

Figure 3.15: Methylation levels of region spanning exon1-intron1 of HLA-B due to HLA-B51 positivity among groups.

Individuals among all four groups, did not show a statistically significant difference due to their HLA-B51 positivity (Figure 3.15).

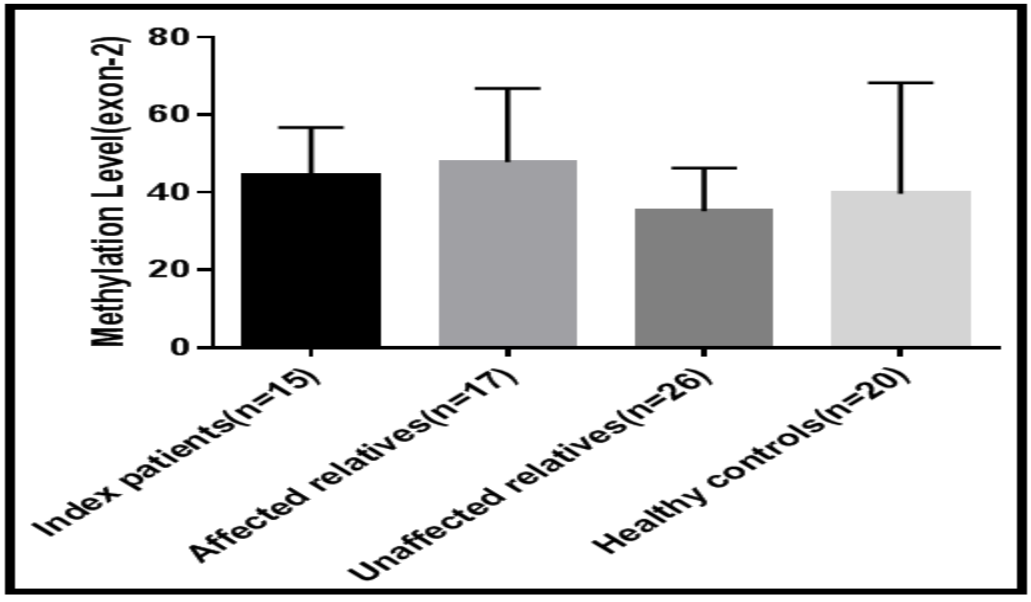

Figure 3.16: Methylation level comparison for exon-2 region of HLA-B among groups.

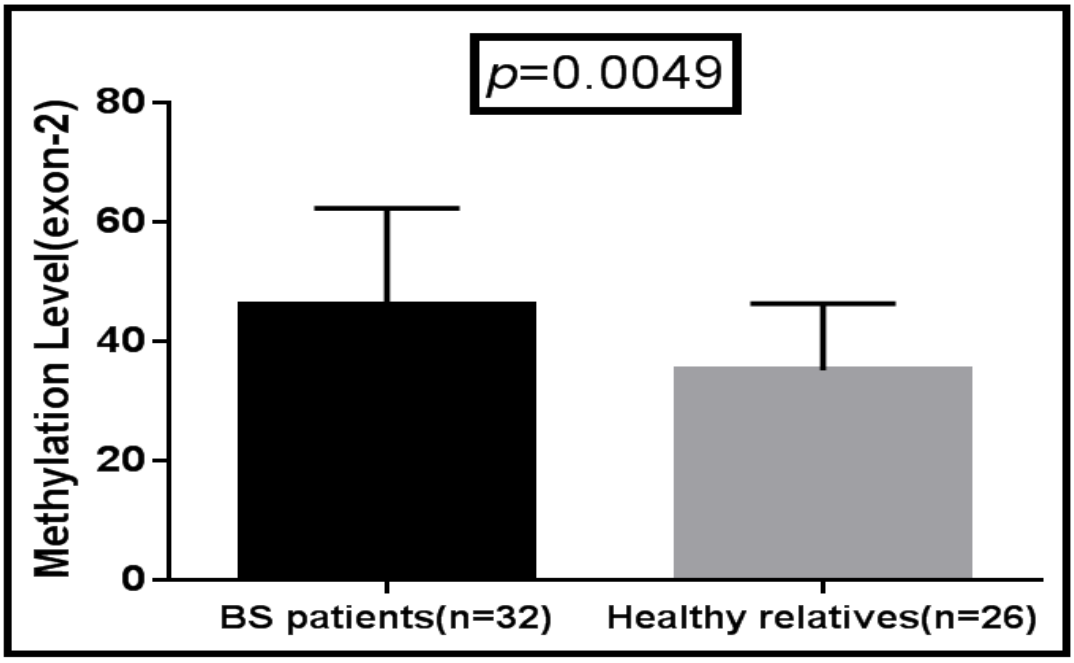

Figure 3.17 : Methylation level comparison of Exon-2 among BS patients and their relatives.

For Exon-2 region of HLA-B gene, methylation levels were statistically higher according to their healthy relatives (p=0.0049) (Figure 3.17).

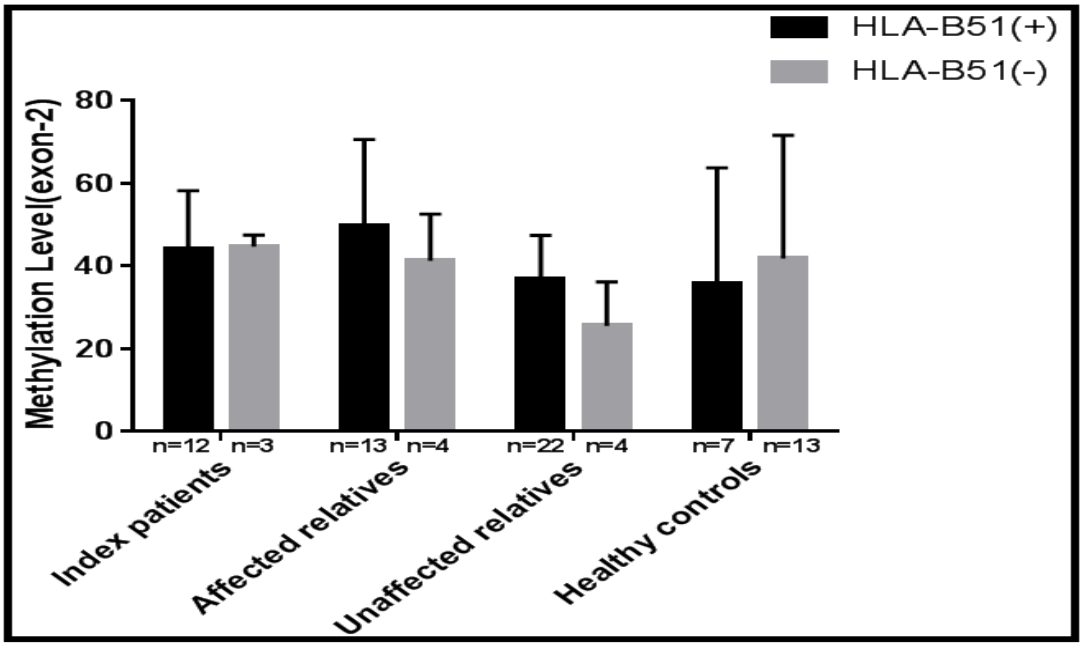

Figure 3.18 : Methylation levels of exon-2 region of HLA-B due to HLA-B51 positivity among groups.

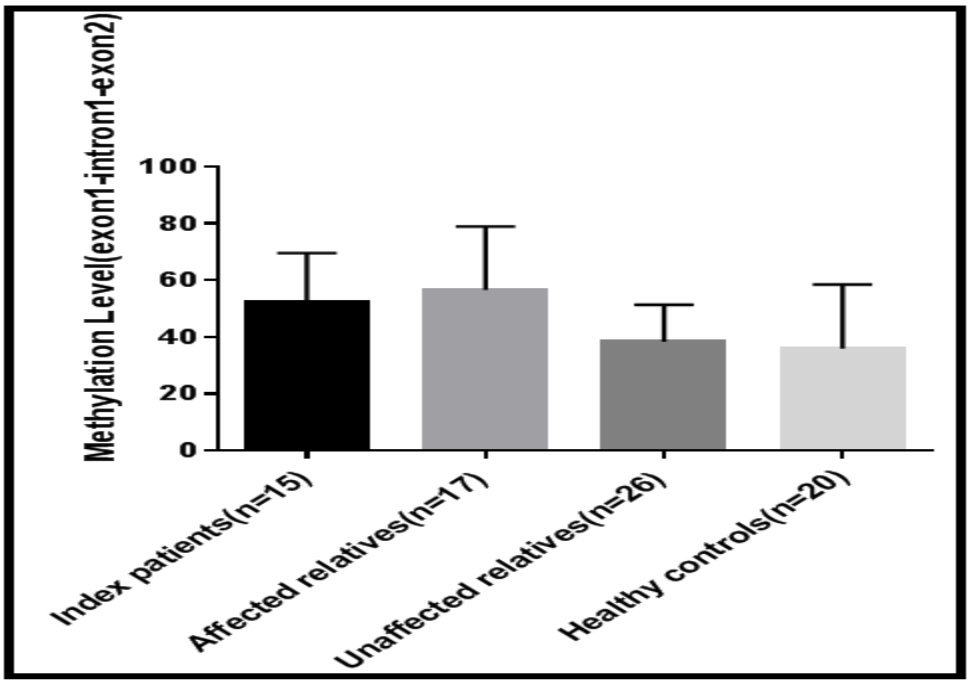

Figure 3.19: Average methylation levels for all regions including exon-1, intron-1 and exon-2 for all groups.

For average methylation, unaffected relatives displayed significantly lower methylation levels when compared with index patients and affected relatives (p=0.0001, p<0.0001, respectively) (Figure 3.19).

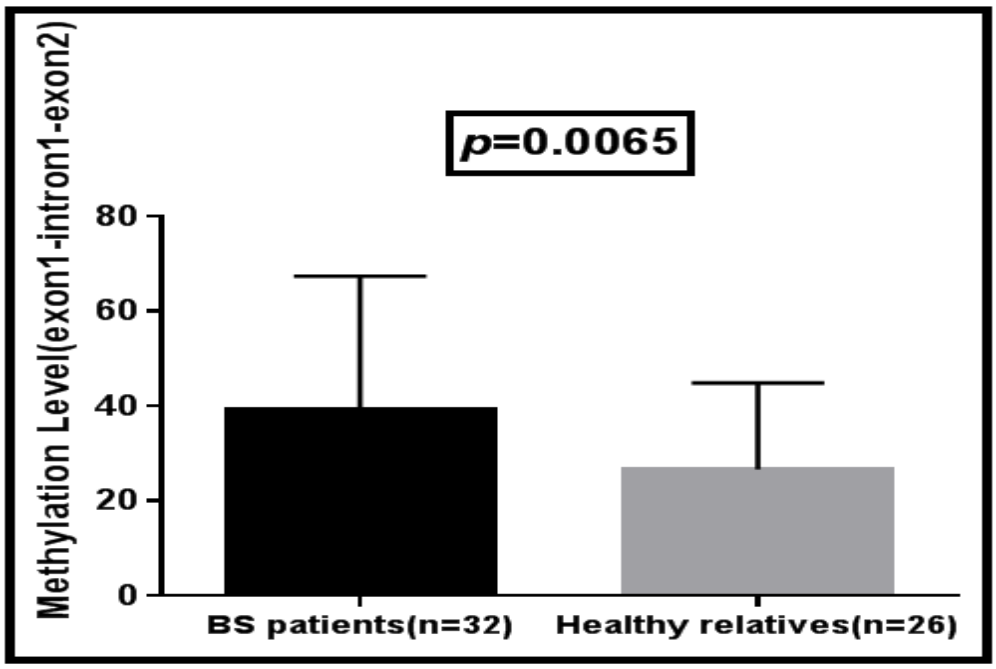

Figure 3.20: Average methylation level comparison among all BS patients and their healthy relatives.

BS patients showed statistically higher methylation levels when compared with healthy relatives in their families (p=0.0065) (Figure 3.20).

Considering the age difference between unaffected relatives, unaffected relatives whose ages are between 16-20 (n=3) were analysed seperately comparing to remaining unaffected relatives (n=23) and BS relatives, the results were also not significantly different.

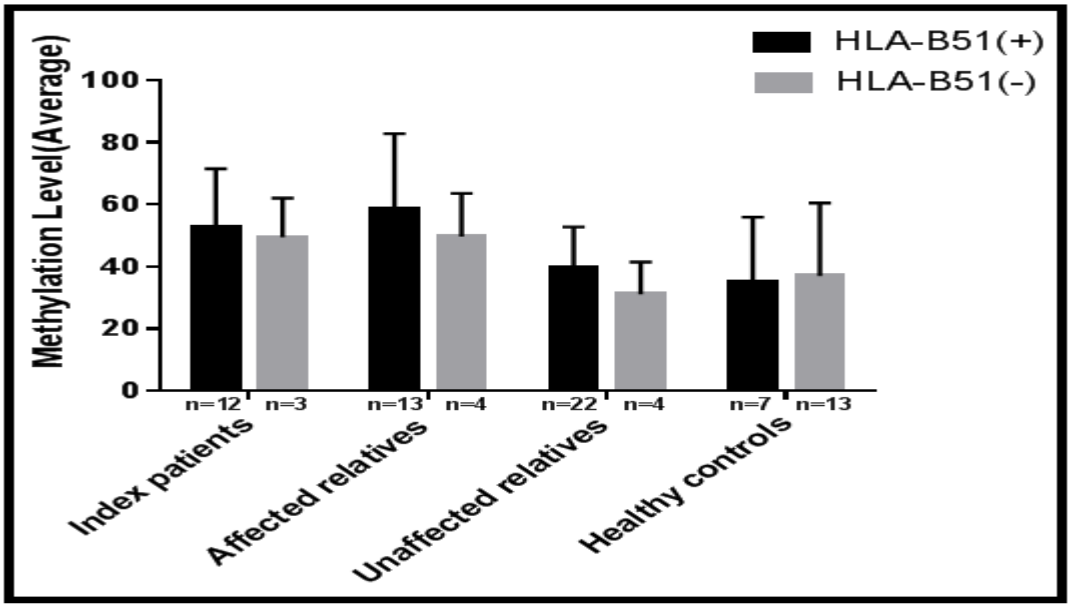

Figure 3.21: Average methylation level differences among groups through HLA-B51 positivity.

Individuals among all four groups, did not show a statistically significant difference for exon-1-intron-1-exon-2 region due to their HLA-B51 positivity (Figure3.21).

4.DISCUSSION

BS risk factors include genetic predisposition which is mainly based on familial aggregation and increased HLA-B51 carrier as well as infectious agents, environmental causes and yet undefined immunological mechanisms. Even though HLA-B51 allele has shown to be the most associated marker for BS, the presence of healthy individuals who carry HLA-B51 allele and similarly BS patients without HLA-B51 allele have led us question other mechanisms for BS causation.

Therefore, HLA-B51 frequency was determined among 15 BS families. HLA-B51 positivity ratio was 13/15 for index patients, 13/17 for affected relatives and 22/26 for unaffected relatives. For healthy controls, who are not related with families, HLA-B51 positivity was 8/25. When BS patients compared with their healthy relatives for HLA-B51 frequency, results did not show statistically significant difference. These findings indicate that, since the family members have similar genotypic predisposition, our results point out possible epigenetic mechanisms independent from HLA-B51 frequency.

Epigenetic mechanisms that control gene expression without changing DNA sequence, have been related with many complex diseases. Also, CpG island presence on HLA-B gene in 1346 nucleotide length, starting from promoter until exon3, strengthen the idea of epigenetic factors' effect on causation of BS. Additionally, our previous studies evidenced phenotypic discordance and significantly higher methylation levels of HLA-B gene exon 1 and exon 2 regions in BS twins when compared with healthy controls. Along with these data, investigating possible genetic and epigenetic roles of HLA-B51 allele in BS patients and healthy relatives in BS families, have become our hypothesis.

Hence, methylation analyses were performed on exon1, intron1 and exon-2 regions of HLA-B gene for 15 BS families including BS patients and healthy individuals. Throughout statistical analyses, average methylation levels manifested statistically significant differences between BS patients and their healthy relatives. BS patients had higher methylation levels compared with healthy individuals in the family (p=0.0065). Nevermore, methylation levels of exon-1 and exon-2 for BS patients did

display the same high methylation patterns seperately when compared to healthy relatives with the *p* values of 0.0001 and 0.0049, respectively.

For both exon1 and exon2 regions , methylation levels were not correlated with HLA-B51 positivity. Methylation levels were not significantly different for HLA-B51(+) and HLA-B51(-) individuals among index patients, relatives with BS and healthy relatives. When BS patients compared to their first, second, third and fourth degree BS relatives, average methylation levels were not statistically different.

As a conclusion for all data, HLA-B51 frequency was not significantly different among BS patients and healthy relatives. However, HLA-B51 frequency for healthy controls was statistically different as being lower according to other three groups (p=0.0112, p=0.0107, p=0.0003). These results suggested that HLA-B51 allele positivity is not the only factor. All the same, BS patients showed statistically significant higher methylation level when compared with all healthy individuals (p=0.0002).These findings indicated that epigenetic factors may be involved in BS causation. Alternatively, other epigenetic mechanisms on HLA-B51 allele could also be examined. Apart from that, other genes that may be in epistasis with HLA-B51 or trans-regulatory elements, might have an effect on disease formation through HLA-B51 gene.

This study requires further researches in certain aspects. For instance, HLA-B51 suballeles can be detected and compared with each other. Recent researches have shown that DNA methylation pattern may act differently through its location on the gene. So as another future aspect, for further understanding DNA methylation's effect on HLA-B gene, expression patterns for HLA-B gene can be determined and compared through methylation levels. Addition to that, in a random population consisting of sporadic BS patients, HLA-B51 frequency and its methylation and expression levels can be determined, thereby sample size would be increased.

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APPENDICES

APPENDIX A

Laboratory Equipment

Balances

Precisa 620C SCS Precisa

BJ 610C

Centrifuges

Sigma 1-13 B. Braun International

Allegra 25R Centrifuge Beckman

Coulter

Pipettes

Gilson Pipetman 20 µL, 200µl, 1000 µl

PCR machine

Techne 512

Vortex

HerdolphReax top

Heat Block

Dri-Block® DB-2D- Bibby Scientific Ltd

Serological Pipette

5, 10, 25 ml Greiner bio-one

CELLSTAR® **Spectrophotometer**

NANODROP-Thermo Scientific

EDTA Blood Tube

VACUTEST- ARZERGRANDE

Examination Gloves

BROCHE

APPENDIX B

Chemicals

Agarose

Applichem

dNTP

Fermentas

EDTA

Applichem

Ethanol

Merck

EtBr

Amresco

$MgCl_2$

Fermentas

Tris-Base

Amresco

qMethyl OneStep Kit

Zymo Research

DNA Isolation Kit for Mammalian Blood

Roche Diagnostics

10X PCR Buffer

Fermentas

APPENDIX C

Preperation of Buffers

TE Buffer
Tris base
10 mM EDTA 1 mM
Add ddH2O 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 ddH2O to 1 liter and adjust the pH to 8.0

Mini Agarose Gel (1%)
Agarose 0.5g
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 1.5g
TBE buffer (1X) 150 mL
Add 4.5 µL EtBr (final concentration: 0.5 mM) before pouring the gel into tray.

APPENDIX D

INFORMED CONSENT FORM

Sağlık Bakanlığı Türkiye İlaç ve Tıbbi Cihaz Kurumu	**İLAÇ, BİYOLOJİK VE TIBBİ ÜRÜNLER BAŞKAN YARDIMCILIĞI KLİNİK İLAÇ ARAŞTIRMALARI DAİRE BAŞKANLIĞI** ASGARİ BİLGİLENDİRİLMİŞ GÖNÜLLÜ OLUR FORMU	**Doküman Adı:** KADB-F14-R.00
		Yayın Tarihi: 09.04.2012
		Sayfa No: 49/3
		Onaylayan: DB

1. Davet edildiğiniz "*Ailesel Behçet Sendromunda HLA-B5 Geninin Genetik ve Epigenetik Analizleri*" isimli çalışma bir araştırma projesidir.

2. Çalışmanın amacı ülkemizde yaygın olarak görülen Behçet Sendromuyla (BS) ilişkili olduğu düşünülen HLA-B5 geninin metilasyon seviyesinin ve bu genin aktivitesinin araştırılmasıdır.

3. Araştırmada gönüllülere uygulanacak herhangi bir tedavi bulunmamaktadır.

4. Araştırmaya katılmayı kabul ederseniz görevlendirilmiş bir hekim tarafından muayene edileceksiniz ve klinik bulgularınız kaydedilecektir. Muayene sonucunda doktorunuz uygun görürse çalışmaya alınacaksınız. Katılan gönüllülerin kollarından biri 2-3ml diğeri 5-6 ml olmak üzere (2 tüp) kan alınacaktır.

5. Sizden alınan kanların beyaz kan hücreleri ayrılarak DNA ve RNA izolasyonu yapılacaktır. Elde edilen DNA örneklerinden HLA-B5 geninin metilasyon analizleri yapılacak, RNA örneklerinden ise HLA-B5 geninin aktivitesine bakılarak Behçet Sendromuna olan etkisi ortaya çıkarılacaktır.

6. Kan alınması sırasında oluşabilecek riskler: 1) İğne batmasına bağlı olarak acı duyabilirsiniz. 2) Düşük bir ihtimal olarak iğne batması sonucu kanamanın uzaması ve enfeksiyon riski bulunmaktadır.

7. Araştırmada gönüllü açısından hedeflenen herhangi bir klinik yarar bulunmamaktadır.

8. Bu çalışmaya katılmanız için sizden herhangi bir ücret talep edilmemektedir. Bunun yanı sıra size bir ödeme yapılmayacaktır.

9. Bağlı bulunduğunuz Sosyal Güvenlik Kurumuna (SGK) herhangi bir ücret ödemeniz gerekmemektedir.

10. Araştırmaya katılımınız sizin isteğinize bağlıdır ve istediğiniz zaman araştırmaya katılmayı reddedebilir veya araştırmadan çekilebilirsiniz.

11. Sizinle ilgili tüm kimlik bilgileri, klinik bulgular ve elde edilecek deneysel bulgular gizli kalacak ve kimseyle paylaşılmayacaktır. Araştırma konularının yayınlanmasında dahi kimlik bilgileriniz kimseyle paylaşılmayacaktır.

12. Araştırma konusuyla ilgili ve araştırmaya katılmaya devam etme isteğiniz etkileyebilecek yeni bilgiler edinildiğinde gönüllü veya yasal temsilcisi bilgilendirilecektir.

13. Sizden bir defaya mahsus olarak kan alınması planlanmaktadır.

14. Araştırmaya 200 kişilik hasta grubunu ve 200 kişilik sağlıklı kontrol grubunu oluşturacak bireylerin katılması planlanmaktadır.

15. Araştırmaya katılıcak gönüllünün kimliğini ortaya çıkaracak kayıtlar gizli tutulacak, araştırma sonuçlarının yayımlanması halinde gönüllünün kimliği gizli kalacaktır.

16. Elde edilen kan örnekleri Behçet Sendromuyla ilişkilendirilmiş HLA-B5 geninin genetik ve epigenetik analizlerinde kullanılacaktır.

17. Çalışmada bulunan analizle İstanbul Teknik Üniversitesi Moleküler Biyoloji ve Genetik Bölümü laboratuarlarında gerçekleştirilecektir. Örnekler hiçbir şekilde yurtdışına gönderilmeyecektir.

Yukarıda gönüllü kişeye araştırmaya katılmadan önce verilmesi gereken bilgileri gösteren metni okudum. Yukarıda konusu ve amacı belirtilen araştırma ile ilgili yazılı ve sözlü açıklamalar yapıldı. Karar vermem için yeterli süre tanındı. Bu koşullarla söz konusu araştırmaya kendi rızamla katılmayı kabul ediyorum. 'Ailesel Behçet Sendromunda HLA-B5 Geninin Genetik ve Epigenetik Analizleri' araştırması kapsamında alınan kan örneklerimin;

- *Sadece yukarıda bahsi geçen araştırmada kullanılmasına izin veriyorum.*
- *İleride yapılması planlanan tüm araştırmalarda kullanılmasına izin veriyorum.*
- *Hiçbir koşulda kullanılmasına izin vermiyorum.*

18. *Söz konusu araştırmaya, hiçbir baskı ve zorlama olmaksızın kendi rızamla katılmayı kabul ediyorum.*

Bu formun imzalı bir kopyası bana verilecektir.

Gönüllünün,
Adı-Soyadı:
Adresi:
Tel-Faks:

Tarih ve İmza:

Velayet veya vesayet altında bulunanlar için veli veya vasinin,
Adı-Soyadı:
Adresi:
Tel-Faks:
Tarih ve İmza:

Açıklamaları yapan araştırıcının,
Adı-Soyadı:
Görevi:
Adresi:
Tel-Faks:
Tarih ve İmza:

Bir sorunla karşılaşıldığında aşağıdaki numaralardan proje sorumlularına ulaşabilirsiniz;
Doç. Dr. Eda TAHİR TURANLI: 0532 524 6521
BSc. Pelinsu KÖPRÜLÜ: 0534 546 86 34

APPENDIX E

Pedigrees continue;

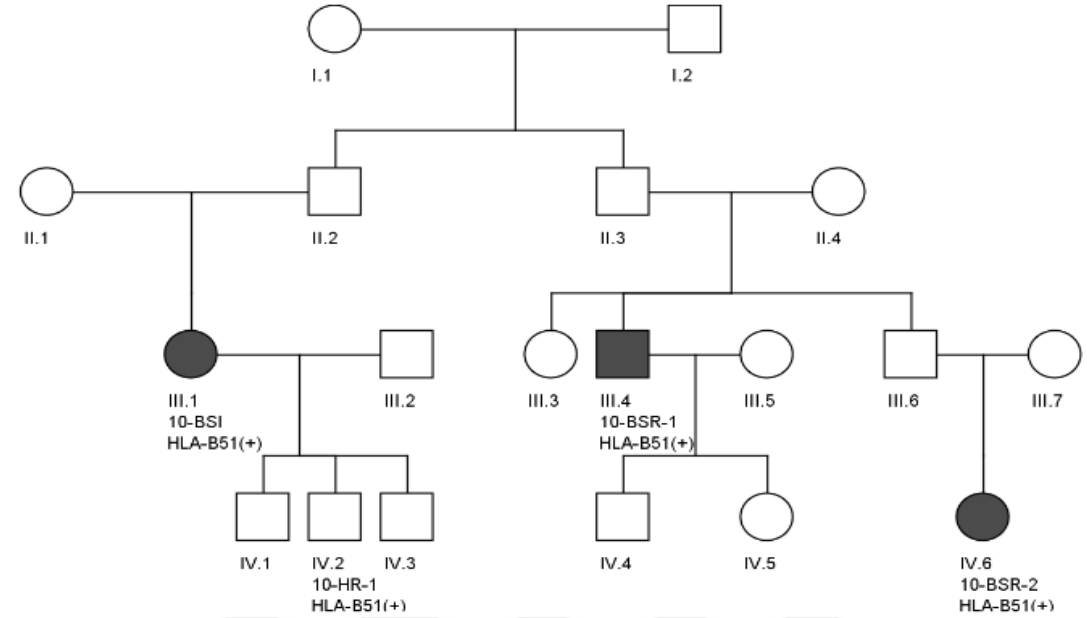

Figure E.1: Family 10: 10-BSI: index patient, HLA-B51(+); 10-BSR-1: third degree relative with BS, HLA-B51(+); 10-BSR-2: third degree relative with BS, HLA-B51(+); 10-HR-1: first degree healthy relative, HLA-B51(+).

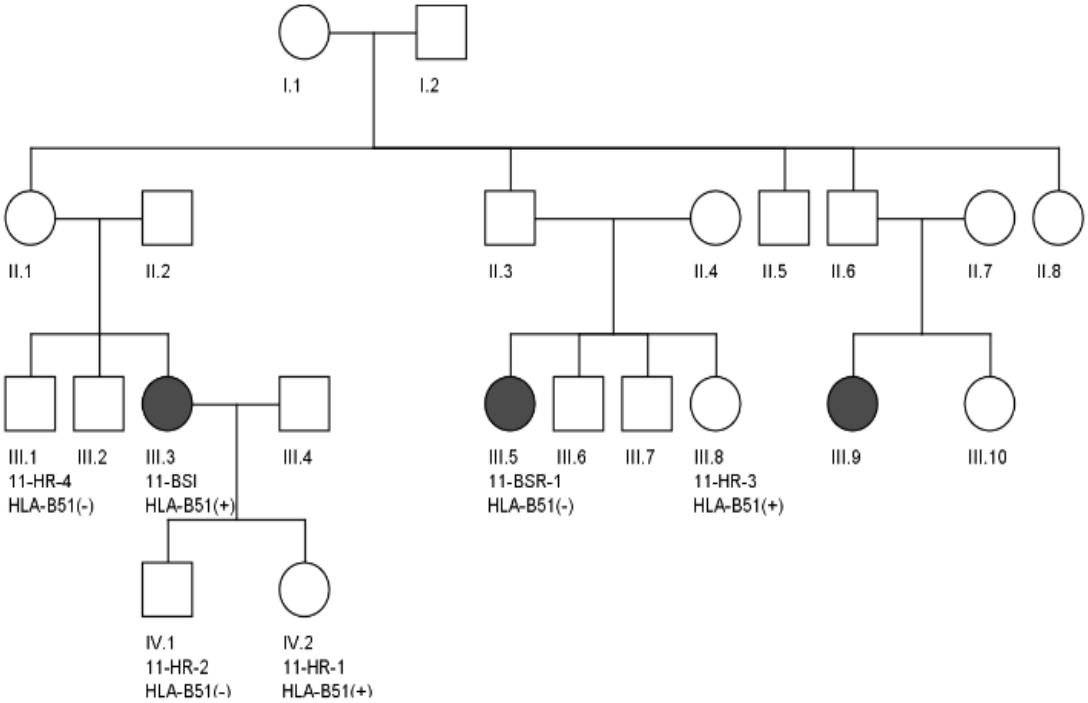

Figure E.2: Family 11: 11-BSI: index patient, HLA-B51(+); 11-BSR-1: third degree relative with BS, HLA-B51(-); 11-HR-1: first degree healthy relative, HLA-B51(-); 11-HR-2: first degree healthy relative, HLA-B51(-); 11-HR-3: third degree healthy relative, HLA-B51(+); 11-HR-4: first degree healthy relative, HLA-B51(-).

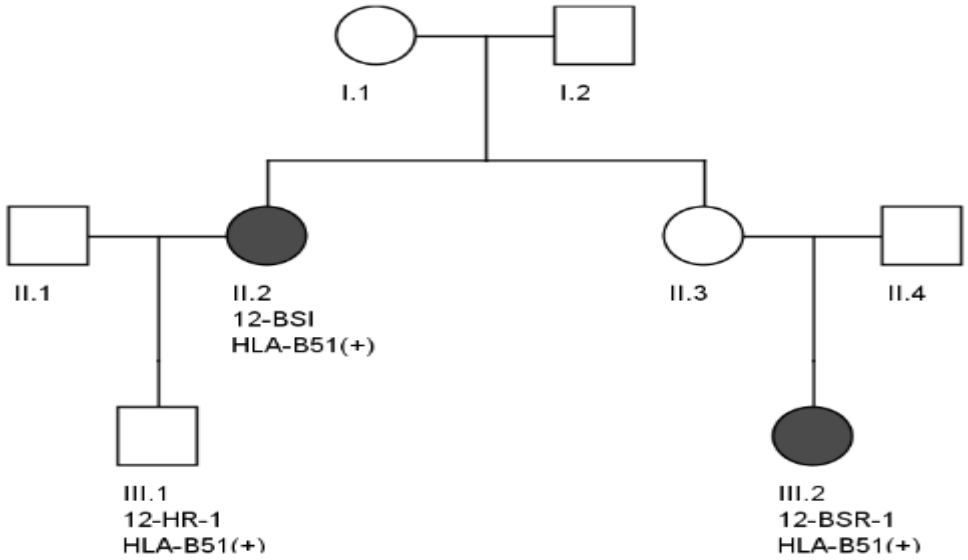

Figure E.3: Family 12: 12-BSI: index patient, HLA-B51(+); 12-BSR-1: second degree relative with BS, HLA-B51(+); 12-HR-1: first degree healthy relative, HLA-B51(+).

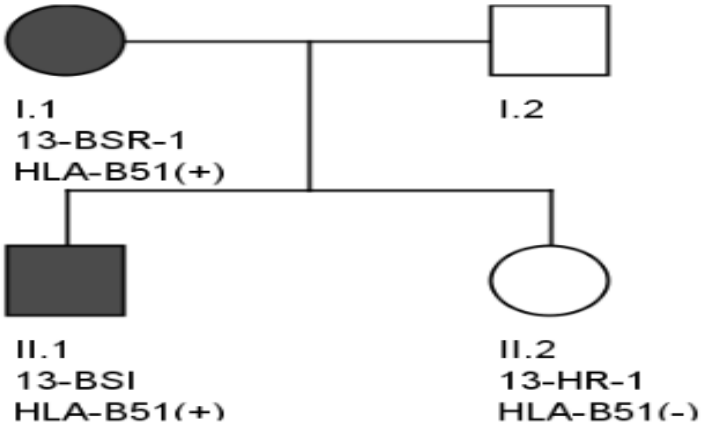

Figure E.4: Family 13: 13-BSI: index patient, HLA-B51(+); 13-BSR-1: first degree relative with BS, HLA-B51(+); 13-HR-1: first degree healthy relative, HLA-B51(-).

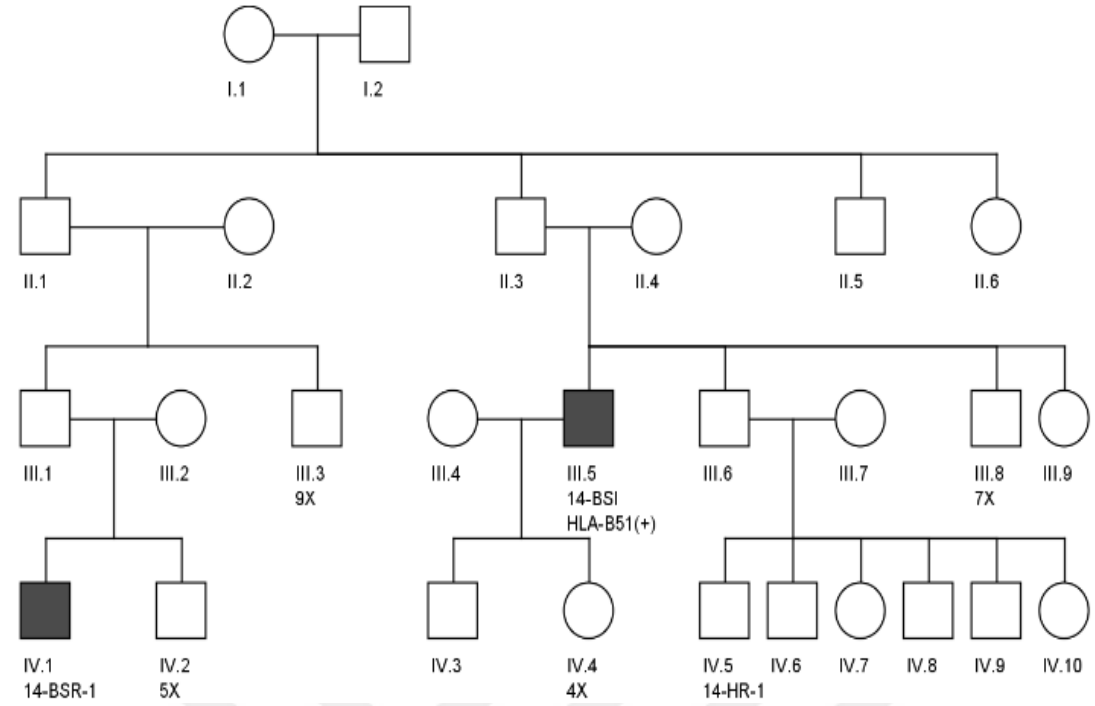

Figure E.5: Family 14: 14-BSI: index patient, HLA-B51(+); 14-BSR-1: fourth degree relative with BS, HLA-B51(+); 14-HR-1: second degree healthy relative, HLA-B51(-).

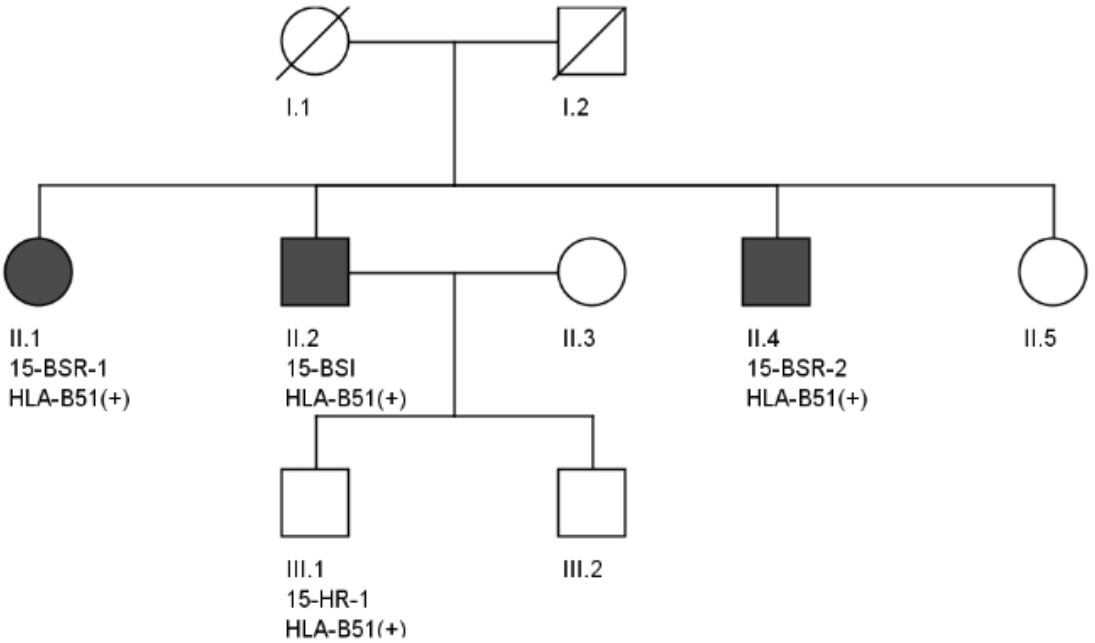

Figure E.6: Family 15: 15-BSI: index patient, HLA-B51(+); 15-BSR-1: first degree relative with BS, HLA-B51(+); 15-BSR-2: first degree relative with BS, HLA-B51(+); 15-HR-1: first degree healthy relative, HLA-B51(+).

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