MUTATION PROFILE OF HEMOPHILIA A PATIENTS WITH INHIBITORS AND ASSOCIATION OF INTERLEUKIN AND CYTOKINE GENE POLYMORPHISMS WITH INHIBITOR DEVELOPMENT

by

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To my parents

and

to Ömer Fidancı

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ABSTRACT

MUTATION PROFILE OF HEMOPHILIA A PATIENTS WITH INHIBITORS AND ASSOCIATION OF INTERLEUKIN AND CYTOKINE GENE POLYMORPHISMS WITH INHIBITOR DEVELOPMENT

Hemophilia A (HA) is an X linked recessive bleeding disorder characterized by qualitative and quantitative deficiency in the factor VIII (FVIII) protein, mainly caused by Factor 8 (*F8*) gene mutations. A severe complication in the replacement therapy of HA patients is the development of allo-antibodies (inhibitors) against FVIII which neutralize the substituted FVIII. Genetic risk factors along with *F8* gene mutations influence the development of inhibitors. Interleukins and cytokines such as IL4, IL5, IL10, TGFB1 and IFNG that are involved in the regulation of B lymphocyte development are possible targets as other genetic risk factors. The aim of this dissertation was to reveal the *F8* gene mutation profile of severe HA patients who developed inhibitors using various methods to assess the possible associations between 9 selected interleukin and cytokine gene polymorphisms with inhibitor development in HA patients with a null mutation in the *F8* gene. The most prevalent mutation in inhibitor patients was intron 22 inversion followed by nonsense mutations and large deletions with major effects on FVIII function. Therefore, severe HA patients were screened for intron 22 inversion to constitute inhibitor (+) and inhibitor (–) patient subgroups to carry out a case-control association study. A significant association with the T-allele of rs2069812 located in IL5 gene promoter and patients with inhibitors was found with a *p*-value of 0.0251. The TT genotype was also significantly associated with the inhibitor (+) patient group with a *p*-value of 0.0082 and OR of about 7, suggesting that the T-allele as the recessive susceptibility allele and C-allele was the dominant protective allele. The present findings are highly informative about the role played by the polymorphisms in genes involved in B lymphocyte development as genetic risk factors in antibody development in severe HA patients with null mutations and paves the way for furthe studies in the field.

ÖZET

İNHİBİTÖRLÜ HEMOFİLİ A HASTALARININ MUTASYON PROFİLİ VE İNTERLÖKİN VE SİTOKİN GEN POLİMORFİZMLERİ İLE İNHİBİTÖR GELİŞİMİ İLİŞKİSİ

Hemofili A, pıhtılaşma faktörlerinden Faktör VIII'in (FVIII) nitelik veya nicelik olarak eksikliğinden kaynaklanan, büyük ölçüde faktör 8 (*F8*) gen mutasyonlarının sebep olduğu X-kromozomuna bağlı çekinik kanama bozukluğudur. Hemofili A hastalarının replasman tedavisinde FVIII'i nötralize eden FVIII antikorların (inhibitör) oluşması ciddi bir komplikasyondur. *F8* mutasyonları ile birlikte başka genetik risk faktörleri de inhibitor gelişimini etkilemektedir. Bunlar arasında B-lenfositlerinin regülasyonunda yer alan IL4, IL5, IL10, TGFB1 ve IFNG gibi interlökin ve sitokinler diğer genetik risk faktörleri olabilecek hedeflerdir. Bu tezin amacı inhibitor geliştiren ağır hemofili hastalarında çeşitli yöntemlerle *F8* mutasyon profilini ortaya çıkarmak ve bunu takiben, FVIII yapılmaması ile sonuçlanan *F8* mutasyonlu inhibitör geliştiren HA hastalarında 9 seçilmiş interlökin ve sitokin gen polimorfizmleri ile inhibitor gelişimi arasındaki ilişkiyi irdelemektir. İnhibitörlü hastalarda en sık rastlanan FVII işlevini önemli ölçüde etkileyen mutasyonlar, sırasıyla, intron 22 inversiyonu, anlamsız mutasyon ve büyük delesyonlardır. Bu sebeple, bir hasta-kontrol ilişkisi çalışması için inhibitor (+) ve inhibitor (-) hasta altgrupları oluşturmak için ağır HA hastalarında intron 22 inversiyonu taranmıştır. IL5 geni promotör bölgesinde yer alan rs2069812'nin T aleli ile inhibitörlü hastalar arasında *p*- değeri 0.0251 olan önemli bir ilişki bulunmuştur. TT genotipinin de 0.0082 *p*- değeri ve OR=7 ile inhibitör (+) grubu ile ilişkili olması T-alelinin çekinik yatkınlık aleli ve C-alelinin baskın koruyucu alel olduğunu düşündürmektedir. Bu bulgular B lenfosit gelişiminde yer alan gen polimorfizmlerinin FVIII yapımı olmayan inhibitörlü ağır HA hastalarında oynadığı rol hakkında önemli bilgi vermekte ve bu alanda ileri çalışmalara önderlik etmektedir.

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A	Adenine
C	Cytosine
E	Exon
G	Guanine
M	Marker
Q	Histidine
R	Arginine
T	Thymine
W	Tryptophan
X^2	Chi square
Xq28	Region 2–8 of the long arm of the X chromosome
Y	Tyrosine
FIX	Factor X
FIXa	Activated factor IX
FV	Factor V
FVa	Activated factor V
FVII	Factor VII
FVIIa	Activated factor VII
FVIII	Factor VIII
FVIII:C	Factor VIII coagulant activity
FVIIIa	Activated factor VIII
FX	Factor X
FXa	Activated factor X
FXI	Factor XI
FXII	Factor XII
FXIIa	Activated factor XII
FXIII	Factor XIII
FXIIIa	Activated factor XIII
GPIb/IIIa	Glycoprotein Ib/IIIa

GPIb/IX	Glycoprotein Ib/IX
Asp	Asparagine
bp	Base pair
BPB	Bromophenol blue
Ca	Calcium
Cl	Chloride
C/EBP	Enhancer binding protein
Ca	Calcium
cDNA	Complementary deoxyribonucleic acid
CEN	Centromere
CNV	Copy number variation
Cp	Cross point
C-terminus	Carboxyl terminus
CSGE	Conformation sensitive gel electrophoresis
Del	Deletion
Df	Degree of freedom
DGGE	Denaturing gradient gel electrophoresis
dH_2O	Distilled water
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotides
dsDNA	Double stranded deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetate
EGF1	Epidermal growth-like domain 1
EGF2	Epidermal growth-like domain 2
ER	Endoplasmic reticulum
ESE	Exonic Splicing Enhancer
EtBr	Ethidium bromide
EtOH	Ethanol
F8	Factor VIII gene
F8A	Factor VIII associated gene a in intron 22
F8B	Factor VIII associated gene b in intron 22

F9	Factor IX gene
Gla	γ-carboxy glutamic acid
Glu	Glutamine
HAMSTeRS	Haemophilia A mutation search test and resource site
His	Histidine
HMWK	High molecular weight kininogen
HNF-4	Hepatic nuclear factor-4
HRM	High Resolution Melting
HUM	Human
IL4	Interleukin 4
IL5	Interleukin 5
IL10	Interlekin 10
kb	Kilo base
kDa	Kilo dalton
LD	Linkage disequilibrium
Mb	Mega base
$MgCl_2$	Magnesium chloride
min	Minute
mRNA	Messenger RNA
NaCl	Sodium chloride
ng	Nano gram
OR	Odds ratio
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
RE	Restriction enzyme
RNA	Ribonucleic acid
rpm	Revolutions per minute
RR	Relative risk
RT-PCR	Reverse Transcriptase PCR
SDS	Sodiumdodecylsulphate
sec	Second
SSCP	Single strand conformation polymorphism
SNP	Single nucleotide polymorphism

Taq	Thermus aquaticus
TBE	Tris-Boric acid-EDTA
TDT	Transmission disequilibrium test
TEL	Telomere
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TGFB1	Transforming growth factor 1
TNF	Tumor necrosis factor
UTR	Untranslated region
UV	Ultra violet
Val	Valine
VBP1	von Hippel-Lindau binding protein 1
VNTR	Variable tandem repeat
vWD	von Willebrand disease
vWF	von Willebrand factor
vWF:Ag	von Willebrand factor antigen
WP	Palade bodies

1. INTRODUCTION

1.1. Hemophilia A

Hemophilia A is an X linked recessive bleeding disorder characterized by qualitative and quantitative deficiency in the coagulation factor VIII (FVIII) protein. The frequency of this disorder is one in 5000 male births. According to the levels of FVIII concentration measured by coagulation assays (FVIII:C) and clinical symptoms, hemophilia A patients are classified as mild, moderate and severe. FVIII activity is < 0.01 IU/ml in severe patients, 0.01-0.05 IU/ml in moderate patients and 0.05-0.4 IU/ml in mild patients (Jacquemin *et al*., 2002). Most severely affected patients need regular replacement therapy. In moderately and mildly affected patients, bleeding is observed after surgery and/or minor injuries. They can lead a normal life and need occasional treatments mainly during and after surgeries (Bolton-Maggs and Pasi, 2003). About two thirds of cases have a family history and one third of cases are sporadic.

The amount of FVIII circulating in plasma is about 100 to 200 ng/ml. In hemophilia A, hemostasis fails in stabilization of the fibrin clot since sufficient amount of thrombin generation cannot be performed and bleeding continues. Depending on the severity of the disease patients are subject to bleeding episodes. Bleeding episodes can be seen into joints, muscles or inner organs. Hemorrhage into central nervous system (CNS) causing death is seen in 20 per cent in hemophilic patients.

In hemophilia A carrier females, the FVIII residual activity is about 50 per cent. Rare homozygous females have similar clinical features with hemizygous male patients (Graw *et al*., 2005). Hemophilia A is transmitted from carrier females to their sons. All daughters of affected patients are carriers of the disease and their sons have a 50 per cent risk of being a patient. Hemophilia in females resulting from abnormal X-inactivation, Turner's syndrome, structural abnormality of the X chromosome or extreme lyonization are also rare cases (Wadelius *et al*., 1993). The molecular basis of hemophilia A has been extensively studied over the last 20 years and the gene coding for the FVIII protein has

been analyzed to elucidate the defective forms of the gene by various groups (Goodeve and Peake, 2003).

There are three main categories of molecular causes of FVIII deficiency. The first major one consists of mutations in the factor 8 (*F8*) gene, the second one comprises mutations in proteins interacting with FVIII protein and a yet third group exists with unkown mutations in the *F8* and modifier genes (Oldenburg and El-Maarri, 2006).

1.2. Factor VIII Protein and Structure

FVIII protein is mainly expressed in sinusoidal endothelial cells, Kupffer cells and to a lesser extent in hepatocytes. Some expression has also been found in glomeruli and tubular epithelial cells in the kidney (Oldenburg and El-Maarri, 2006).

FVIII is a cofactor that plays an essential role in the activation of FX by FIXa in the presence of calcium ions and phospholipids. FVIII circulates as an inactive precursor that is activated at the time of haemostatic challenge, via the intrinsic or extrinsic pathways of the coagulation cascade (Thompson, 2003) (Figure 1.1). In circulation, FVIII interacts with von Willebrand factor (vWF). During activation it leaves the vWF and acts as a cofactor for factor IX (FIX) and participates in the formation of Xase complex (Oldenburg *et al*., 2004).

FVIII is a glycoprotein synthesized as a mature single chain polypeptide of 2351 amino acids. After 19 amino acids signal peptide is removed, it is secreted as a 300 kilo dalton (kDa) protein consisting of three A domains A1, A2 and A3, two C domains C1 and C2 and a unique B domain (Wang *et al*., 2003). FVIII is a dimeric protein, consisting of a light chain, which is 80 kDa, and a heavy chain that is 90-250 kDa. The light chain contains A3, C1, and C2 domains; the heavy chain contains A1, A2 and part of B domains. Three small amino acid sequences: a1, a2 and a3 connect A1 to A2, A2 to the N terminal of the B domain and the carboxyl terminal of the B domain to A3, respectively (Table 1.1). Thus, the FVIII sequences are arranged in the order NH2-A1-a1-A2-a2-B-a3-A3-C1-C2-COOH (Vehar *et al*., 1984) (Figure 1.2)

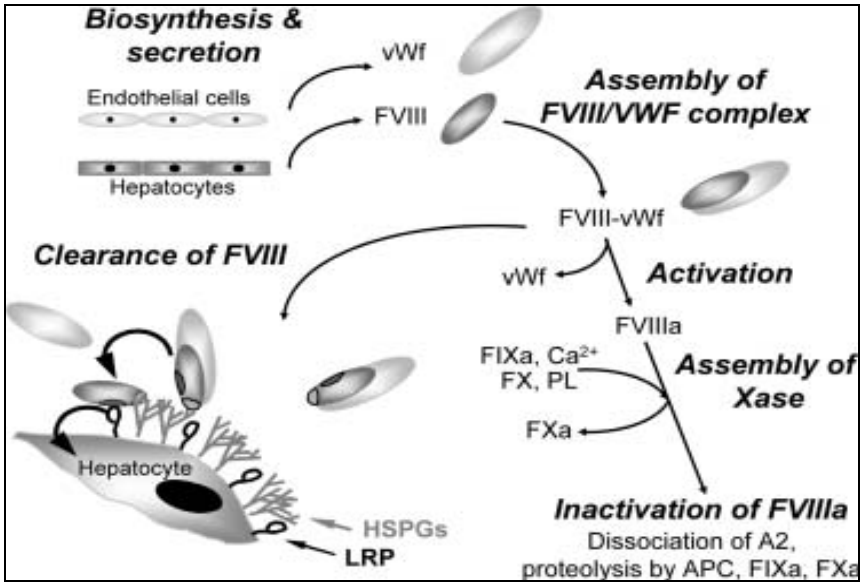


Figure 1.1. The life cycle of FVIII protein (Oldenburg *et al*., 2004)

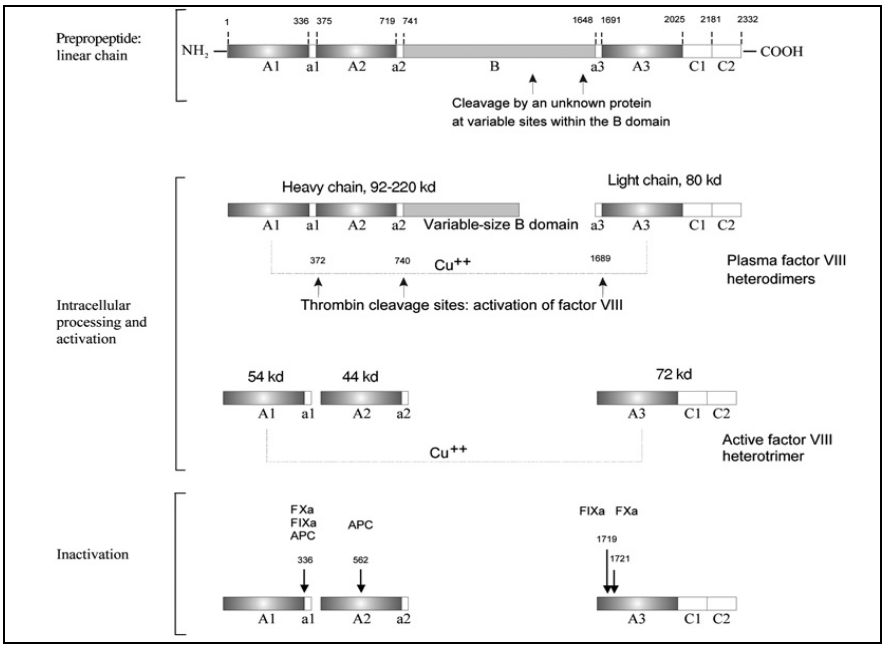


Figure1.2. The linear structure of factor VIII protein (Oldenburg and El-Maarri, 2006)

The three A domains of FVIII are homologous with FV and ceruloplasmin. A2 domain contains sequences that are binding sites for FIXa (residue 558-565). In this site FIX binds to FVIII with its protease domain to form the full tenase complex. FIXa can also bind to FVIII from A1 (residue 337-372) and A3 (residue 1811-1818). Bindings of FVIII to phospholipids are mediated with the C2 domain. In addition C2 domain contains important binding sites for vWF and FXa. The other important interaction site for FX is on

the A1 (residue337-372) (Figure 1.3) (Saenko *et al*., 1999). B domain of FVIII is the largest part of the FVIII and shows a little similarity with FV B domain sequences. The function of this domain is not well understood and just before secretion, it is cleaved from the protein at variable sites (Pittman *et al*., 1994).

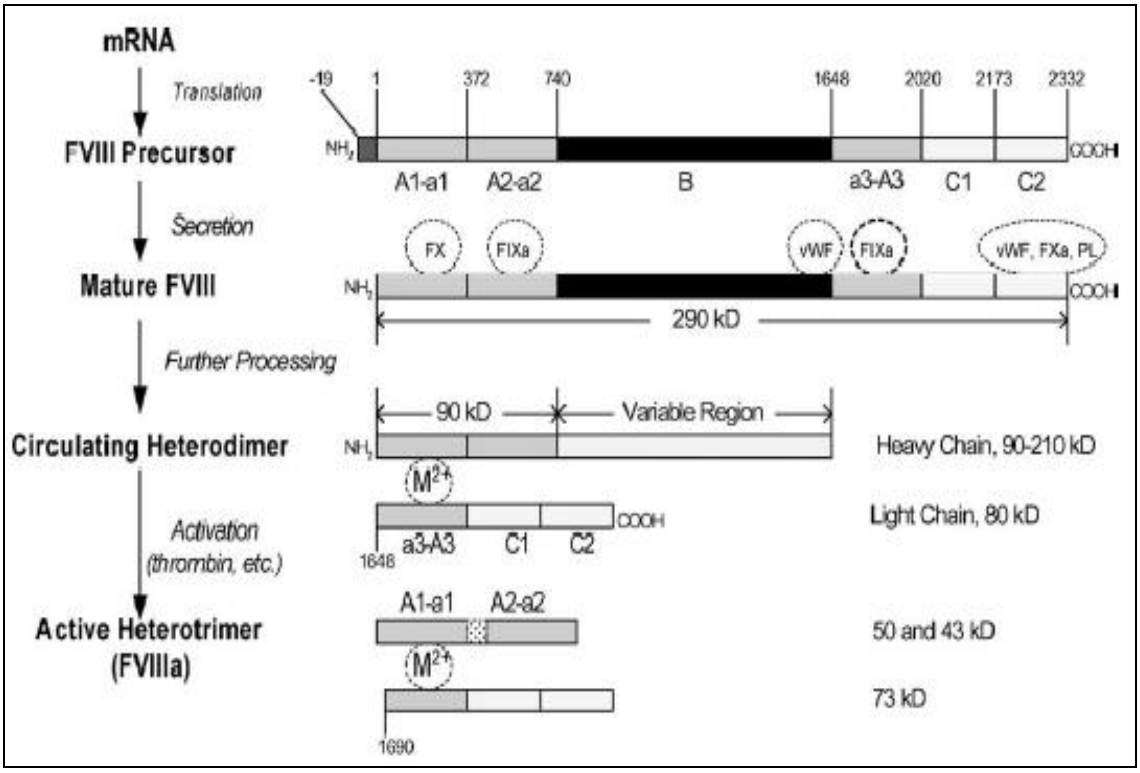


Figure1.3. Protein-protein interactions of FVIII

The activation of FVIII begins with the cleavage of thrombin from a1 (residue 372), a2 (residue 740) and a3 (residue 1689) of the protein. With this cleavage process FVIII leaves its bulky B domain and form its light and heavy chain. In the presence of magnesium (Mg^{2+}) the chains are associated and circulate in plasma as a heterodimer complex (Jacquemin *et al*., 2002).

Table 1.1. Factor VIII protein domains (Vehar *et al*., 1984)

Domain	Amino Acid Number	Nucleotides	Exons	Interaction with Other Proteins
A1	1-336	58-1066	1-8	FX
a1	337-374	1067-1118	8	
A2	375-719	1181-2215	8-14	S5558-Q565 FIX Protease Domain
a2	720-740	2216-2278	14	
B	741-1648	2279-5002	14	
a3	1649-1690	5003-5128	14	vWF
A3	1691-2025	5129-6133	14-20	E1181-K1818 FIX FGF1 Domain
C1	2026-2180	6134-6598	20-23	
C2	2181-2332	6599-7054	24-26	vWF, FXa, Phospholipid surface

1.3. Factor 8 Gene

The *F8* gene, (MIM# 306700) cloned in 1984 and localized to the distal band on the long arm of the X chromosome to Xq28, is one of the largest genes spanning 186 kilo base (kb) with 26 exons. Size of exons ranges from 69 to 3106 base pairs (bp). Exon 14 which encodes the B domain is the largest exon. The organization of the gene is very complex due to its large intronic sequences that vary from 0.2 kb to 32 kb.

Intron 22, the largest intron contains two genes, *F8A* and *F8B* located in the int22h-1 region (Figure 1.4). Int22h-1 has two homologous copies at about 500 kb upstream to *F8* gene. There is another inverted repeat region in intron 1 (int1h) and at about 140 kb upstream to the gene. Both int22h-1 and int1h are involved in intragenic homologous recombinations giving rise to inversion mutations.

The mRNA of the *F8* gene is approximately 9.5 kb and alternative splicing is not seen except exclusion of exon 19. The mRNA codes for the 2332 amino acid-protein FVIII (Thompson, 2003). *F8A* is a single exon gene transcribed in opposite direction to the *F8* gene. *F8B* is transcribed in the same direction to the *F8* gene (Graw *et al*., 2005). The two genes share a CpG island playing a role as a bi-directional promoter (Oldenburg and El-Maarri, 2006). In order to understand the function of the *F8B* gene, transgenic and chimeric mice have been used in expression studies. Interestingly, these mice showed growth retardation, microcephaly and severe ocular defects (Valleix *et al*., 1999). *F8A* has a product that interacts with a protein called Huntington-associated protein (HAP40). The significance of this interaction is not understood yet.

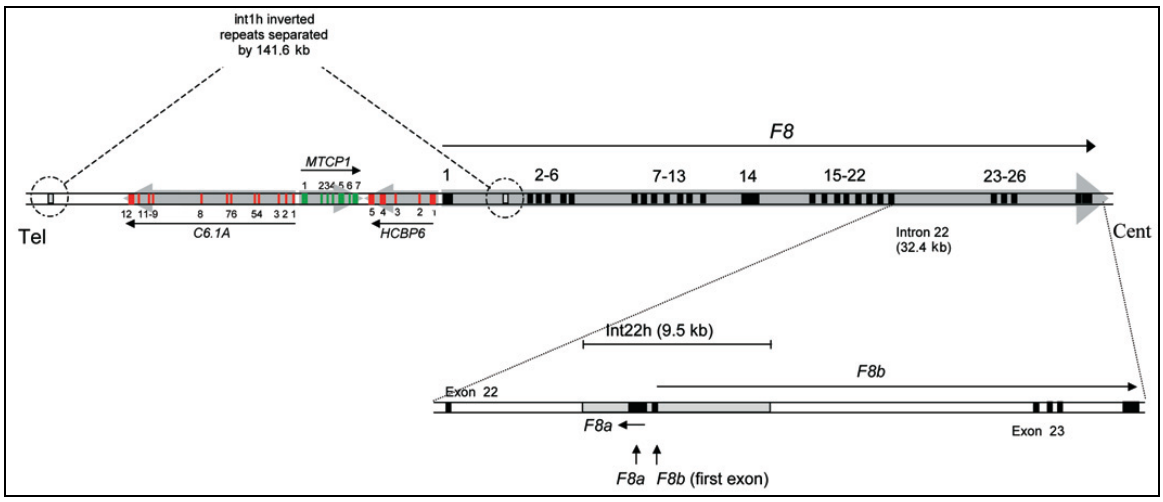


Figure 1.4. Genomic organization of the *F8* gene at Xq28 (Oldenburg and El-Maarri, 2006)

1.4. Molecular Pathology of Hemophilia A

1.4.1. Mutations in the *F8* Gene

Mutations in the *F8* gene leading to variable phenotypes of hemophilia A were described after the gene was cloned and an extensive database of the *F8* gene mutations has been established. This continuously updated resource is available online at the Haemophilia A Mutation Search Test and Resource Site (HAMSTeRS), URL http://europium.mrc.rpms.ac.uk. Mutations described in the *F8* gene are heterogenous and

can be grouped as gene rearrangements, point mutations, deletions and insertions (Goodeve and Peak, 2003).

The *F8* gene is prone to rearrangements caused by intrachromosomal homologous recombination between sequences within introns and homologous copies oriented in opposite directions out of the *F8* gene leading to inversions and causing severe hemophilia A. Up to date two different inversions have been described. Intron 22 inversion detected with Southern blot technique (Lakich *et al*., 1993) occurs between int22h-1 region (9.5 kb) in intron 22 and one of its two homologous inverted copies (int22h-2, int22h-3) that are telomeric to and 400 kb away from the *F8* gene (Naylor *et al*., 1996). With these extragenic copies two main types of inversion occur. If recombination occurs between int22h-1 and int22h-2, it is referred as the proximal type of inversion; if recombination occurs between int22h-1 and int22h-3, it is referred as the distal type of inversion. These events reverse the orientation of exons 1-22 and separate them from exons 23-26 (Figure1.5). This type of inversion affects 40-45 per cent of severe hemophilia A patients (Naylor *et al*., 1995). Another similar inversion involving repeated homologous regions of intron 1 was also reported (Bagnall *et al*., 2002) A 1041-bp sequence (int1h-1) of the intron 1 was found to be duplicated (int1h-2) and oriented in the opposite direction 140 kb apart from the *F8* gene between *C61A* and von Hippel-Lindau binding protein 1(*VBP1*) genes. This inversion separates exon 1 and partial intron 1 from exons 2-26 in about two per cent of severe hemophilia A patients (Bagnall *et al*., 2002) (Figure 1.5).

Deletions in *F8* gene vary from one base to several kilo bases and even encompass the whole gene. It has been proposed that large deletions are caused by the recombination in Alu elements in the *F8* gene. Depending on the size of the deletion, disease severity and disruption of protein function vary (Salviato *et al*., 2002). Like deletions, insertions can be large or as small. Deletion and insertion can occur at the same time, for example; with the insertion of LINE-1 element into intron 20, a large deletion of the *F8* gene (20.7 kb) is determined (Van de Water *et al*., 1998).

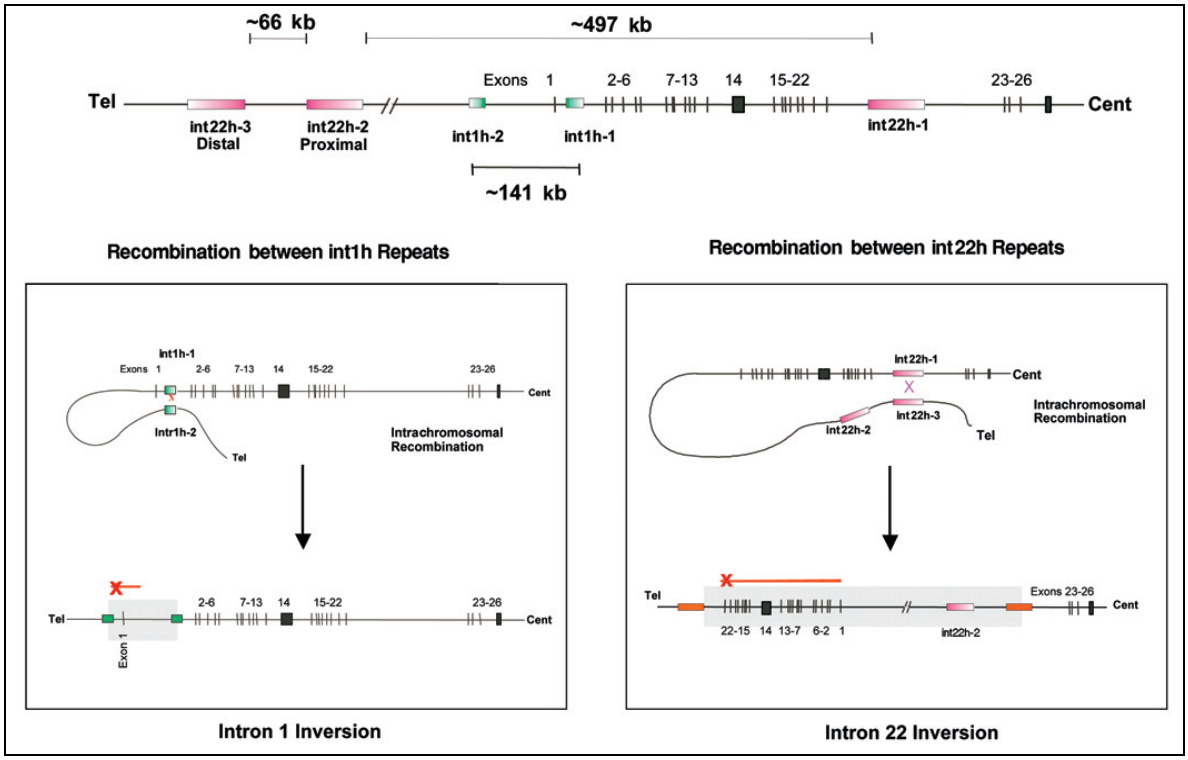


Figure 1.5. Mechanisms of intron 1 and intron 22 inversions (Oldenburg and El-Maarri, 2006)

According to HAMSTeRS database, up to date 897 unique mutations of different types have been reported in all exons of the *F8* gene. Approximately half of the point mutations occur in the CpG sites of the *F8* gene (Oldenburg and El-Maarri, 2006). The severity of hemophilia A resulting from missense mutations depends on the evolutionarily conservation of the amino acid substituted and its location in the FVIII protein. Nonsense mutations resulting in a truncated FVIII protein are associated with severe cases of hemophilia A. The message is usually destructed at the transcription level by non-sense decay (Goodeve and Peake, 2003). Splice site mutations causing the destruction or creation of mRNA splice sites are associated with variable severity of hemophilia A cases. These mutations can cause exon skipping (Gau *et al*., 2003).

1.4.2. Mutations in Interacting Proteins

In blood circulation, FVIII protein interacts with intracellular and extracellular proteins. Mutations in these proteins can cause FVIII deficiency, FV/FVIII combined deficiency or Hemophilia A like phenotype. FVIII protein interacts with chaperone

immunoglobulin binding protein (BiP) in endoplasmic reticulum. Mutations in this protein can cause hemophilia A phenotype (Graw *et al*., 2005).

In blood FVIII protein interacts with vWF (von Willebrand factor). This protein protects FVIII against proteolytic cleavage by thrombin or protein C. Mutations in the interaction sites of FVIII and vWF can cause hemophilia A like phenotype. This disease is called Type 2N (Normandy) von Willebrand disease (vWD) (Nishino *et al*., 1989).

1.4.3. Unknown Mutations

In 2 per cent of hemophilia A patients, mutations in *F8* gene can not be identified. It has been proposed that large intronic regions of *F8* gene can include some mutations causing abnormal splicing, gene rearrangements, inversions or deletions. mRNA studies of *F8* gene has not been explanatory of the hemophilia A phenotype in these cases (El-Maarri, *et al*., 2005). The existence of modifier gene effects is another possibility to explain hemophilia A phenotype in patients who do not have mutations in the *F8* gene. It should be kept in mind that polymorphisms detected in such patients could be important since synonomous single nucleotide polymorphisms (SNP) found in coding regions in *F8* gene could be affecting FVIII protein and protein translation kinetics (Komar, 2010).

1.4.4. Mutation Detection Strategy for Hemophilia A Patients

Mutation detection studies within hemophilia cohorts showed that most patients have defects in their essential *F8* gene regions. The basic strategy for mutation detection is to test for inversion mutations in severe cases and then screen for point mutations. Because of the large size of the *F8* gene, it is necessary to screen the gene region in 30 separate amplifications (Bowen, 2002). Chemical mismatch cleavage, denaturing gel gradient electrophoresis and automated direct DNA sequencing are the main methods for the screening of exonic mutations in *F8* gene (Vidal *et al.*, 2001) (Naylor *et al*., 1991).

Currently, molecular diagnosis of severe hemophilia A can be performed with an initial screening for intron 1 and intron 22 inversions by multiplex PCR, southern blot techniques, long PCR and inverse PCR respectively. Inversion negative patients are then

screened for point mutations predominantly on epitopes (inhibitor targets and functionally most significant regions) indicating A2, A3 and C2 domains by PCR amplifications of exons 8-20 and 24-26 and complete DNA sequencing. If point mutations are not observed in these regions, rest of the coding regions of the *F8* gene are then amplified and sequenced.

1.4.5. Copy Number Variation

Copy number variation (CNV) is a segment (1 Kb to several Mb) of DNA that has variable number of copies in the genome. CNVs are the genomic variants like the deletions, insertions, duplications and inversions that contribute to genetic diversity in human and other mammals (Iafrate *et al*., 2004). In other words, CNV is a DNA segment larger than 1 kb and is in different copy number in comparison with a reference genome (Redon *et al*., 2006). It has been proposed that CNV can cause fork stalling during replication (Lee et al., 2006). Factors like size, orientation, percentage similarities and the genetic distance can cause region specific repeats to be rearranged resulting in CNVs. CNVs can be detected by cytogenetic techniques like fluorescent in situ hybridization, qPCR and array comparative genomic hybridizations array (CGH).

Up to date, 5 variations for *F8* gene were detected. Variant 23331 were detected by Levy *et al*., 2007 by genome sequencing (Sanger dideoxy technology) in an individual. Variants 97109, 97110, 83871 and 83872 were found by Matsuzaki *et al*., 2009 by using custom high density oligonucleotide array in whole genome at 200 bp resolution. In this study 90 Yoruba Nigerian DNA samples were studied. Like the other genetic variants, CNVs have been found to be associated with some human diseases. Two models can be proposed for CNV-phenotype associations. The first model copy number polymorphisms (CNP) define the multiple allelic variants in genomic structure and associated with biological functions in immunity, drug response (Cooper et al., 2007). For example, copy number changes that affect the gene or the functional elements' dosage influence HIV-1/AIDS susceptibility (Gonzalez et al., 2005) and glomerulonephritis in systemic lupus erythematosus (Aitman et al., 2006). The second model is the CNVs that are in fewer allelic states. They delete or duplicate large genomic segments. Non-allelic homologous recombinations between low-copy repeats or segmental duplications give rise to these

variants. It has been reported that some rare CNVs are found in patients with autism and schizophrenia rather than those of healthy people (Sebat *et al*., 2007) (Walsh *et al*., 2008).

1.5. Inhibitors in Hemophilia A

Treatment of hemorrhages in patients with severe hemophilia A is performed with plasma-derived or recombinant FVIII replacement therapy. The major complication of this therapy is the development of antibodies (inhibitors), which inhibit FVIII activity. Inhibitor formation occurs in 20-30 per cent in severe hemophilia A patients. According to their immunological setting, these inhibitors are allo-antibodies when they appear in transfused hemophilic patients, and auto-antibodies in patients with auto-immune disease. Quantification of inhibitors is performed with the Bethesda assay that detects the level of inhibitors in patients' plasma. The level of inhibitors is measured as Bethesda Unit (BU/ml). Patients with >5 BU/ml and <5 BU/ml are defined as "low" responder (LR) and "high" responder (HR) patients, respectively, by the FVIII/IX Subcomittee of the International Society of Hemostasis and Thrombosis (ISTH) (Key, 2004). Inhibitor measurement was started in 2004 in Turkey.

1.6. Risk Factors for Inhibitor Development of Hemophilia A

Both genetic and non-genetic factors play crucial roles in the development of inhibitor against FVIII protein. Potential genetic factors are listed in (Table1.2) (Oldenburg *et al*., 2004). Genetic factors include mutations or polymorphisms within the *F8* gene, and some immune response genes like major histocompatibility complex (MHC) class I /II, interleukins and cytokines (Oldenburg *et al*., 2002). It has been found that there are some potential environmental factors that influence the development of inhibitors. These factors include FVIII therapy (age, dosage), immune system challenges (bacterial/viral infection, large muscle bleeds), antenatal FVIII exposure to maternal FVIII (Santagostino *et al*., 2005).

1.6.1. *F8* Genotype and Inhibitor Development

F8 gene mutations were shown to be a decisive risk factor in inhibitor development (Oldenburg, 2001). However, the same type of *F8* gene mutation can be seen in hemophilia A patients both with and without inhibitors. *F8* mutations can be divided into two groups in terms of their effect on the production of FVIII. One group is comprised of severe molecular defects – so called null mutations- because they do not produce any FVIII protein. These mutations include large deletions, inversions and nonsense mutations and lead to a severe hemophilia A phenotype (Zhang *et al*., 2009). Patients with this type of mutations develop inhibitors with greater than 30 per cent prevalence. The second group contains small deletions; missense and splice site mutations that result in loss of function, but not complete absence of the FVIII protein. Patients with these types of mutations have moderate or mild phenotype and develop inhibitors with less than 10 % prevalence (Oldenburg *et al*., 2002).

According to data taken from HAMSTeRS, patients with large deletions, affecting more than one domain of the FVIII protein are at the highest risk of inhibitor development (75 per cent). Nonsense mutations on the light chain cause the increase of inhibitor development much more than those on the heavy chain. The third high risk mutation is the intron 22 inversion with an inhibitor risk about 30-35 per cent (Figure 1.6).

Small deletions and insertions cause frameshift formation with a subsequent stop codon. However, the risk of inhibitor formation in this type of mutations is much lower than those of nonsense mutations. This situation was explained by the endogenous restoration of the reading frame by polymerase errors during DNA replication/RNA transcription in patients with small deletion/insertion mutations that were located at stretches of adenines. Polymerase errors cause the production of small amounts of endogenous FVIII protein. Thus, this endogenous FVIII protein production prevents inhibitor development (Young *et al*., 1997). Despite being the main mutation type of mild hemophilia A patients, missense mutations are also observed in severe hemophilia A patients and interestingly, patients with missense mutations may also develop inhibitors. Missense mutations in the C1 and C2 domains of FVIII protein have 4-fold higher risk of

inhibitor formation which states that immunogenicity of these domains are higher than other domains or epitopes (Suzuki *et al*., 1997).

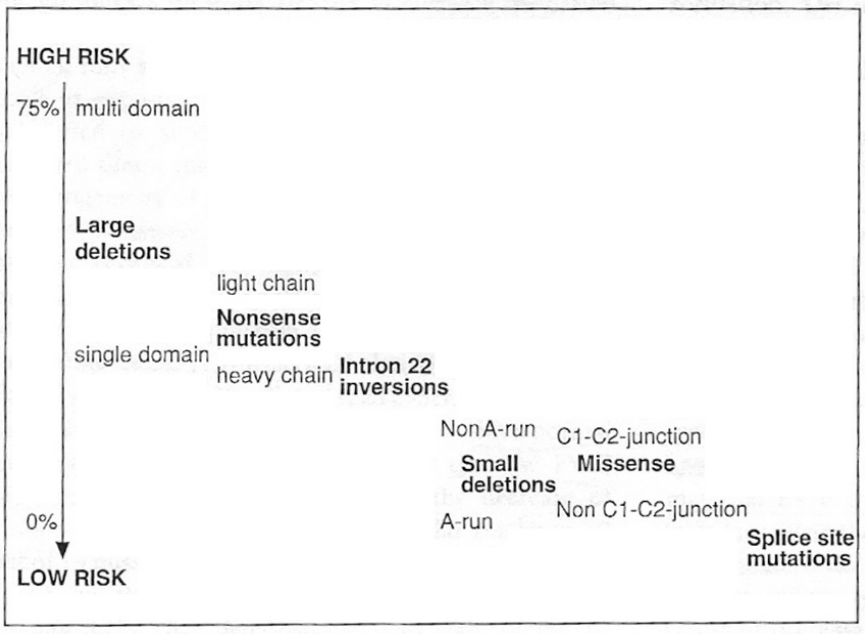


Figure1.6. Mutation types and risk of inhibitor development (Oldenburg *et al*., 2002)

1.6.2. FVIII Antibody Epitopes (Inhibitor Targets)

In the intrinsic blood coagulation cascade, FVIII protein makes major interactions with other coagulation proteins. FVIII inhibitors, which develop during the replacement therapy, interfere with important interactions of FVIII at different stages of its functional pathway. Recently, some inhibitor targets on FVIII protein so-called FVIII antibody epitopes have been characterized. A2, A3 and C2 domains of FVIII protein were found to be mostly immunogenic and A1 and B domains to be poorly immunogenic (Zhang *et al*., 2009). Inhibitor epitopes are located on the a1 region (amino acids 351-365), the A2 domain (amino acids 484-508), the a3 domain (amino acids 1687-1695), the A3 domain (amino acids 1778-1823) and the C2 domain (amino acids 2181-2243 and 2248-2312) (Figure 1.7) (Oldenburg *et al*., 2004).

FVIII inhibitors can be classified as neutralizing, non-neutralizing and catalytic antibodies. Neutralizing antibodies interfere with functional interactions of FVIII. These inhibitors interact with domains of FVIII mentioned above. Epitopes (amino acids 2181-

2243 and 2248-2312) on C2 domain of FVIII protein include phospholipid (PL) binding sites. Interaction of inhibitors on these sites interferes with FVIII protein binding to PL and prevents assembly of Xase complex. These residues and a3 domain residues are also important for binding to vWF. Interference in vWF binding causes the destabilization of FVIII and lead to proteolytic inactivation by activated protein C, FXa and FIXa (Kaveri *et al*., 2009).

Blocking of A2 domain of FVIII and FIX interaction by inhibitors with specific to A2 domain inactivates the conversion FX to FXa. Non-neutralizing antibodies bind to functionally non-significant epitopes of FVIII and cause a decrease in the half-life of FVIII protein. Catalytic antibodies have hydrolytic activity on exogenous FVIII protein. The catalyzed FVIII protein fragments vary between 70kDa and <30kDa in different patients. It was found that cleavage sites are located on the A1, A2, B, A3 and C1 domains of FVIII protein, which form the outer core of the FVIII molecule being accessible to inhibitors. (Ananyeva *et al*.,2003).

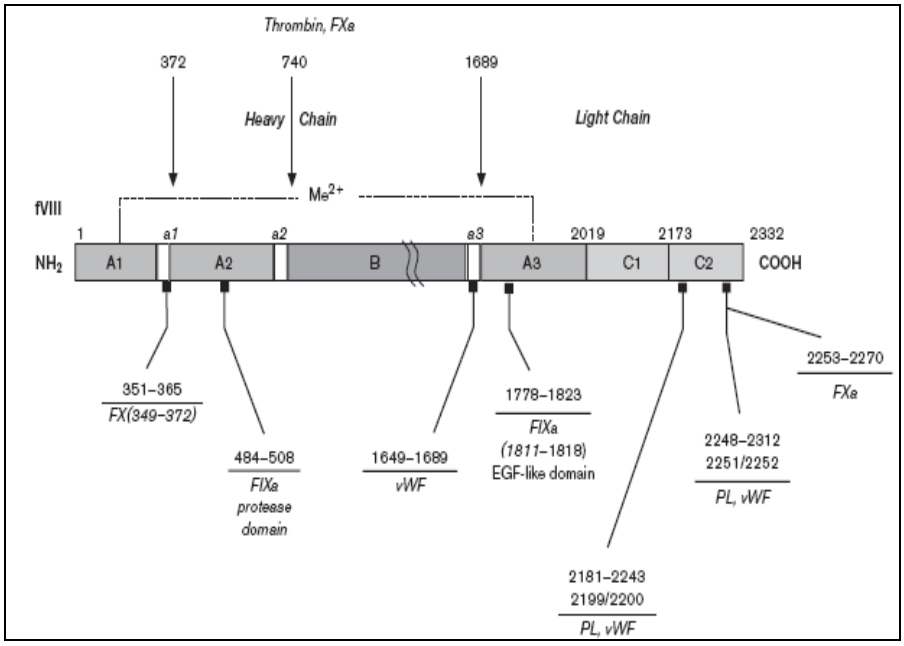


Figure1.7. Factor VIII domains and epitopes (Ananyeva *et al*., 2003)

1.6.3. Immune Response Genes as an Other Genetic Risk Factor for Inhibitor Development

The observation that African-American patients with severe hemophilia A have two fold increased risk of inhibitor formation compared with a white population group indicates that genetic risk factors other than *F8* gene mutations exist for inhibitor development (Key, 2004).

MHC class II genes DQ, DR, and DP play important role in the presentation of extracellular antigens, such as exogenous FVIII to the patient's immune system. Whereas, the MHC class I genes like A, B, C are responsible for the presentation of intracellular antigens, including endogenously truncated or immunologically altered FVIII. According to MHC class I and II genotyping studies which were recently done on a homogenous group of European patients with intron 22 inversion with inhibitor formation, some risk alleles and protective alleles were identified (Table 1.2) (Oldenburg *et al*., 2004). A3, B7, C7, DQA01102, DQB0602, DR15 were suggested as "risk" alleles as they occurred more often in inhibitor than non-inhibitor patients, and C2, DQA0103, DQB0603, DR13 alleles were suggested as "protective" alleles as they occurred less often in inhibitor than in non-inhibitor patients. However, these associations were not confirmed or excluded by other population studies such as Malmö International Brother Study (MIBS) (Zhang et al., 2009). The conclusion was that polymorphisms, especially in HLA DQ and DR loci do not make a strong contribution to the risk of inhibitor formation. These MHC class I/II alleles belonged to extended haplotypes that were also frequent and less frequent, respectively in the normal population. Haplotype inheritance can mask those MHC class I/II alleles that determine the risk of or protection from inhibitor formation.

Other genetic risk factors include some immune response genes like MHC genes and cytokine genes. The synthesis of inhibitors depends upon the activation of CD4+ (helper) T cells specific for FVIII. When FVIII is endocytosed by an antigen-presenting cell (APC), intracellular proteolytic degradation results in the generation of short component peptides. One or more of these peptides then act as T-cell epitope(s) when presented to cognate T-cell receptors on CD4+ (T helper) cells by major histocompatibility complex (MHC) II molecules located on the APC surface. In order for full and efficient activation of T helper

cells to occur, recognition of the MHC II presented peptide by the T-cell receptor must be supported by additional co-stimulatory signals between the APC and T cell. Engagement of the T-cell receptor (signal 1) without subsequent co-stimulation (signal 2) leads to failure in activation of T-helper cells.

In the presence of appropriate co-stimulation and cytokine environment however, naive CD4+ cells (Th0 cells) induce to differentiate into T helper 1 (Th1) or T helper (Th2) clones. Th1 cells are classically associated with cell-mediated functions and the synthesis of complement binding IgG subclasses (IgG1 and IgG2), whereas Th2 cells are important in the synthesis of non-complement binding antibodies (IgG4 and IgE), and providing help to B cells. Cytokines secreted by the effector Th1 [such as interleukin 2 (IL2) and interferon-γ (IFNG) and Th2 (such as IL4, 5 and 10) clones then direct B-cell synthesis of antibodies, which, in the case of FVIII, may function as inhibitors. However, Th2 cells can also down regulate B cell antibody synthesis under certain circumstances (Key *et al*., 2004) (Astermark, 2006) (Figure 1.8).

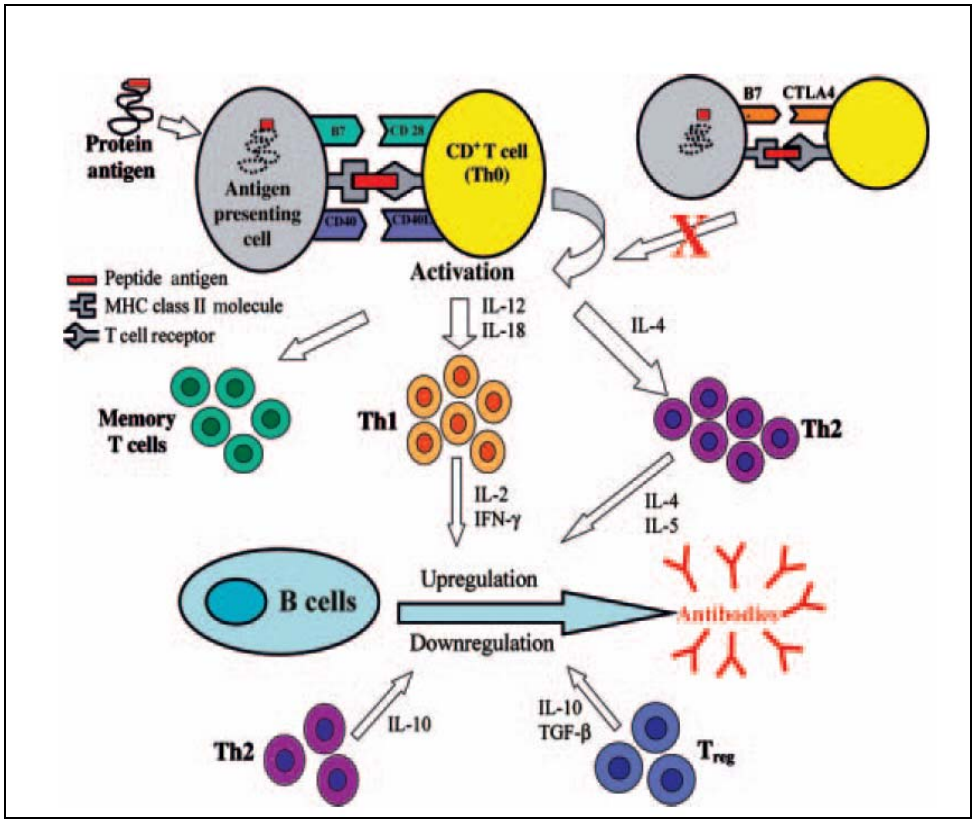


Figure 1.8. Activation of CD4+ T helper cells and B cells for antibody production (Key, 2004)

Table 1.2. Common and rare MHC Class I and Class II alleles in severe hemophilia A pateints with intron 22 inversion and inhibitor formation (Oldenburg *et al*., 2004)

Risk Alleles	**Inhibitors**	**Non-inhibitors**	**Relative Risk**
A3	11(%38)	9 (%21)	2.2
B7	14 (%48)	8 (%19)	4.0
C7	17 (%59)	16 (%38)	2.3
DQA0102	20 (%35)	16 (%19)	2.2
DQB0602	18 (%31)	12(%14)	2.7
DR15	19(%33)	17 (%20)	1.9
Protective Alleles			
C2	1(%3.4)	6 (%14)	0.2
DQA0103	1(%1.7)	12 (%14	0.1
DQB0603	0	6 (%7)	0.1
DR13	1 (%1.7)	9 (11)	0.1

Cytokines are directly or indirectly involved in antibody mediated-immune response. They may be determinants for the immune response. According to recent studies, IL1 and IL4 gene polymorphisms for example, were not found to be associated with inhibitor formation (Astermark *et al*., 2006).

On the other hand, it was found that there was a strong association between an allele with 134 bp in one of the CA repeat microsatellites (IL10.G) located in the promoter region of the IL10 gene, and development of inhibitors. The association was consistent in the subgroup of families in severe hemophilia A with inversions. IL10 is the first gene located outside the causative factor VIII gene mutation to be associated with inhibitor development. IL10 is secreted by Th2 cells and promotes B cells to differentiate and produce antibodies. It is proposed that the allele 134 is associated with enhanced secretions of IL10 in patients. The SNP in the promoter region of tumor necrosis factor alpha (TNF-α) has a strong link between inhibitor formations in hemophilia A siblings in the MIBS study (Astermark *et al*., 2006). A C/T SNP in the promoter region of the gene cytotoxic T-

lymphocyte associated protein-4 (CTLA-4) was found to be associated with inhibitor formation with 31.2 per cent of T allele- carriers (p=0.012) (Astermark *et al*., 2006).

1.7. Association Study

Association studies are used in genetics to test whether allele or genotype frequencies are different between healthy and patient groups. If there is an association, a specific genetic locus, allele or a SNP is observed frequently in an individual or a group not by chance. Genetic association studies can be performed by case-control designs, family based designs and quantitative trait association (Hirschhorn and Daly, 2005). If the case and control groups are not well matched because of the ethnicity and geographical origins, this situation can cause false-positive association due to population stratification. To avoid this stratification, two independent control groups could be used. Alternatively family based association studies can be performed. The most commonly used test for this association is transmission disequilibrium test (TDT) (Bakker *et al*., 2005) (Cordell and Clayton, 2005).

In the light of these data, it can be postulated that, the genetic variants like SNPs other than in IL10 and TNFA genes and CNVs in immune response genes may have role or may be associated with inhibitor formation in severe hemophilia A patients.

2. PURPOSE

A major complication in the replacement therapy of 20-30 per cent of severe hemophilia A patients is the development of allo-antibodies (inhibitors) against FVIII which neutralize the substituted FVIII. Several studies have shown that genetic factors, such as the type of *F8* gene mutation, race and immune response genes constitute decisive risk factors for the development of inhibitors.

The patients with common intron 22 inversion have a risk of about 30-35 per cent. Intron 22 inversions constitute a homogenous type of mutation with a well known functional consequence, that is, the lack of *F8* mRNA, and therefore, are suitable for studying other genetic risk factors in patients who develop inhibitors. Inhibitor patients with an apparently severe defect can also be included in this homogenous group provided that they are known to lack complete *F8* mRNA or protein due to the type of the causative mutation.

The aim of the study is first to investigate the *F8* gene mutation profile of severely affected patients who developed inhibitors and estimate the risk of inhibitor development due to mutation types among Turkish patients.

Secondly, the aim is to select the group of hemophilia A patients with homogenous mutations (mainly intron 22 inversions) to test whether other genetic factors are involved in inhibitor development by:

- Constituting a group of patients who develop inhibitors and lack *F8* mRNA
- Constituting an other group formed from hemophilia A patients without inhibitors and who lack *F8* mRNA
- Genotyping and comparing the frequencies of selected SNPs in interleukin and cytokine genes in the two groups and in comparison to a control group without hemophilia A phenotype.

3. MATERIALS

3.1. Patient and Control Samples

The peripheral blood samples from 34 severe hemophilia A patients with inhibitors for the first study and 256 severe hemophilia A patients for the second study were collected in the various hematology clinics within the country. The diagnosis of hemophilia A was based on clinical and hematological data. One-stage clotting assay was used for measurement of FVIII activity (Sigma Diagnostic, St. Louis, USA). All measurements were performed in duplicate. Mean ± SD values for FVIII were 113.98 ± 33.86 U/dl in controls. Values over 150 U/dl were accepted as high. The clinical criteria of Eyster were used to determine disease severity (Eyster et al, 1980). The clinical forms of patients were shown in Appendix A. The clinical data of patients of first study are shown in Appendix B and the clinical data of patients of second study are shown in Appendix C.

The Ethics Committee of Ege University Medical School approved two studies and informed consent of the patients was obtained from all analyzed subjects.

3.2. Oligonucleotide Primers and Probes

F8 gene specific primer sequences used in amplification of all exons and introns, were obtained from Williams *et al.,* 1998 and Schwaab *et al.,* 1997. *F8* gene RT_PCR primer sequences were obtained from El-Maari *et al*., 2005. New intron and exon specific primers were designed by our laboratory. All primers were purchased from IDT, USA.

3.2.1. Primer Pairs for Mutation Detection in the *F8* Gene

Sequences of *F8* exon and 5’ and 3’ UTR region specific primers, intron 1 inversion primers were given in Table 3.1.

Table 3.1. Primer pairs for mutation detection in the *F8* Gene

Primer Name	Primer Sequence
F8 P1-F	F-5’ GAG CTC ACC ATG GCT ACA TTC
F8 P1-R	R-5’ TCC TGT CAC TCC TCT TCT CAG
F8 P2-F	F-5’ AGG TCA GGA GAA AGG GCA TG
F8 P2-R	R-5’ CCC ACT GGA TTG CTC AGC AC
F8 E1-F	F-5’ AAT CCT ATC GGT TAC TGC TTA
F8 E1-R	R-5’AGC ATC ACA ACC ATC CTA AC
F8 E2-F	F-5’ TGG AAG CAT TAC TTC CAG CT
F8 E2-R	R-5’AAC TGC AAC CTC AAG ATT GG
F8 E3-F	F-5’ TGC TTC TCC ACT GTG ACC T
F8 E3-R	R-5’ ATC TAG TAA ATG TTA AGA AAT ACA
F8 E4-F	F-5’ GTA CAG TGG ATA TAG AAA GGA C
F8 E4-R	R-5’ GAT TCA GTT GTT TGT ACT TCT C
F8 E5-F	F-5’ CTT ACT GTC AAG TAA CTG ATG
F8 E5-R	R-5’ CTT CAT TCC TGA ACA GTA ATG
F8 E6-F	F-5’ TCC CAC TTA TTG TCA TGG AC
F8 E6-R	R-5’ TAC AGA ACT CTG CAG AAC AT
F8 E7-F	F-5’ GGC AAG AGC TGT TGG TTT G
F8 E7-R	R-5’ TGT CCA GTA AAT TTT ATT AAA AGT
F8 E8-F	F-5’ CCA TAT AGC CTG CAG AAC AT
F8 E8-R	R-5’ CTG ATG CTG AGC TAT GTT AG
F8 E9-F	F-5’ CTA ACA TAG CTG AGC ATC AG
F8 E9-R	R-5’ AGA TAT GTC CAT TGG AGA CAA
F8 E10-F	F-5’ CTA GCC TCA AAT TAC TAT AAT G
F8 E10-R	R-5’ ACT TTA GAC TGG AGC TTG AG
F8 E11-F	F-5’ TGC GAC TTT AGC TTC CAC TT
F8 E11-R	R-5’ ACT GAC CTA TAT TGC AAA CCA
F8 E12-F	F-5’ TGC CAT CGC TTT CAT CAT AG
F8 E12-R	R-5’ CAT TCA TTA TCT GGA CAT CAC
F8 E13-F	F-5’ AAC AAT CTA CTT TTT TGG AAG A
F8 E13-R	R-5’ CCT CCA GCA AGA GAA TGC TA
F8 E14A-F	F-5’ GAC CTG TGA TAT AAT GAT A
F8 E14A-R	R-5’ GAA AAA GTC TCA TAT TTG GC
F8 E14B-F	F-5’ CCT TGG TTT GCA GAC AGA AC
F8 E14B-R	R-5’ TGT ATT ATC AGT ACC TGC TG
F8 E14C-F	F-5’ AGC AAC AGA GTT GAA GAA AC
F8 E14C-R	R-5’ CTA ATA TAT TTT GCC AGA CT
F8 E14D-F	F-5’ AAC AAA ACT TCC AAT AAT TC
F8 E14D-R	R-5’ AGA GTT CTT TCC ATG AGT CC
F8 E14E-F	F-5’ CCC CAR TCC ACC AGA TGC AC
F8 E14E-R	R-5’ ATC TTG AAG TAC TGG AGC AT
F8 E14F-F	F-5’ TAC ATA CAG TGA CTG GCA CT
F8 E14F-R	R-5’ GAC CAC TGG GTT GAG GTG TC
F8 E14G-F	F-5’ CAC GCA ACG TAG TAA GAG AG
F8 E14G-R	R-5’ GCC AAC CTC TCT TTG ATC AC
F8 E14H-F	F-5’ TAT AGA AAG AAA GAT TCT GG
F8E14H-R	R-5’ CAG GTC TGT TTG CTT CAT TC
F8 E14J-F	F-5’ CCC RAC GGA AAC TAG CAA TG
F8 E14J-R	R-5’ TCT TCA TTT CAA CTG ATA TG
F8 E14K-F	F-5’ AGG ACT GAA AGG CTG TGC TC
F8 E14K-R	R-5’ AAG AGT TTC AAG ACA CCT TG
F8 E15-F	F-5’ AGA TGA AGT GGT TAA CTA TGC
F8 E15-R	R-5’ GTG GGA ATA CAT TAT AGT CAG
(F means forward, R means reverse, P means promoter, E means exon)	

Table 3.1. Oligonucleotide primer pairs used in *F8* gene PCR and DNA sequencing (continued)

Primer Name	Primer Sequence
F8 E16-F	F-5' AGC ATC CAT CTT CTG TAC CA
F8 E16-R	R-5' TCA GTA GAT TCC AGA ATG ACA
F8 E17-F	F-5' TGT CAT TCT GGA ATC TAC TGA
F8 E17-R	R-5' CAC TCC CAC AGA TAT ACT CT
F8 E18-F	F-5' AGA GTA TAT CTG TGG GAG TG
F8 E18-R	R-5' CTT AAG AGC ATG GAG CTT GT
F8 E19-F	F-5' GCA AGC ACT TTG CAT TTG AG
F8 E19-R	R-5' AGC AAC CAT TCC AGA AAG GA
F8 E20-F	F-5' ACG TTG AGT ACA GTT CTT GG
F8 E20-R	R-5' ACT AAT AGA AGC ATG GAG ATG
F8 E21-F	F-5' TCT CTG ATT TCT CTA CTT ACT TGG
F8 E21-R	R-5' GTG ATA CAT TTC CCA TCA TTG
F8 E22-F	F-5' AAA TAG GTT AAA ATA AAG TGT TAT
F8 E22-R	R-5' TGG AAG CTA AGA GTG TTG TC
F8 E23-F	F-5' GTC TTA TGT AGA TGT TGG ATG
F8 E23-R	R-5' AGT CTC AGG ATA ACT AGA ACA
F8 E24-F	F-5' CAG TGG AAG CTG CTC AGT AT
F8 E24-R	R-5' CCC ATA ACC AAA CTT CCT TG
F8 E25-F	F-5' AGT GCT GTG GTA TGG TTA AG
F8 E25-R	R-5' TTG CTC TGA AAA TTT GGT CAT A
F8 E26-F	F-5' CCA ATA AAT GCT ATC TTT CCT C
F8 E26-R	R-5' CTG AGG AGG GAG AGG TGA
F8 E26A-F	F-5' TCG CTA CCT TCG AAT TCA CC
F8 E26A-R	R-5' GGC CTA ACT TTT CAG GGA AGA
F8 E26B-F	F-5' TCA GTC CTG CAT TTC TTT
F8 E26B-R	R-5' GAG ATA AGA ATG TGC CCC TCA
F8 E26C-F	F-5' CCT GAT CAA GCA TGG AAC AA
F8 E26C-R	R-5' TCT TTC TTG GCC ATC ACA AA
F8 E26D-F	F-5' GAA TCC CTA AGT CCC CTG AAA
F8 E26D-R	R-5' TGG TGA TAT GGC AGA CTG GA
F8 E26E-F	F-5' TGT GGA AAT ATG AGG AAA ATC CA
F8 E26E-R	R-5' TCA GAT AAG GGT CAA GCA GGA
F8 E26F-F	F-5' GGA TCC CAA TCT GAG AAA AGG
F8 E26F-R	R-5' TCA GTG CCC CTA TTT GTT TT
Intron1 9-F	F-5' GTT GTT GGG AAT GGT TAC GG
Intron1 9c-R	R-5' CTA GCT TGA GCT CCC TGT GG
Int1h-2F	F-5' GGC AGG GAT CTT GTT GGTAAA
Int1h-2R	R-5' TGG GTG ATA TAA GCT GCT GAG CTA
(F means forward, R means reverse, E means exon)	

3.2.2. Primers for Exon and Intron-Specific Amplifications of the *F8* Gene

Some intronic and exonic primers were designed for the unamplified regions for patients suspected to have large deletions. Sequences of *F8* intron 18 and intron 19 region specific primers were also given in Table 3.2.

Table3.2. Primer sequences designed for exon and intron-specific amplifications of the *F8* gene

Primer Name	Primer Sequence
F8 E7-F	F-5’ GGATGCCACAGGAAATCAGT
F8 E7-R	R-5’ TTGGTGGGAAGAGATATGACAA
F8 IVS10-F	F-5’ AAGAGACTTGAGCATCACAGATTG
F8 IVS10-R	R-5’ TTGCAGTGGAATCTAATTGGC
F8 E13-F	F-5’ TGCAGTTGTCAGTTTGTTTGC
F8 E13-R	R-5’ GGGTTTTCCATCGACATGAA
F8IVS18A-F	F-5’ GGACATGTGTTCACTGTACGAAA
F8IVS18A-R	F-5’ TGGATGACTACTGGTGCCCT
F8IVS18B-F	F-5’ CTTTCCTAGGGAAAAGGAATTT
F8IVS18B-R	R-5’ TGCAGTGGCACTTTCATAGC
F8IVS18C-F	F-5’ GCAAGACCCCCATCTCTACA
F8IVS18C-R	R-5’CTCCCTTTTCCAATGCAGAC
F8IVS18D-F	F-5’ AATTACAATGTCCATCCCAAGA
F8IVS18D-R	R-5’ TGCAGGTGAACACTGAGGG
F8IVS18E-F	F-5’ TTCTCAGCCCTCAGTGTTCA
F8IVS18E-R	R-5’ CCAAATTCCAGCTTTGGATG
F8IVS19A-F	F-5'GCTGGGATGAGCACACTTTT
F8IVS19A-R	R-5'GGGTTAATCTCCAAACTCTTCCA
IVS means intervening sequence variation E means exon, F means forward, R means reverse	

3.2.3. Primers for Reverse Transcriptase-PCR Analysis of the *F8* Gene

Reverse transcriptase (RT)-PCR amplification was performed in two rounds of PCR using a nested approach and the specific 12 primers pairs were used. Sequences of primers were given in Table 3.3.

Table 3.3. RT-PCR Primers

Primer Name	Primer Sequence
Ex1–8/1	F-5'CTT CTC CAG TTG AAC ATT TG
Ex1–8/4	R-5'TCA GCA GCA ATG TAA TGT AC
Ex1–8/2	F-5'GCA AAT AGA GCT CTC CAC CT
Ex1–8/R	R-5'TGG CCA GAC TCC CTT CTC TA
Ex1–8/F	F-5'TTG AAT TCA GGC CTC ATT GG
Ex1–8/3	R-5'GAG CGA ATT TGG ATA AAG GA
Ex8–14/1	F-5'AAG TAG ACA GCT GTC CAG AG
Ex8–14/4	R-5'CTA GGG TGT CTT GAA TTC TG
Ex8–14/2	F-5'AGA AGC GGA AGA CTA TGA TG
Ex8–14/R	R-5'GAG AGG GCC AAT GAG TCC TGA
Ex8–14/F	F-5'TGC CTG ACC CGC TAT TAC TC
Ex8–14/3	R-5'AGA AGC TTC TTG GTT CAA TG
Ex14–19/1	F-5'GGG AAA TAA CTC GTA CTA CT
Ex14–19/4	R-5'AAC TGA GAG ATG TAG AGG CT
Ex14–19/2	F-5'AGT CAG ATC AAG AGG AAA TTG
Ex14–19/R	R-5'CCA GCT TTT GGT CTC ATC AA
Ex14–19/F	F-5'CCT GCT CAT GGG AGA CAA GT
Ex14–19/3	R-5'GAT TGA TCC GGA ATA ATG AAG
Ex19–26/1	F-5'TGA GAC AGT GGA AAT GTT AC
Ex19–26/4	R-5'TTG CCT AGT TAT ATT GGA AG
Ex19–26/2	F-5'AGC ATA AGT GTC AGA CTC CC
Ex19–26/R	R-5'TCG AGC TTT TGA AGG AGA CC
Ex19–26/F	F-5'GCC ATT GGG AAT GGA GAG TA
Ex19–26/3	R-5'AGT TAA TTC AGG AGG CTT CA
Ex means exon, F means forward, R means reverse	

3.2.4. Primers for Long PCR Analysis of the *F8* Gene

Long-PCR analysis used in the detection of large deletions in the *F8* gene. Sequences of primers were given in Table 3.4.

3.2.5. Primers for the *F8* gene Intron 22 Inversion Mutation Analysis

In order to detect intron 22 inversion mutation, Southern Blot, inverse PCR and subsycling long PCR analyses were used. Sequences of intron 22 probe specific, inverse and long PCR primers were given in Table 3.5.

Table 3.4. Oligonucleotide primer pairs used for long PCR analysis of the *F8 g*ene

Primer Name	Primer Sequence
F8 E13-F F8 E26A-R	F-5’ TGCAGTTGTCAGTTTGTTTGC R-5’TCA R-5’ GGC CTA ACT TTT CAG GGA AGA
F8 E1-F F8 E9-R	F-5’ AAT CCT ATC GGT TAC TGC TTA R-5’ AGA TAT GTC CAT TGG AGA CAA
F8 E6-F F8 E7-R	F-5’ TCC CAC TTA TTG TCA TGG AC R-5’ TTGGTGGGAAGAGATATGACAA
F8 E13-F F8 E14A-F	F-5’ TGCAGTTGTCAGTTTGTTTGC R-5’ GAA AAA GTC TCA TAT TTG GC
F8 E12-F F8 E13-R	F-5’ TGC CAT CGC TTT CAT CAT AG R-5’ GGGTTTTCCATCGACATGAA
F8 E9-F F8 E11-R	F-5’ CTA ACA TAG CTG AGC ATC AG R-5’ ACT GAC CTA TAT TGC AAA CCA
(E means exon, F means forward, R means reverse)	

Table 3.5. Oligonucleotide primer pairs used for intron 22 inversion mutation analysis

Primer Name	Primer Sequence
F8 Probe a-F F8 Probe a-R	F-5’ TGGAGCCCAAACAGCTATCT R-5’ AAGTACTCCTGGGAGGCTTT
F8-IU F8-ID F8-ED	F-5’CCTTTCAACTCCATCTCCAT F-5’ACATACGGTTTAGTCACAAGT F-5’TCCAGTCACTTAGGCTCAG
F8Int22-P F8Int22-Q	F-5’GCCCTGCCTGTCCATTACACTGATGACATTATGCTGAC F-5’GGCCCTACAACCATTCTGCCTTTCACTTTCAGTGCAATA
F8Int22-A F8Int22-B	F-5’CACAAGGGGGAAGAGTGTGAGGGTGTGGGATAAGAA F5’CCCCAAACTATAACCAGCACCTTGAACTTCCCCTCTCATA
(F means forward, R means reverse)	

3.2.6. Primers for PCR Amplification of SNPs in Immune Response Genes

Nine SNPs and VNTR regions’ PCR amplification were optimized and sequenced for 10 healthy Turkish individuals to see their genotypes and use them for genotyping assays as controls. Sequences of 9 SNPs and VNTR region specific primers were given in Table 3.6.

Table 3.6. Oligonucleotide primer pairs used for 9 SNPs and VNTR region

Primer Name	Primer Sequence
Rs2241715	F-5'TTATCTTCCGTGGCTTCACC R-5' GGTTACTCAGCAAACCCCAA
Rs1800871	F-5' TCAACTTCTTCCACCCCATC R-5' GGCACATGTTTCCACCTCTT
Rs1554286	F-5' TCCTGCCCTTAGGGTTACCT R-5' AACGCCTGCTCAAAGAGAAA
Rs3024496	F- 5'ACAGCTCCAAGAGAAAGGCA R-5' TGTCACCCTATGGAAACAGC
Rs2069812	F-5' TCCCCCTTACTACTGGGACC R-5' TTGTTGAAGATAAAGAGGGAAGG
Rs2069705	F-5' CAGAGCAAGACTCCGTCTCA R-5' TACAACACTTTGGGATGGCA
Rs1861494	F-5' AGATGAGGAAACTGAGCCCC R-5' TTCGTGTTTGGGTGACTTTG
Rs2243267	F-5' GTCTTTCTAACCGCTCTGCC R-5' ACTTTTTAAAATAGGGGTCAACTG
Rs2243282	F-5' GCTAGAGATGATGGTGGCGT R-5' AACTCTTGATCTTCTGCTGAACC
VNTR	F-5' GCTAGAGATGATGGTGGCGT R-5' AACTCTTGATCTTCTGCTGAACC
(F means forward, R means reverse)	

3.2.7. Primers and Hybridization Probes for the Association Study

For nine SNPs primers and hybridization probes were designed. Figure 3.1-3.9 shows the sequences of the two alternative primer pairs and probes, positions of each primer and probe and also Tm of each oligonucleotide. The primers and probes were obtained from TIBMOLBiol, Germany.

938335	hu interferon, gamma (IFNG) (rs2069705)		AC007458	Tm
rs2069705 F	TggTTCAAACCCACTTTgCATT	S	100334-355	60,1°C
rs2069705 S	gCAAACTTgATTCCTgACTCCTCTAC	S	100396-421	58,9°C
rs2069705 A	ggTgACAgAgCAAgACTCCgTC	A	100680-659	59,8°C
rs2069705 R	gCCACTgCACTCCAgCCT	A	100699-682	60,1°C
Anc rs2069705	gCCAgTTTTACAggTAAggAgACTgAgTCA-FL	A	100493-464	63,9°C
rs2069705 wt	640-gAAgATTTAAgAAgCTAACTCACAATCA p	A	100461-434	56,6°C

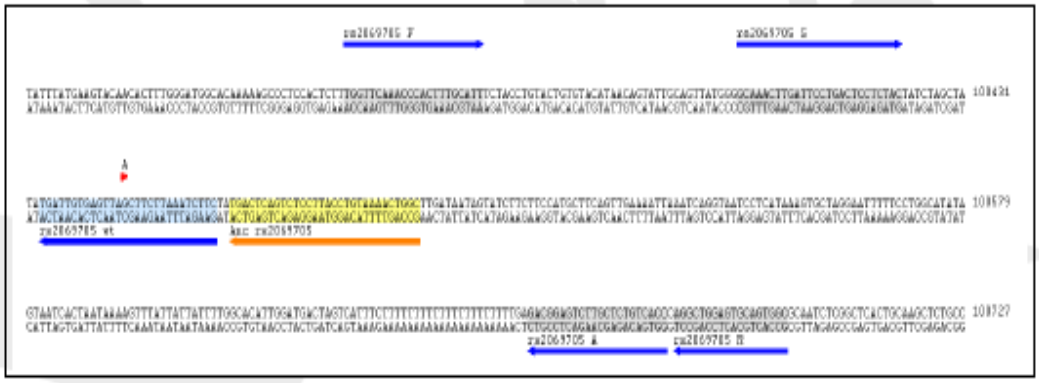


Figure 3.1. The design of primer and probes for rs2069705

938335	hu IL-10 C-819T		Z30175	Tm
0819 F*	TCATTCTATgTgCTggAgATgg	S	-907-886	54,7°C
0819 R*	TgggggAAgTgggTAAgAgT	A	-699-718	57,5°C
0819 [C]	ggTgATgTAACATCTCTgTgCCTC-FL	S	-829-806	57,8°C
0819 Anc	640-TTTgCTCACTATAAAATAgAgACggTAggg p	S	-803-774	61,5°C

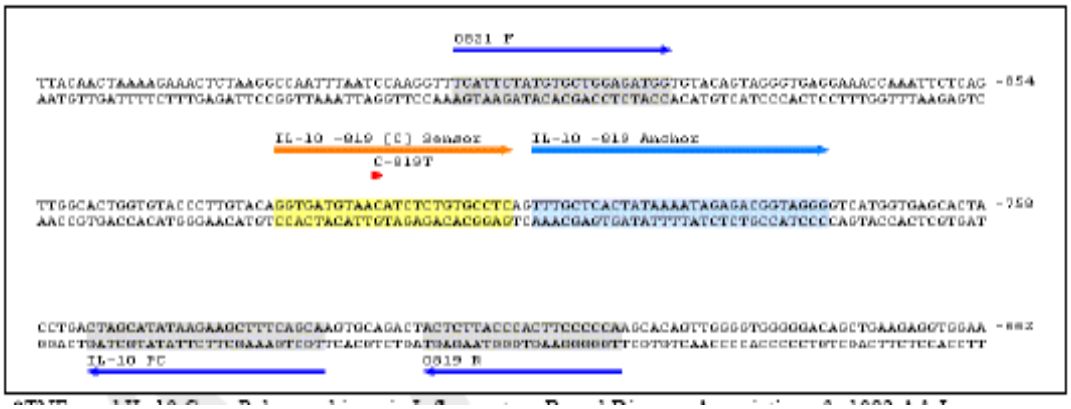


Figure 3.2. The design of primer and probes for rs1800871

939232	hu IL-10 rs3024496 T→C		U16720	Tm
4496-fw	CATCTACAAAgCCATgAgTgAgTT	S	7756-7779	55,8°C
4496-se	ggCgACTCTATAgACTCTAggACAT	S	7846-7870	54,8°C
4496-as	ATCgTTCACAgAgAAgCTCAgTA	A	8022-8000	54,2°C
4496-rev	gTgTCACCCTATggAAACAgC	A	8103-8083	55,9°C
4496Sen C	CCTTATTgTACCTCTCTCATAgAATATT-FL	S	7934-7961	52,4°C
4496Anch	640-TACCTCTgATACCTCAACCCCCATTTCTA p	S	7965-7993	63,1°C

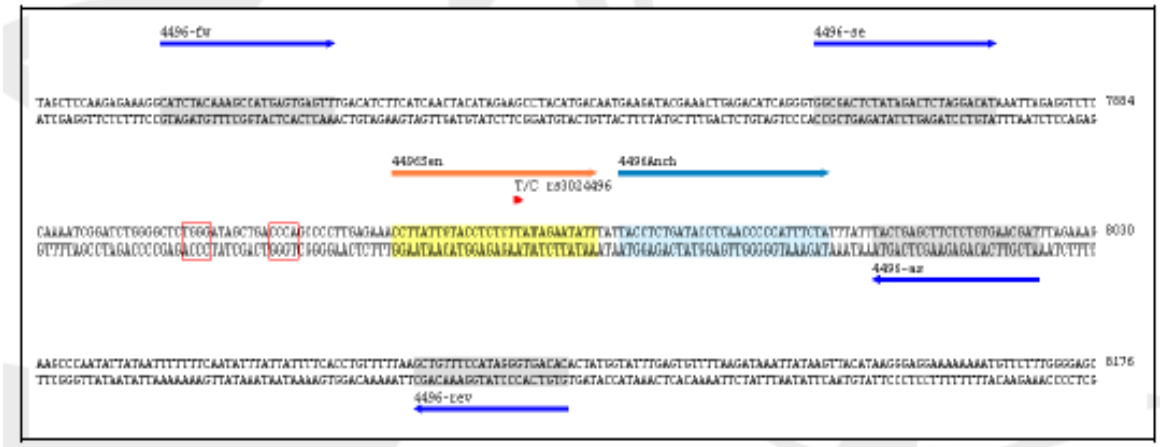


Figure 3.3. The design of primer and probes for rs3024496

939232	hu interleukin 10 (IL10) (rs1554286)		AL513315	Tm
10 F	CTCTgCCAgTCTgTgTCTTT	S	5272-5291	52,7°C
10 S	gTCTgTggATgTgAgTgTCC	S	5297-5316	52,2°C
10 A	CAgACATCAAggCgCAT	A	5453-4537	52,6°C
10 R	CCTggAggAggTgATgC	A	5491-5475	53,6°C
Sensor mut	CAgATCAgTTCTTTCCCTTgCAgC-FL	A	5374-5351	61,5°C
Anchor 10	640-gCCCCCAAAATACCATCTCCTACAgACCA p	A	5349-5321	69,3°C

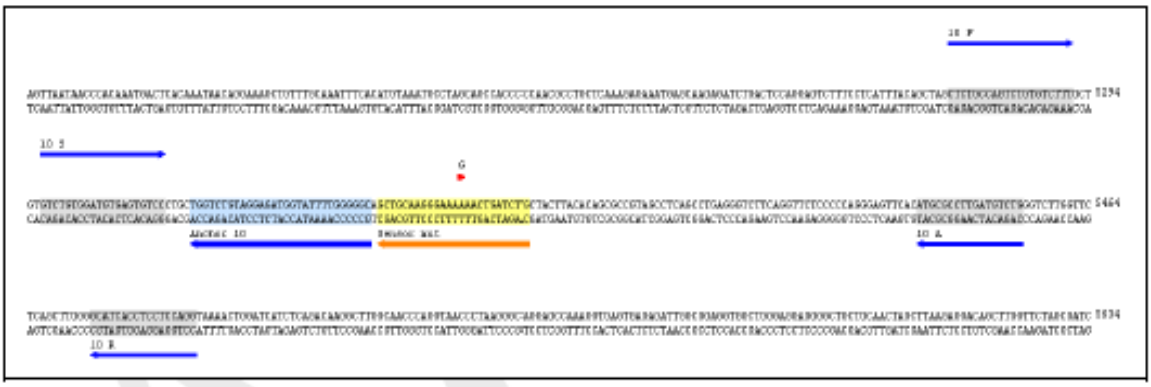


Figure 3.4. The design of primer and probes for rs1554286

939232	hu TGFB1 (rs2241715)		AC011462	Tm
rs2241715 F	TCTTTCTgTCCACgCATggg	S	79899-918	60,1°C
rs2241715 S	CCTggTTTTTgTCTCTCCgACTA	S	79922-944	58,2°C
rs2241715 A	gggTTggAgggTgATgCAg	A	80043-025	60,0°C
rs2241715 R	gggTgTgAAAAgAgACTggTgAg	A	90252-230	58,7°C
rs2241715 T	CCACggTCCTgTTgCCTC-FL	S	79972-989	59,6°C
Anc rs2241715	64 0-TCTCCgTCTCTgACATCTCCCCgCC p	S	79991-015	69,6°C

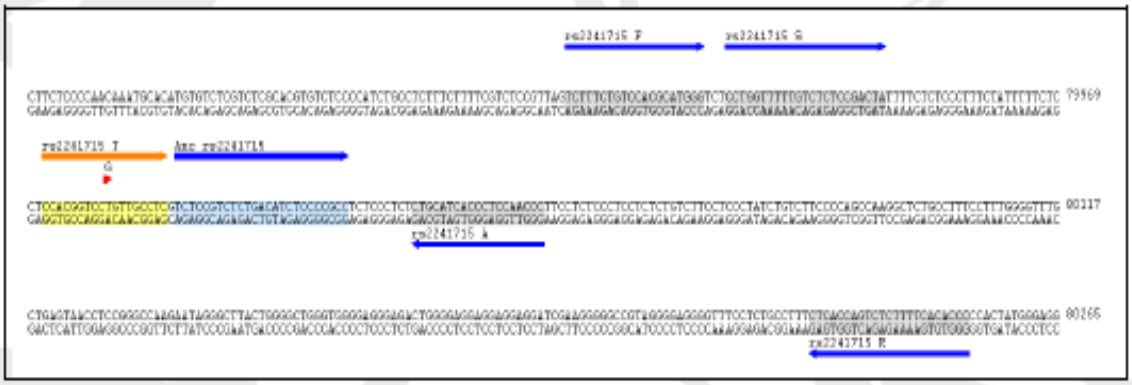


Figure 3.5. The design of primer and probes for rs2241715

939232	hu IL5 (C-703T)		AF353265	Tm
IL5 F	CCTggTCACAgTTCAAgg	S	804-821	51,9°C
IL5 S	TgTgACCCTTgTCAgAAAgAg	S	829-849	54,1°C
IL5 mis	TgAggTCTCAAgATgATgT XTCAg	A	964-942	54,3°C
Sensor C	gAACAgAATACATACAgATCCAggAgT-FL	S	915-941	55,7°C

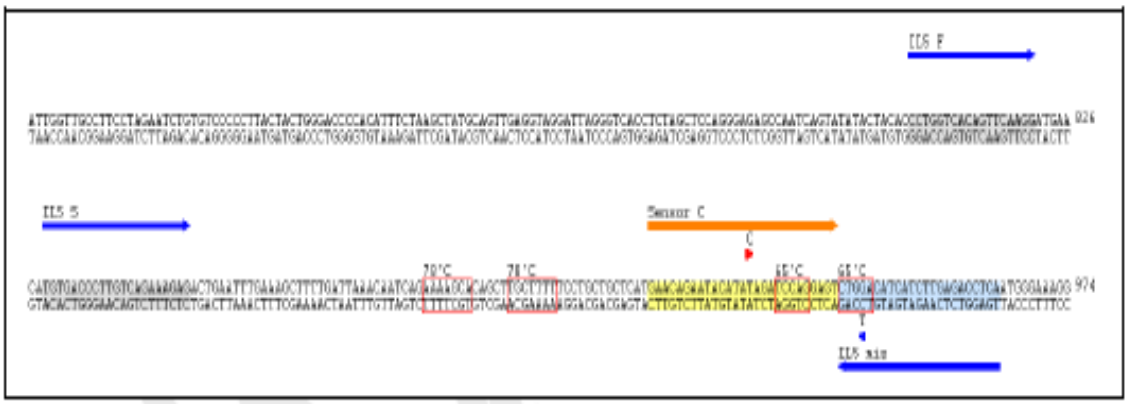


Figure 3.6. The design of primer and probes for rs2069812

940269.1	hu IFNG rs1861494 C= 35% EU; T=65%EU		AF375790	Tm
rs1861494 F	CAAAgCCAgTAAgAgAATCgCT	S	3558-3579	56,7°C
rs1861494 S	gCTggCATTgTAACTATTgCg	S	3602-3622	57,0°C
rs1861494 A	TCTTTCCATTAAgACAgACAgCC	A	3771-3749	55,6°C
rs1861494 R	gTgATTCATCACAgTTCCTTggT	A	3841-3819	55,9°C
Sen. [C]	gTACTCCCCgCTTCTTCCTC--FL	A	3685-3666	56,8°C
Sen. [T]	gTACTCCCTgCTTCTTCCTC--FL	A	3685-3666	52,4°C
Anc.	640-CCTACTTCCTCTTCACTggATTTgTCAACT--PH	A	3664-3635	63,2°C

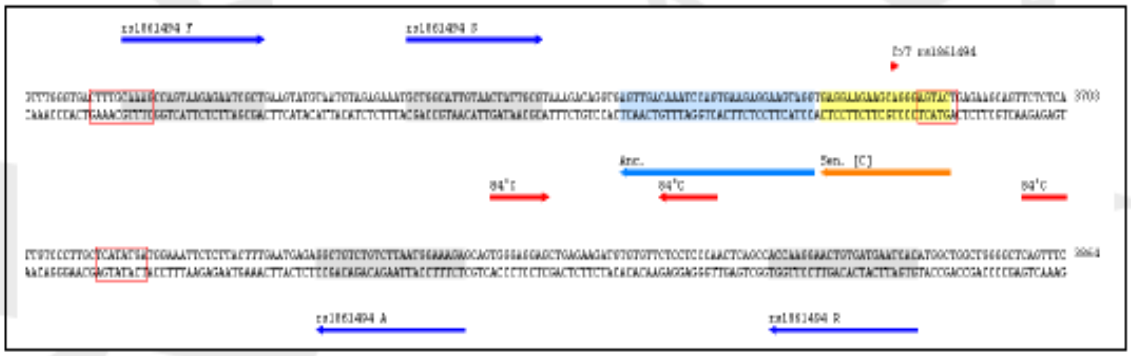


Figure 3.7. The design of primer and probes for rs21861494

940269.2	hu IL-4 rs2243282 A= 17% EU; C=83%EU		M23442.1	Tm
rs2243282 F	TggACAAATggAgCAgTTgAg	S	7894-7914	56,9°C
rs2243282 S	TgAggTgAACAgATTTgggATATg	S	7911-7934	57,1°C
rs2243282 A	TgggCAggAACAAAgAggTC	A	8024-8005	58,8°C
rs2243282 R	CCTCCAgCCCTgCCTTg	A	8093-8077	59,8°C
Anc.	TTCCTCCCCTgTATCTTACCCCC--FL	A	8003-7977	61,9°C
Sen. [C]	640-ACAACCgATCTgTCAgCAAAT--PH	A	7975-7955	54,3°C
Sen. [A]	640-ACAACCTATCTgTCAgCAAATCT--PH	A	7975-7953	53,5°C

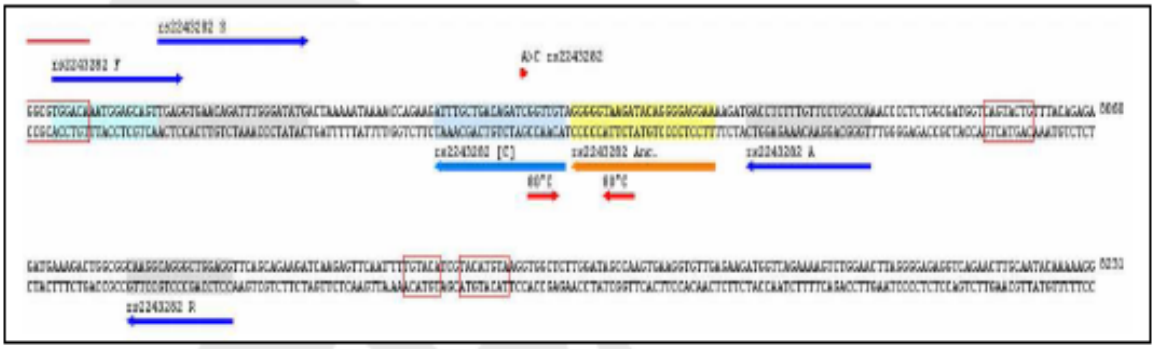


Figure 3.8. The design of primer and probes for rs2243282

940269.3	hu hu IL-4 rs2243267 C= 16% EU; T=84%EU		M23442.1	Tm
rs2243267 F	CgTTAAAATATgAACTgCATTCCTAg	S	5211-5236	55,8°C
rs2243267 S	ATgAACTgCATTCCTAgAATTTAgC	S	5220-5244	55,4°C
rs2243267 A	gCATTTCCTAAgCCTTCggT	A	5420-5401	57,3°C
rs2243267 R	TCCCACCAgCCAgAggT	A	5554-5538	57,1°C
Sen. [C]	TACAgAACCAAAAATCTgCCACgA--FL	S	5302-5325	60,6°C
Sen. [G]	TACAgAAgCAAAAATCTgCCACgA--FL	S	5302-5325	60,8°C
Anc.	640-gCCTgACAAATgACAgTACCACTgTgCA--PH	S	5331-5358	67,7°C

Figure 3.9. The design of primer and probes for rs2243267

3.2.8. Primers for DNA Sequence Analysis of the IL5 Gene

The primer sequences that were used in DNA analysis of promoter region and 4 exons of the IL5 gene were shown in Table 3.7.

Table 3.7. Oligonucleotide primers for the IL5 gene

Primer Name	Primer Sequence
Promoter1	F-5’ TCCCCCTTACTACTGGGACC R-5’ TTGTTGAAGATAAAGAGGGAAGG
Promoter2	F-5’ TGAGCCAATACCTTCCCTCTT R-5’ CCCCACATTTGCATTTCTTA
Promoter3	F-5’ AAATGTGGGGCAATGATGTA R-5’ GCAGTGCCAAGGTCTCTTTC
Exon1	F- 5’ GGAACCATCACAAATGATTACC R-5’ CAAACGCAGAACGTTTCAGA C
Exon2	F-5’ TCACAGCCACCCATATGAAA R-5’ TGCTGGTGTGCTGTAAAAATG
Exon3	F-5’ AACTTACTTTTTGGCCGTCA R-5’ CATTGCAGAATGGACAATGG
Exon4	F-5’ CCAGCAGCAAATTGAACAGT R-5’ CATTGACGGCCAAAAAGTAA
(F means forward, R means reverse)	

3.3. Enzymes

Table 3.8. Taq DNA polymerases used in PCR

DNA Polymerase	Unit
Taq DNA polymerase	5 U/µl, (Promega, USA)
ExTaq DNA Polymerase	5 U/µl, (Takara, Japan)

3.3.1. Restriction Enzymes

Table 3.9. Restriction enzymes used in this thesis

Restriction Enzymes	Unit
BclI	10 U/µl New England BioLabs
BslI	10 U/µl, (Promega, USA)
NsiI	10 U/µl, (Promega, USA)

3.3.2. Ligation Enzyme

Table 3.10. Ligation enzyme used in this thesis

Ligation Enzyme	Unit
T4	10 U/µl New England BioLabs

3.4. Buffers and Solutions

3.4.1. DNA Extraction

Table 3.11. Buffers used in this thesis

Buffers	Concentrations
Lysis Buffer	155 mM NH_4Cl 10 mM $KHCO_3$ 1 mM Na_2EDTA (pH 7.4)
Nuclease Buffer	10 mM Tris-HCL (pH 8.0) 400 mM NaCl 2 mM Na_2EDTA (pH 7.4)
Sodiumdodecylsulphate (SDS)	10 per cent SDS (w/v) (pH 7.2)
Proteinase K	20 mg/ml in H_2O
Sodium Chloride (NaCl)	5 M NaCl
Ethanol (EtOH	Absolute EtOH, Riedel de Haen, Germany
TE Buffer	20 mM Tris-HCl (pH 8.0) 1 mM Na_2EDTA (pH8.0)

3.4.2. Southern Blot Analysis

Table 3.12. Southern blot solutions used in this thesis

Solutions	Concentrations
Depurination Solution	0.25 M HCl
Denaturation Solution	1.5 M NaCl 0.5 M NaOH
Neutralization Solution	0.5 M Tris-HCl (pH 7.4) 0.01 M Na_2EDTA (pH 8.0)
DNA Transfer Solution (10X SSC)	1.5 M NaCl 150 mM Sodium Citrate pH 7

Table 3.12. Southern blot solutions used in this thesis (continued)

Solutions	Concentrations
Prehybridization Solution (5X SSC)	1 per cent (w/v) Blocking Reagent 0.1 per cent N-lauroylsarcosine 0.02 per cent SDS
Hybridization Solution	5 μg DIG-labeled probe diluted in 10 ml of Prehybridization Solution
Washing Solution 1 (2 X SSC)	containing 0.1 per cent SDS
Washing Solution 2 (0.1 X SSC)	containing 0.1 per cent SDS
Buffer 1	10 mM Maleic Acid 150 mM NaCl Adjusted to pH 7.5
Buffer 2	1 percent Blocking Reagent in Buffer 1
Buffer 3	100 mM Tris-HCl (pH 9.5) 100 mM NaCl 50 mM $MgCl_2$

3.4.3. Polymerase Chain Reaction (PCR)

Table 3.13. PCR buffers used in this thesis

Buffers	Concentrations
10X $MgCl_2$ Free Buffer	100 mM Tris-HCl 500 mM KCl Promega, USA
Magnesium Chloride ($MgCl_2$)	25 mM in dH2O Promega, USA
Deoxyribonucleotides (dNTP)	25 mM of each dNTP Promega, USA
Dimethylsulphoxide (DMSO)	Stock solution Sigma, Germany

3.4.4. Agarose Gel Eletrophoresis

Table 3.14. Agarose gel electrophoresis buffers used in this thesis

Buffers	Concentrations
10X TBE Buffer	0.89 M Tris-Base 0.89 M Boric acid 20 mM Na_2EDTA (pH 8.3)
20X TAE Buffer	96.8 gr Tris 22.84 ml Acetic acid 40 ml 0.5 M EDTA
Ethidium Bromide (EtBr)	10 mg/ml Sigma, Germany
1 or 2 per cent Agarose Gel	1 or 2 per cent agarose in 0.5X TBE Buffer, containing 0.5ug/m Ethidium bromide
0.7 per cent Agarose Gel	0.7 per cent agarose in 1X TAE Buffer Containing 0.5ug/ml Ethidium Bromide
10X Loading Dye	2.5 mg/ml Bromophenol Blue (BPB) 1 per cent SDS in glycerol
DNA Ladder	100 bp, MBI Frementas, Lithuania 500 bp O'Range Ruler, MBI Fermentas λ/HindIII DNA Marker, Promega, USA

3.5. Kits

3.5.1. Kits for Light Cyler 480

Table 3.15. Kits for Light Cyler 480 used in this thesis

Kits	Concentrations
Probes Master	2X Faststart Taq DNA polymerase (Roche) Reaction Buffer dNTP mix, 6.4mM $MgCI_2$
Genotyping Master	5X Faststart Taq DNA polymerase (Roche) Reaction Buffer dNTP mix, 15mM $MgCI_2$
High Resolution Melting	2X Faststart Taq DNA polymerase (Roche) Reaction Buffer dNTP mix, 25mM $MgCI_2$ High resolution melting dye
Syber Green I Master	2X Faststart Taq DNA polymerase (Roche) Reaction Buffer dNTP mix, 10mM $MgCI_2$ Syber Green I dye

3.5.2. Kits for MagNA Pure Compact Instrument

Table 3.16. Kits for MagNA Pure Compact instrument used in this thesis

Kits	Concentrations
MagNA Pure Compact DNA Isolation Kit	Whole blood large volume 32 reactions
MagNA Pure Compact RNA Isolation Kit	Whole blood 500µl of fresh blood 32 Reactions

3.5.3. Kits for Long PCR and RT-PCR

Table 3.17. Kits for long PCR and RT-PCR used in this thesis

Kits	Concentrations
Expand Long PCR Template System	10X Expand Long PCR Reaction Buffer , 17.5mM $MgCI_2$ (Roche)
Abgene Extensor Long PCR Master Mix	2X Extensor Long PCR Master Mix (Thermo Fischer)
Titan One Tube RT-PCR Kit	5X RT-PCR Buffer, 10mM dNTP mix 7.5mM $MgCI_2$, (Roche)

3.6. Equipments

Table 3.18. Equipments used in this thesis

Equipment	Model
Autoclave	Model MAC-601 Eyela, Japan
Balances	Electronic Balance Model CC081 Gec Avery, UK
Centrifuges	Centrifuge 5415C Eppendorf, Germany Universal 16R Beckman Coulter Hettrich, Germany
Deep Freezers (-20°C)	Bosch, Germany
Documentation System	BioDoc Video Documentation System
Electrophoretic Equipments	Horizon 58, Model 200 (BRL, USA)
Light Cycler 480	Multiplate well 96 (Roche, Germany)
MagNA Pure Compact DNA Isolation Instrument	(Roche, Germany)
Magnetic Stirrer	Chiltern Hotplate Magnetic Stirrer

Table 3.18. Equipments used in this thesis (continued)

Equipment	**Model**
Ovens	Microwave Oven (Vestel, Türkiye) EN400 (Nuve, Türkiye)
Power Supplies	ECPS 3000/150 Constant Power Supply Pharmacia, Sweden
Spectrophotometer	NanoDrop ND-1000, Thermo, USA
Thermocyclers	Techne (Progene, UK) Techne Gradient (Progene, UK)
UV Transilluminator	Chromato-Vue Transilluminator
Water Bath	Köttermann, Laborteknik (Germany)

4. METHODS

4.1. DNA Extraction and Quality Control Analysis

4.1.1. DNA Extraction from White Blood Cells

Ten ml blood samples from patients and their relatives were collected into K_2EDTA containing tubes to prevent coagulation before DNA extraction. They were stored at 4ºC if DNA was to be extracted within or -20ºC. For DNA extraction, the samples were taken into sterile Falcon centrifuge tubes, and after addition of 30 ml ice cold lysis buffer they were kept at 4ºC for 15 minutes to allow lysis of leukocyte membranes. In order to collect the nuclei, the samples were centrifuged at 5000 rpm for 10 minutes. Centrifugation step was performed at 4ºC. The supernatant was discarded and centrifugation was repeated with 10 ml lysis buffer to wash the nuclear pellet. Then, the DNA was extracted using NaCl (salting out) extraction. In NaCl extraction method, nuclear pellet was resuspended in 3 ml of nuclei lysis buffer in order to lyse nuclear envelope of leukocytes. After the addition of proteinase K (150 µg/ml) and SDS (0.14 per cent), the samples were incubated at 37ºC overnight or 56ºC for 3 hours for degradation of cellular proteins. Five ml of sterile distilled water was added to the mixture, and the protein residues were salted out by adding 5 ml of 5 M NaCl. The samples were centrifuged at 5000 rpm at room temperature for 25 minutes. The supernatant was taken into a sterile Falcon tube and DNA was precipitated with two volumes of absolute EtOH. The precipitated DNA was fished out with a pipette. After evaporation of ethanol, DNA was dissolved in TE buffer in an eppendorf tube and stored at 4ºC (Miller *et al*., 1988).

4.1.2. DNA Extraction by MagNa Pure Compact Instrument

MagNA Pure Compact Instrument is a new robotic system which extracts nucleic acids (DNA and RNA) from blood or tissue samples. The extraction principle relies on the specific binding affinity of magnetic beads to DNA. Following lysing of cell membrane and nuclear envelope, DNA binds to magnetic beads; thus, it is isolated from remnants of the cell. DNA is ‘freed’ of beads during elution step. MagNA Pure Compact Nucleic Acid

Isolation large volume kit was used in this study. This kit requires 1 ml of blood sample. Final elution volume is 200 µl of DNA.

4.1.3. Qualitative Analysis of DNA by Agarose Gel Electrophoresis

Genomic DNA was analyzed on 1 per cent agarose gel which was prepared by dissolving 1 g of agarose in 100 ml 0.5 X TBE buffer. Agarose was dissolved in TBE buffer by boiling in microwave and cooled down to 56 ^{o}C. EtBr, which intercalates DNA and causes DNA to be visualized under UV light, was added into the solution with a final concentration of 0.5 µg/ml. Then the homogeneous mixture was poured onto eletrophoresis plate and left to polymerize at room temperature. In order to load the DNA samples into the wells of the agarose gel, 1 µl of DNA was mixed with 9 µl of loading dye 1X BPB. The gel was run at 150 volts and visualized under UV light.

4.1.4. Quantitative Analysis of DNA by Spectrophotometer

DNA concentrations were also measured by a spectrophotometer. 50 µg of double stranded DNA has an absorbance of 1.0 at 260 nm (OD_{260}). Each sample was diluted to a factor and the absorbance was read at 260 nm in a spectrophotometer. The concentration was calculated by the following equation:

50 µg/ml x OD_{260} x dilution factor = concentration of genomic DNA (µg/ml)

The concentration and purity of DNA was also measured by the nanodrop spectrophotometer.

4.2. Detection of Intron 22 Inversion in The *F8* Gene

4.2.1. Southern Blot Analysis

Southern blot analysis of the intron 22 inversion mutation involves the following steps.

4.2.1.1. Restriction Endonuclease Digestion. Five μg of genomic DNA was digested with 10 units (U) of BclI restriction enzyme in a total volume of 20 μl overnight. To check whether the digestion was complete, 3-μl aliquot of the digestion was mixed with 1 μl of 10 X loading buffer, electrophoresed on a 1 per cent agarose gel in 0.5 X TBE buffer, containing ethidium bromide (EtBr) and visualized on a UV transilluminator. In order to terminate the digestion reaction, 5 μl of stop loading dye was added

4.2.1.2. Gel Electrophoresis. The BclI-digested DNA fragments were resolved by agarose gel electrophoresis in 350 ml of 0.7 per cent gel in 1 X TAE buffer at 75 V for overnight. Lambda (λ) phage DNA digested with restriction enzyme HindIII was used as a size marker. The gel was stained with EtBr, and DNA fragments were visualized under UV light and photographed. Subsequently restriction fragments were transferred to a nylon membrane by Southern blotting as described in the following section

4.2.1.3. Southern Blotting. Before the transfer of DNA from the gel to the membrane, the gel was sequentially treated with 0.25 M HCl for depurination of DNA, denaturation, and neutralization solutions, each for 15 min. Meantime, the system that would be used for the transfer was prepared. The DNAs were transferred to positively charged nylone membrane (Roche) with using the model 785 vacuum blotter machine (Bio-Rad) for 2 hours. Firstly, a nylon membrane 0.5 cm bigger than each border of the precut window on the window gasket was cut a sheet of filter paper the same size as the nylon membrane. The precut nylon membrane was wetted in double distilled water by slowly lowering the membrane at a 45 degree angle to the water. Then, the membrane and the filter paper were wetted in the 10X SSC transfer solution. The wetted filter paper was placed on the porous vacuum plate.The wetted membrane was placed on top of the filter paper. The bubbles were removed by rolling a 10 ml glass pipet over the membrane. The Reservoir Seal O-ring was wetted with water.The window gasket was placed on top of the membrane/filter paper. Gently the gel was placed, well side up, on top of the Window Gasket. The gel must overlap the window. Bubbles were removed by using a 10 ml glass pipet. As a final check, the gel edges was overlapped the window gasket by at least 5 mm. The sealing framewas locked onto the four latch posts. the vacuum source was started and slowly the bleeder was turned valve clockwise until the gauge read at 5 in. of Hg. The gel was transferred for 120

minutes at 5 inches Hg. After 2 hours, the membrane was fixed at 120 ˚C for 30 minutes for further use.

4.2.1.4. Amplification of F8A Probe. A 892 bp of F8A probe was synthesized by PCR amplification. Primer sequences are given in Table 3.1. PCR amplification was performed on 100 ng of genomic DNA with 1X Mg^{2+} Free Reaction Buffer, 2,5mM Mg^{2+}, 0.5 mM of each dNTP, 0.2mM of each oligonucleotide primer, and 2.5 U Taq DNA Polymerase (Promega). 40 amplification cycles were performed at 94˚C for 30 seconds, 55.5˚C for 30 seconds, and 72˚C for 2 minutes. The PCR products were checked on 2 per cent agarose gel. 5 μl aliquots of each product were mixed with 5μl 1X loading dye and run at 150V with a 100 bp DNA ladder. The products were visualized under UV light.

4.2.1.5. Labeling of Probe**.** The F8A probe and λ phage DNA were labeled with digoxigenin-11-dUTP (DIG) using the random primed method (Boehringer Mannheim's DIG DNA Labeling Kit). Three μg of DNA template diluted in dH_2O to a total volume of 15 μl was heat-denatured for 10 min, and chilled on ice. Then 2 μl of hexanucleotide mix, 2 μl of dNTP labeling mix and 2 U of Klenow enzyme were added to a final concentration of 1 X, and the reaction tube was incubated at 37°C overnight. In order to terminate the labeling reaction, 0.8 μl of 0.2 M EDTA was added. Prior to hybridization, the yield of DIG-labeled DNA probe was estimated in a direct detection assay, and the success of the labeling reaction was confirmed. The reaction mix was stored at -20°C for further use.

4.2.1.6. Pre-Hybridization and Hybridization. The Southern blot was placed in a rolling tube containing 10 ml of pre-hybridization solution, and allowed to pre-hybridize at 65°C for two hours. The corresponding 3 μg of labeled probe and 0.5 μg of labeled λ DNA were heat-denatured in boiling water for 10 min and subsequently was pipetted into the pre-hybridization solution in the rolling tube. Hybridization was carried out at 65°C overnight.

4.2.1.7. Colorimetric Detection with NBT and X-Phosphate. Blots were sequentially washed with washing solutions 1and 2 in rolling tubes under stringent conditions to get rid of non-specific hybridization. Later each blot was transferred to a plastic container. Fifty ml of 1 per cent blocking reagent solution was added to eliminate background, and the

container was shaken for 30 min. The blocking reagent solution was discarded, and blots were then shaken in the solution containing antibody-digoxigenin conjugated to alkaline phosphatase. After discarding the antibody solution, each blot was placed in a nylon bag and colorimetric alkaline phosphatase substrates, and 75 mg/ml of NBT and 50 mg/ml of X-phosphate were added. The bag was sealed and left in dark to allow formation of color precipitates.

4.2.2. Genotyping Using Inverse PCR

Although southern blot analysis is robust and can be used to identify all types of inversions, this technique is labor-intensive and time consuming. Inverse PCR technique as an alternative rapid tool will be used for intron 22 inversion detection. Inverse PCR involve three steps: (a) BclI restriction; (b) self-ligation of restriction fragments, providing BclI rings; and (c) standard multiplex analysis by use of a set of three primers.

One μg of genomic DNA was digested with 10U of Bcl I enzyme at 55 °C for over night. Restriction products were ligated with 3U of T4 ligase enzyme at 16°C for overnight. Ligated products were used as a template for inverse PCR. Intron 22 inversion was detected according to the size of PCR products. Ligated products amplified under standard PCR conditions using primers IU, ID and ED. PCR was performed with 2μl of eluted T4 product in a volume of 25μl, containing 0.5 U of Taq DNA polymerase, 0.2 μM each primer, 200 μM dNTP, 5 mM $MgCl_2$, and standard Taq polymerase buffer. The initial denaturation step of 2 min at 94 °C was followed by 35 thermocycles of 94 °C for 30 s, 55.2 °C for 1 min, and 72 °C for 90 s; final extension was at 72 °C for 5 min.

4.2.3. Subcycling Long PCR Analysis

This method for detection of the FVIII gene intron 22 inversion removes the requirement for Southern Blotting. In subcycling long PCR, intron 22 inversion was detected according to the size of PCR products. Four primer pairs, P, Q A and B were used to amplify the PCR products in a combination. Schematic representaion of primer combinations were given in Figure 4.1. Use of the ABgene Extensor PCR kit simplifies the reaction set up procedure. PCR procedure took around 13 hours to perform. PCR protocol

was performed according to the procedure of Liu *et al*., 1998. PCR programme were shown in Figure 4.1.

94 ^{0}C for 2 minutes initial denaturation

Then 10 cycles consisting of:

94 ^{0}C for 12 seconds
Four subcyles per main cycle consisting of:

60 ^{0}C for 120 seconds

65 ^{0}C for 120 seconds

Remaining 20 cycles:

94 ^{0}C for 12 seconds

Four subcyles per main cycle consisting of:

60 ^{0}C for 120 seconds plus an additional 3 seconds added per cycle

65 ^{0}C for 120 seconds plus an additional 3 seconds added per cycle

Figure.4.1. Conditions of subcycling PCR

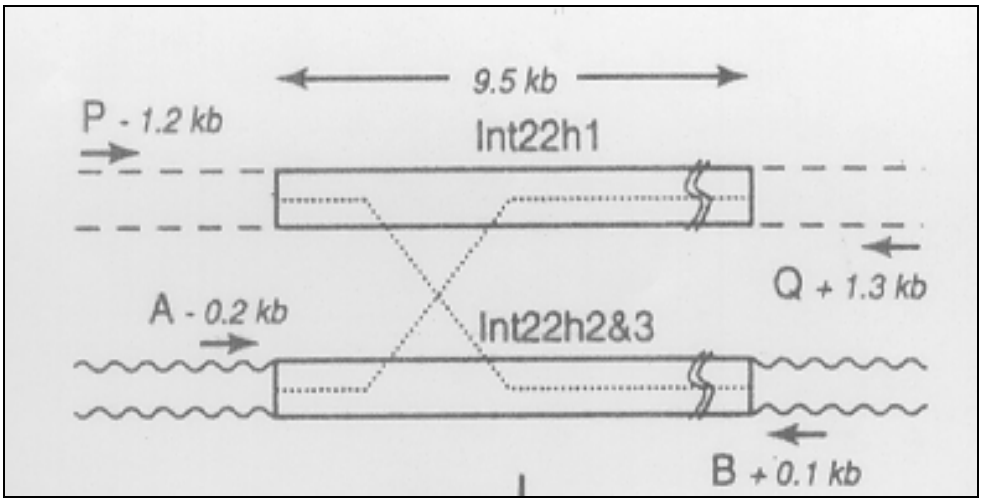


Figure 4.2. Schematic representation of primer pairings in intron 22 inversion (Liu *et al*., 1998)

4.3. Detection of Intron1 Inversion Mutation

PCR amplifications of int1h-1 and int1h-2 regions in intron1 of F8 gene, were performed on 100 ng of genomic DNA with 1X Mg^{2+} free reaction buffer, 2,5mM $MgCl_2$, 0.5 mM of each dNTP, 200 ng of each primer, 5% DMSO and 2.5 U Taq DNA Polymerase in a 25µl volume. 35 amplification cycles were performed at 94 ˚C for 30 seconds, 55.5 ˚C for 30 seconds, and 72 ˚C for 2 minutes. The primers used for PCR assays are shown in Table 3.1. Amplifications of int1h-1 and int1h-2 regions were checked on 0.8 per cent agarose gels by loading 8 µl of the PCR product in 5 µl of 1X loading dye and run at 175 volts with a 500 bp DNA ladder for 20 minutes. The gels were visualized under UV light and documented.

4.4. Point Mutation Detection

4.4.1. PCR Amplifications of the *F8* Gene

A2, A3 and C2 domains coding of F8 exons were optimized and and amplified initially.). Each PCR reaction was prepared in a 25µl volume, containing 1X Mg^{2+} free reaction buffer, 2 or 2,5 mM of $MgCl_2$, 0.2 mM of each dNTP, 10 µM of each primer, 0.5 or 1 U of Taq polymerase and 50 ng of genomic DNA at exon-specific conditions shown in Table 4.1. PCR conditions of these amplifications were optimized like the following PCR programme.

94ºC for 2 minutes (Initial denaturation)	
94ºC for 30 seconds (Denaturation) 45.6-54ºC for 30 seconds (Annealing) 72ºC for 45 seconds (Extension)	35 cycles
72ºC for 5 minutes (Final extension)	

Figure 4.3. Conditions of PCR for *F8* gene

Amplifications of exons 8-14A, 14K-20 and 24-26 of F8 were checked on 2 per cent agarose gels by loading 5 µl of the PCR product in 5 µl of 1X loading dye and run 175 volts with a 100 bp DNA ladder for 15 minutes. The gels were visualized under UV light and documented.

A1 and C1 domains of *F8* coding exons were optimized and amplified as indicated above. Amplifications of exons 1-7, 14B-14J and 21-23 exons of *F8* were checked on 2 per cent agarose gels by loading 5 µl of the PCR product in 5 µl of 1X loading dye and run 175 volts with a 100 bp DNA ladder for 15 minutes. The gels were visualized under UV light and documented.

Primer sequences of non-coding 5' and 3' UTR regions of *F8* gene were designed by using "workbench primer3" programme on internet. Optimal primers were checked by BLAST not to have unspecific annealing.

4.4.2. Purification of PCR Products for DNA Sequencing

Before sending the exon samples to DNA sequencing, they were purified by using QIAquick PCR purification kit. Five volumes of buffer PB was added to 1 volume of the PCR sample and mixed. A QIAquick spin column was placed in a 2 ml collection tube for each sample. Then, the solutions were applied to the column and centrifuged for 1 minute. The flow-through was discarded and 0.75 ml buffer PE was added to the column and centrifuged for 1 minute. After discarding the flow-through, column was placed back in the same tube and centrifuged for an additional1 minute. Then, column was placed to a new 1.5 ml eppendorf tube, to elute DNA 50 µl of buffer EB was added to the center of the QIAquick membrane and centrifuged for 1 minute. After elution, purified PCR products were checked on 2 per cent agarose gel and visualized under UV light.

4.4.3. DNA Sequencing

Sufficient amount of purified exon products were sent to İontek, İstanbul and Macrogen, South Korea for automated sequencing. The results were obtained online as ABI document.

Table 4.1. PCR conditions for each exon and intron of *F8* gene

Exon Number	Annealing Tm (^{o}C)	Mg Concentration (mM)	PCR Size (bp)
F8-P1	56.2	2.5mM	539
F8-P2	56.2	2.5mM	600
F8-1	53	2mM	430
F8-2	57.2	2mM	277
F8-3	51.6	2mM	346
F8-4	53	2mM	319
F8-5	53	2mM	280
F8-6	53	2mM	424
F8-7	53	2mM	434
F8-8	54	2mM	548
F8-9	53	2mM	417
F8-10	54.7	2mM	347
F8-11	54.7	2mM	445
F8-12	53	2mM	320
F8-13	53	2mM	477
F8-14A	53	2mM	518
F8-14B	53	2mM	392
F8-14C	53	2mM	499
F8-14D	53	2mM	350
F8-14E	53	2mM	501
F8-14F	57.2	2mM	381
F8-14G	53	2mM	440
F8-14H	51.7	1.5mM	345
F8-14J	53	2mM	483
F8-14K	54.2	2mM	347
F8-15	53	2mM	349
F8-16	53	2mM	526
F8-17	53	2mM	492
F8-18	53	2mM	413
F8-19	53	2mM	342
F8-20	53	2mM	313

Table 4.1. PCR conditions for each exon and intron of *F8* gene (continued)

Exon Number	Annealing Tm (^{o}C)	Mg Concentration (mM)	PCR Size (bp)
F8-21	53	2mM	152
F8-22	54	2.5mM	280
F8-23	53	2mM	350
F8-24	53	2mM	343
F8-25	53	2mM	373
F8-26A	53	2mM	394
F8-26B	53	2mM	487
F8-26C	53	2mM	432
F8-26D	53	2mM	498
F8-26E	53.9	2mM	558
F8-26F	53	2mM	500
Int1h1	55.5	2.5mM	1900
Int1h2	55.5	2.5.mM	1300
F8-IU-ID	57.2	2.5mM	483
F8-IU-ED	57.2	2.5mM	559
F8-ProbeA	60.3	2.5mM	792

4.5. Detection of Large Deletions

4.5.1. Absolute and Relative Quantification Analysis of the *F8* Gene

Quantitative PCRs (qPCR), assay was used to confirm large deletions and determine the heterozygosity of mothers by detecting the relative amount of template syber green dye which binds to double stranded DNA was used in qPCR analysis. Absolute quantification using the “Fit Points Method” is an analysis used to quantify the target sequence and reference sequence and gives a concentration value. Relative quantification compares these target and reference sequences’ concentrations and gives a ratio. Real-time qPCRs were performed with a LightCycler 480 instrument and LightCycler 480 SYBR Green I Master kit and target and reference sequence-specific primers, Target sequence was F8 regions and reference sequence was exon 6 of the sodium channel 1 alpha (SCN1A) gene. (Ex6F-5’ CACACGTGTTAAGT, Ex6R-5’ AGCCCTCAAGTAT)

The efficiency of the experiments varied 1.54-2.2 and the error, which is the measure of the accuracy of the quantification result based on the standart curve, was less than 0.2. Concentrations of DNA samples of patient, patient's mother, normal male and normal female were calculated and their initial concentrations were equated to 20ng/µl. PCRs of samples were triplicated to avoid pipetting error. Serial dilutions of DNA sample of a normal female were used as a standard for target and reference sequences. Table 4.2 shows the optimization conditions of CNV analysis in light cycler 480.

Table 4.2. The optimized conditions for qPCR analysis in light cycler 480

Programme name	**Tm**	**Acquisition mode**	**Time (hh:mm:ss)**	**Ramp rate (^{o}C/s)**	**Cycle**
Pre-incubation	95 ^{o}C	None	00:10:00	4.4	1
Amplification	95 ^{o}C	None	00:00:10	4.4	32
	60-63 ^{o}C	None	00:00:15	2.2	
	72 ^{o}C	Single	00:00:20	4.4	
High resolution Melting	95 ^{o}C	None	00:00:05	4.4	1
	40 ^{o}C	None	00:01:00	2.2	1
	65 ^{o}C	None	00:00:01	1	1
	97^{o}C	Continous	-	-	10
Cooling	40 ^{o}C	None	00:00:10	2.2	1

4.5.2. Analysis of the mRNA of the *F8* Gene

In order to check cDNA sequence abnormalities, splicing errors or new gene rearrangements of patients without any *F8* mutation, RT-PCR studies were done.

4.5.2.1. Total RNA Extraction. Total RNA was isolated from fresh blood samples of patients by using MagNApure Compact Total RNA isolation kit. 500µl of fresh peripheral

blood sample was required for this analysis. 200µl of total RNA extract was isolated. The concentration of total RNA product was about 10ng/ul.

4.5.2.2. cDNA Synthesis and Reverse Transcriptase-PCR Amplification. cDNA synthesis from 10 ng/µl total RNA and reverse transcriptase (RT)-PCR was done by using Titan One Tube RT-PCR Kit. RT-PCR amplification was performed in two rounds of PCR using a nested approach and the specific 12 primers pairs were used as described. (El-Maarri, *et al*., 2005) (Figure 4.2). The first PCR was performed for the amplification of four regions of F8 mRNA (A, B, C and D). These four regions were used as templates for the second nested PCR to amplify the total of eight regions of *F8* gene exons. Table 4.3 shows the PCR conditions of these amplifications were optimized like the following PCR programme.

50ºC for 30 minutes (for cDNA synthesis)	
94ºC for 2 minutes (Initial denaturation)	
94ºC for 30 seconds (Denaturation)	10 cycles
55.7ºC for 30 seconds (Annealing)	
68ºC for 1 minute (Extension)	
94º C for 30 seconds (Denaturation)	
55.7ºC for 30 seconds (Annealing)	25 cycles
68ºC for 1 minute (+5 seconds for each cycle) (Extension)	
68ºC for 7 minutes (Final extension)	

Figure 4.4. Conditions of RT-PCR

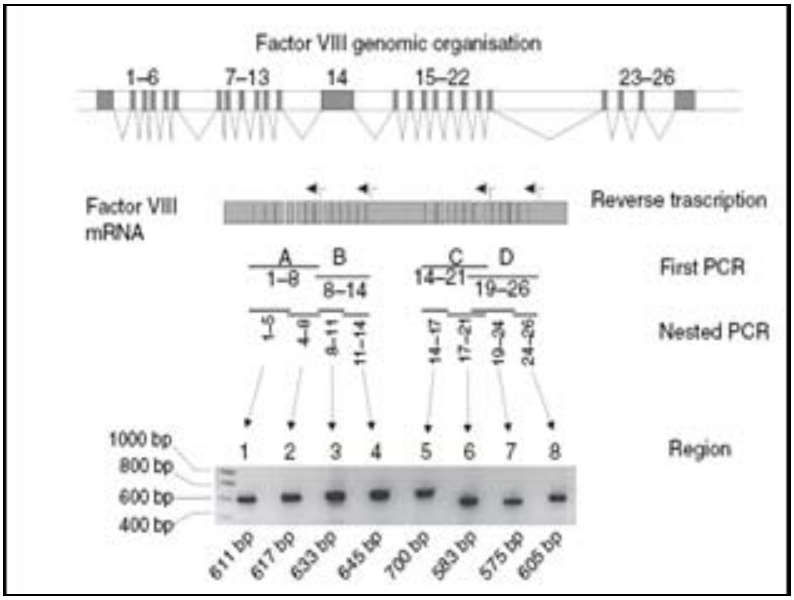


Figure 4.5. Schematic diagram of the genomic organization of the *F8* gene (El-Maarri *et al*, 2005)

Table 4.3. The optimized conditions for RT-PCR analysis of *F8* Gene

Exon Number	**Annealing** Tm **(°C)**	**Mg Concentration**
Ex1–8	55.7	1.5mM
Ex8–14	55.7	1.5mM
Ex14–19	55.7	1.5mM
Ex19–26	55.7	1.5mM
Ex1-5	55.7	2mM
Ex4-8	55.7	2mM
Ex8-11	55.7	2mM
Ex11-14	55.7	2mM
Ex14-17	55.7	2mM
Ex17-21	55.7	2mM
Ex19–24	55.7	2mM
Ex24–26	55.7	2mM
Ex means exon		

4.5.3. Long PCR Analysis

Long PCR were performed with Expand Long Template PCR System kit on 100 ng of genomic DNA. Each 1X reaction buffer contains 2,5mM Mg^{2+}, 250 µM dNTP, 300 µM

specific primers. The first 10 PCR cycles were performed with denaturation at 94°C for 10 seconds, annealing at 55 °C for 30 seconds, extension at 68 °C for 20 minutes. The next 20 cycles were performed with denaturation at 94°C for 10 seconds, annealing at 55 °C for 30 seconds and extension at 68 °C for 20 minutes with 20 seconds increments in each 20 cycles. Table 4.4 shows the PCR conditions of these amplifications

Table 4.4. The optimized conditions for Long PCR analysis of *F8* Gene

Exon Number	Annealing Tm (^{o}C)	Mg Concentration
13-26	55	2.5
1-9	55	2.5
6-7	55	2.5
13-14A	55	2.5
12-13	55	2.5
9-11	55	2.5

4.5.4. PCR Amplification of Intron 18 and Intron 19 of *F8* Gene

Intron 18 and intron 19 regions of F8 gene were separated and optimized. Each PCR reaction was prepared in a 25µl volume, containing 1X Mg^{2+} free reaction buffer, 2 or 2,5 mM of $MgCl_2$, 0.2 mM of each dNTP, 10 µM of each primer, 0.5 or 1 U of Taq polymerase and 50 ng of genomic DNA at intron-specific conditions shown in Table 4.5.

Table 4.5. PCR conditions for intron 18 and intron 19 of *F8* gene in this study

Exon Number	Annealing Tm (^{o}C)	Mg Concentration	PCR Size (bp)
IVS18A	55	2mM	380
IVS18B	55	2mM	436
IVS18C	55	2mM	450
IVS18D	55	2mM	450
IVS18E	55	2mM	496
IVS19A	55	2mM	394
IVS19B	55	2mM	383
IVS means intervening sequence variation			

4.5.5. Karyotype Analysis

Two patients' fresh blood samples were collected into heparine containing tube and Premed Laboratory, Turkey, performed their karyotype analyses.

4.5.6. Comparative Genomic Hybridization Analysis

In order to detect large deletions or gene rearrangements in *F8* gene, CGH analysis was done by using custom designed NimbleGen array 3X720K (3X720.000 probes). genomic regions of interest in designed array were given in Table 4.6. The CGH analysis service was provided by NimbleGen providers in Iceland. The CGH protocol involves independently labeling test and reference genomic DNA using a NimbleGen dual-Color DNA labeling kit and co-hybridization of these DNAs to a NimbleGen CGH array using a NimbleGen Hybridization System (Figure 4.3).

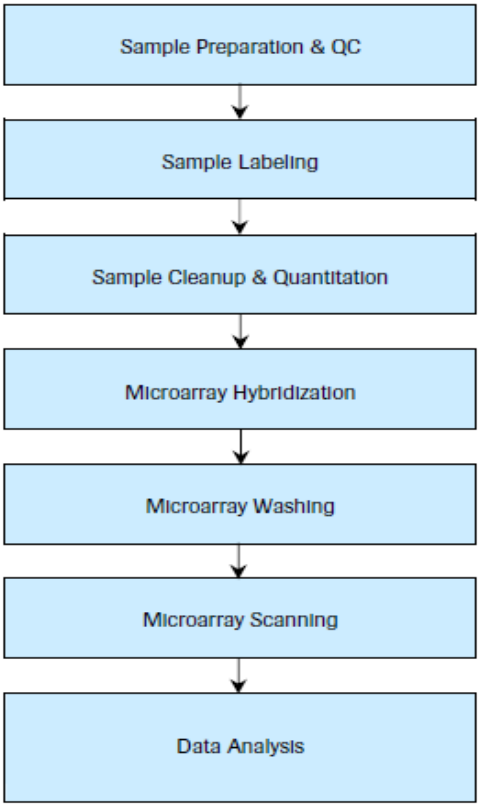


Figure 4.6. Workflow for NimbleGen CGH analysis

After scanning, NimblegGen software produces three files for CGH data, segmentation PDF plot, showing CGH data in single panel rainbow plots or multi-panel plots, GFF files containg the log2 ratio of Cy3 and Cy5 for each probe plotted versus genomic position and data summary files containing a summary of predicted segments. Data analysis was performed by using SignalMap programme provided by Roche Applied Science Inc., GFF files (normalized, averaged or unaveraged) were imported to SignalMap to view data in individual sample. In order to see gene annotations and variants specific to design, design and variation GFF files were also imported. After importation, CGH data were displayed in SignalMap software showing cytogenic ideogram known genes and normal CNVs from database of genomic variants (Figure 4.4).

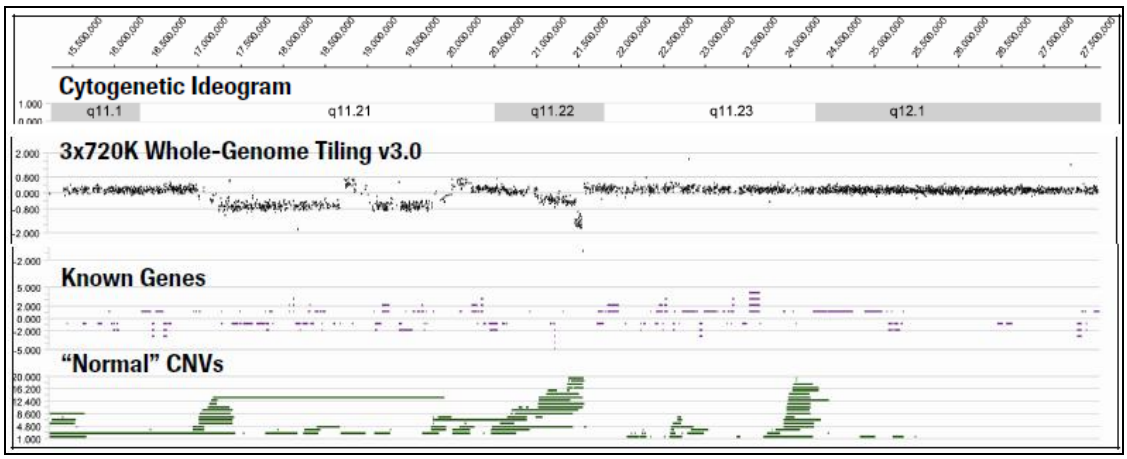


Figure 4.7. A cytogenetic ideogram model in SignalMap programme

Table 4.6. Genomic regions of interest probed in the NimbleGen array design

Gene	Chromosome	Cytogenic Band	Gene Location	Ref. Seq.	DNA Strand
TGFB1	19	19q13.2-q13.1	41,895,651-41,860,816	NM_000660	-
IL4	5	5q31.1	132,009,373-132,018,368	NM_172348	-
IFNG	12	12q14	68,547,550-68,554,521	NM_000619	-
IL10	1	1q31-q32	206,939,948-206,946,839	NM_000572	+
IL5	5	5q31	131,876,136-131,880,214	NM_000879	-
GABRA5	15	15q11.2-q12	27,111,058-27,195,003	NM_000810	+
GABRG3	15	15q12	27,215,517-27,779,134	NM_033223	+
SCN1A	2	2q24.3	166,844,671-166,931,149	NM_006920	-
(SCN1B+ LGI4)	19	19q13.11	35,520,534-35,627,178		+
SCN2A	2	2q23-24	166,150,341-166,248,818	NM_021007	+
KCNQ2	20	20q13.3	62,036,542-62,104,993	NM_172107	-
KCNQ3	8	8q24	133,140,257-133,494,004	NM_004519	-
EPM1	21	21q22.3	45,192,832-45,197,259	NM_000100	-
EPM2A	6	6q24	145,945,446-146,058,122	NM_005670	-

Table 4.6. Genomic regions of interest probed in the NimbleGen array design (continued)

Gene	Chromosome	Cytogenic Band	Gene Location	Ref. Seq.	DNA Strand
EPM2B	6	6p22.3	18,119,718-18,123,851	NM_198586	-
GABRR1	6	6q13-q16.3	89,886,223-89,928,496	NM_002042	-
KCNJ10	1	1q22-23	160,007,033-160,040,961	NM_002241	-
KCNJ9	1	1q21-23	160,050,360-160,060,212	NM_004983	+
ALDH5A1	6	6p22.2-22.3	24,494,197-24,538,434	NM_170740	+
SLC1A7	1	1p32.3	53,551,856-53,609,289	NM_006671	-
GABRA4	4	4p12	46,919,919-46,996,580	NM_000809	-
KCNAB1	3	3q26.1	156,007,776-156,257,927	NM_172159	+
GABRD	1	1p36.3	1,949,768-1,963,192	NM_000815	+
CACNB4	2	2q22-23	152,688,288-152,956,593	NM_001005746	-
GABRR2	6	6q13-16.3	89,966,239-90,025,967	NM_002043	-
CHRNA7	15	15q14	32,321,726-32,462,233	NM_000746	+
ME2	18	18q21	48,404,435-48,475,258	NM_002396	+
NLGN4X	X	Xp22.32-p22.31	5,807,084-6,146,888	NM_020742	-
LGI2	4	4p15.2	24,999,471-25,033,414	NM_018176	-
LGI1	10	10q24	95,516,566-95,558,915	NM_005097	+
EFHC1	6	6p12.3	52,283,994-52,361,581	NM_018100	+
CHRNA4	20	20q13.2-13.3	61,973,665-62,010,489	NM_000744	-
CHRNB2	1	1q21.3	154,539,257-154,553,351	NM_000748	+
KCNA1	12	12p13.32	5,018,073-5,028,420	NM_000217	+
JRK	8	8q24.3	143,737,875-143,752,401	NM_003724	-
HCN1	5	5p12	45,258,353-45,697,220	NM_021072	-
HCN2	19	19p13.3	588,893-618,157	NM_001194	+
GRIK1	21	21q22.11	30,908,256-31,313,282	NM_175611	-
OPRM1	6	6q24-25	154,330,636-154,441,594	NM_001145279	+
F8	X	Xq28	154,063,070-154,251,998	NM_000132	-
GABRA1	5	5q34-35	161,273,197-161,327,963	NM_000806.5	+
GABRB2	5	5q34-35	160,714,436-160,976,125	NM_021911	-
GABRG2	5	5q31.1-q33.1	161,493,648-161,583,544	NM_198903	+
GABRB3	15	15q12	26,787,695-27,019,927	NM_021912	-
GABBR1	6	6p21.31	29,569,006-29,601,962	NM_001470	-
GABBR2	9	9q22.33	101,049,366-101,472,175	NM_005458	-
CACNA1A	19	19p13.2-p13.1	13,354,996-13,442,147	NM_001127222	-
CACNA1G	17	17q22	48,637,449-48,705,542	NM_198396	+
CACNA1H	16	16p13.3	1,202,241-1,272,771	NM_021098	+
CACNA1I	22	22q13.1	39,965,758-40,086,738	NM_021096	+
TRAK1	3	3p25.3-p24.1	42,131,746-42,268,267	NM_001042646	+
CACNA2D	3	3p21.3	50,399,233-50,541,892	NM_006030	-
	2	2q33-36	220,362,637-220,503,533		

4.6. Case-Control Association Analysis

4.6.1. Selection of the SNPs at Immune Response Genes for Association Study

Nine SNPs and one VNTR region in genes IL2, IFNG, IL4, IL5, IL10 and TGFB1 were selected considering the average heterozygosity frequency near to 0.5 in different populations. Data were taken from HapMap and NCBI.

4.6.2. Optimization of SNP Primer and Hybprobe Probes

HybProbe probes are the sequence-specific hybridization probes labeled with fluorescent dyes that bind close together on a single DNA strand. One HybProbe Probe is labeled with the fluorescent donor dye fluorescein, the other one is labeled with an acceptor dye. After cooling to the annealing temperature PCR primers and HybProbe Probes hybridize to their complementary regions. The donor dye now comes into close proximity to the acceptor dye. Energy emitted from the donor dye excites the acceptor dye. The PCR instrument measures the light. After annealing to their target sites, the primers are elongated by thermostable DNA polymerase.

Reactions were optimized by preparing mixture in 20 µl volume containing 1 X probe master mix with 3.2 mM Mg^{2+}, faststart taq DNA polymerase, reaction buffer and dNTP mix, 0.25 pmol of each primer pairs (F, R and S, A) 0.2 pmol of anchor and sensor probes and 60 ng of genomic DNA. In order to increase the flouresence of the peaks asymmetric primer pairs were also used for the optimization with 1:5 or 1:2 ratio of the reduced primer that is in the same orientation with the sensor. The optimization conditions for amplification and melting curve analysis is shown in Table 4.7.

4.6.3. Hardy-Weinberg Equilibrium for Turkish Healthy Individuals

In order to avoid the complication like genotyping errors and population stratification 100 Turkish healthy individual samples were checked whether they were in Hardy-Weinberg equilibrium by the Haploview programme. The Hardy-Weinberg equations are as follows:

- $p+q=1$ where p is the frequency of dominant allele and q is the frequency of recessive allele.

- $p^2 + 2pq + q^2 = 1$ where p^2 is the predicted frequency of homozygous dominant individuals and 2pq is the predicted frequency of heterozygous individuals, and q^2 is the predicted frequency of homozygous recessive ones in the population.

4.6.4. Case Control Association Analysis

The genotyping results of two patients groups were analyzed by the Haploview programme 4.1. According to the analysis, p values of SNPs' allele and genotype frequencies lower than 0.05 were accepted to be significant and associated to the disease.

Table 4.7. Optimization conditions for hybprobe probes in light cycler 480

Programme name	**Tm**	**Acquisition mode**	**Time (hh:mm:ss)**	**Ramp rate (^oC/s)**	**Cycle**
Pre-incubation	95 ^oC	None	00:05:00-00:10:00	4.4	1
Amplification	95 ^oC	None	00:00:10	4.4	45
	52-58 ^oC	None	00:00:15	2.2	
	72 ^oC	Single	00:00:01	4.4	
Melting curve	95 ^oC	None	00:02:00	4.4	1
	40 ^oC	None	00:02:00	2.2	
	80 ^oC	Continous	-	-	
Cooling	40 ^oC	None	00:00:30	1.5	1

4.7. Analysis of IL5 Gene

4.7.1. DNA Analysis by High Resolution Melting

High Resolution Melting (HRM) is a novel, post-PCR method, enabling to analyze genetic variations (SNPs, mutations, methylations) in PCR products. It allows to study the thermal denaturation of a double-stranded DNA in much more detail. After amplification process a melting curve analysis follows where a sequence that has a variation melts at a different Tm and classified as a different group than the wild type. HRM reactions were optimized by preparing the mixture in 20 µl volume containing 1 X master mix with faststart taq DNA polymerase, reaction buffer, dNTP mix and high resolution melting dye, 0.2-0.5 mM of Mg^{2+}, 0.2-0.5 pmol of each primer pairs and 20-40 ng of genomic DNA. The optimization conditions for amplification and melting curve analysis is shown in Table 4.8.

Table 4.8. The optimized conditions for HRM analysis in light cycler 480

Programme name	Tm	Acquisition mode	Time (hh:mm:ss)	Ramp rate (^{o}C/s)	Cycle
Pre-incubation	95 ^{o}C	None	00:10:00	4.4	1
Amplification	95 ^{o}C	None	00:00:10	4.4	45
	Touchdown	None	00:00:15	2.2	
	72 ^{o}C	Single	00:00:10-00:00:16	4.4	
High Resolution Melting	95 ^{o}C	None	00:01:00	4.4	1
	40 ^{o}C	None	00:01:00	2.2	1
	65 ^{o}C	None	00:00:01	1	1
	95 ^{o}C	Continous	-	-	25
Cooling	40 ^{o}C	None	00:00:10	2.2	1

4.7.2. DNA Analysis by Direct DNA Sequencing.

The promoter regions 2 and 3, exons 1, 2 and 4 of IL5 gene were analyzed by direct DNA sequencing. PCR products were purified and sequenced at Macrogen, Korea.

4.7.3. Copy Number Variation Analysis by Quantative PCR

Quantitative PCRs (qPCR), assay was used to detect copy number of the IL5 gene rs2069812 region in two patient groups. The relative amount of template syber green dye which binds to double stranded DNA was used in qPCR analysis. Absolute quantification using the "Fit Points Method" is an analysis used to quantify the target sequence and reference sequence and gives a concentration value. Relative quantification compares these targets and reference sequences' concentrations and gives a ratio. Real-time qPCRs were performed with a LightCycler 480 instrument and LightCycler 480 SYBR Green I Master kit and target and reference sequence-specific primers, Target sequence was IL5 rs2069812 region and reference sequence was exon 6 of the sodium channel 1 alpha (SCN1A) gene.

Concentrations of DNA samples of patients, normal male and normal female were calculated and their initial concentrations were equated to 20ng/µl. PCRs of samples were triplicated to avoid pipetting error. Serial dilutions of DNA sample of a normal female were used as a standard for target and reference sequences. Table 4.9 shows the optimization conditions of CNV analysis in light cycler 480.

Table 4.9. The optimized conditions for qPCR analysis in light cycler 480

Programme Name	**Tm**	**Acquisition mode**	**Time (hh:mm:ss)**	**Ramp rate (^{o}C/s)**	**Cycle**
Pre-incubation	95 ^{o}C	None	00:10:00	4.4	1
Amplification	95 ^{o}C	None	00:00:10	4.4	32
	55-63 ^{o}C	None	00:00:20	2.2	
	72 ^{o}C	Single	00:00:10	4.4	
High Resolution Melting	95 ^{o}C	None	00:00:05	4.4	1
	40 ^{o}C	None	00:01:00	2.2	1
	65 ^{o}C	None	00:00:01	1	1
	97^{o}C	Continous	-	-	10
Cooling	40 ^{o}C	None	00:00:10	2.2	1

5. RESULTS

5.1. The Mutation Profile of Severely Affected Hemophilia A Patients who Develop Inhibitors

5.1.1. Screening of Hemophilia A Patients with Inhibitors for Intron 22 Inversion

Thirty HR patients and 4 LR patients were initially tested for intron 22 inversion by southern blot analysis. The schematic presentation of the southern blot pattern of intron 22 inversion mutation is given in Figure 5.1. A healthy individual has bands of sizes 21.5 kb, 16 kb and 14 kb representing intragenic int22h-1 and extragenic int22h-2 and int22h-3 repeat regions, respectively (lane 1). In the proximal pattern of intron 22 inversion, the band sizes are altered to 20 kb, 16 kb and 15.5 kb (lane 2). Whereas, the band sizes are 20 kb, 17.5 kb and 14 kb in the distal pattern of intron 22 inversion (lane 3). Distal and proximal intron 22 inversions were detected in 13 (38 per cent) and 3 (9 per cent) patients, respectively, and therefore, represented approximately 50 percent of pathological mutations. In addition, two new intron 22 inversion patterns were observed in two patients. One patient (268HA993) had a southern blot pattern that was missing the bands for extragenic copies int22h-2 and int22h-3 (Figure 5.2). In patient 267HA992 the band corresponding to the repeat region in intron 22 (int22h-2) was absent (Figure 5.3). These patients were suspected to have deletions of the corresponding Southern blot bands. Our results confirmed that intron 22 inversion mutation is the major mutation type in severe hemophilia A patients with inhibitors as originally shown by other groups.

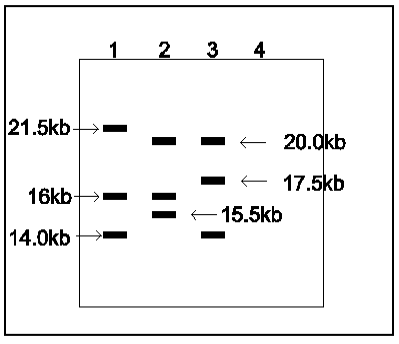


Figure 5.1. The schematic presentation of the southern blot pattern of intron 22 inversion

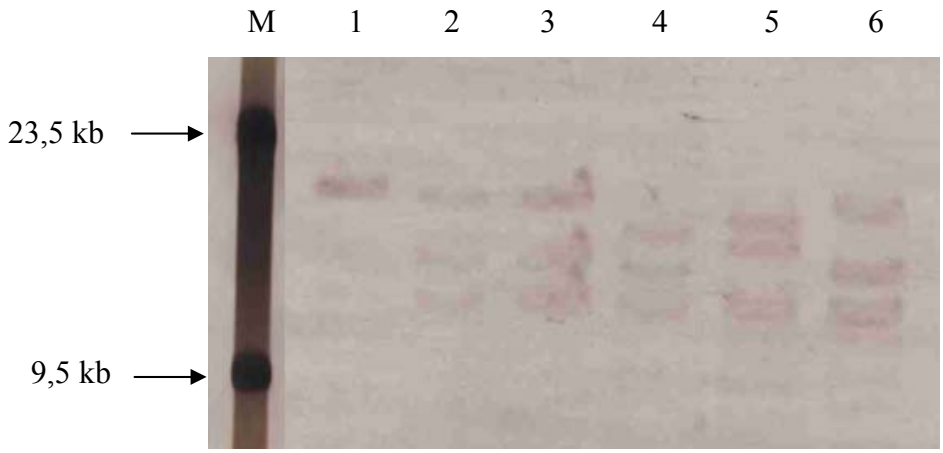


Figure 5.2. Southern blot pattern of patient 268HA993 (lane1) for intron 22 inversion. M indicates Lambda/HindIII DNA marker, lane 2-4 and 6 indicate normal pattern, lane 5 indicates distal pattern

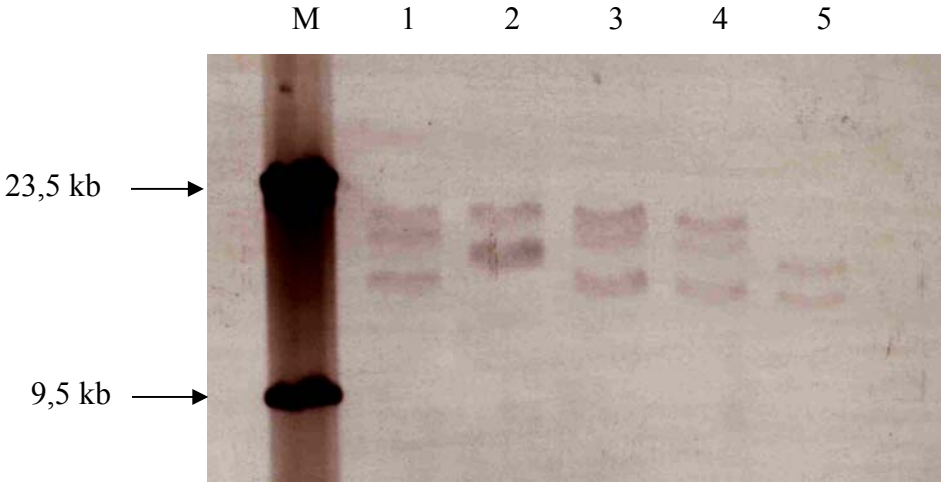


Figure 5.3. Southern blot pattern of patient 267HA992 (lane 5) for intron 22 inversion. M indicates Lambda/HindIII DNA marker, lane 1, 3 and 4 indicate normal pattern, lane 2 indicates proximal pattern

5.1.2. Screening of Hemophilia A Patients with Inhibitors for Intron 1 Inversion

Inhibitor patients without intron 22 inversions were subsequently tested for intron 1 inversion mutation. In order to analyze intron 1 inversion by PCR amplifications primers specific for int1h-2 (int1h-2F, int1h-2R) plus the primer 9F were used yielding a 1300 bp product from normal DNA and a 1900 bp product in presence of the inversion. Only 1 patient (278HA1003) had the rare intron 1 inversion mutation (Figure 5.4).

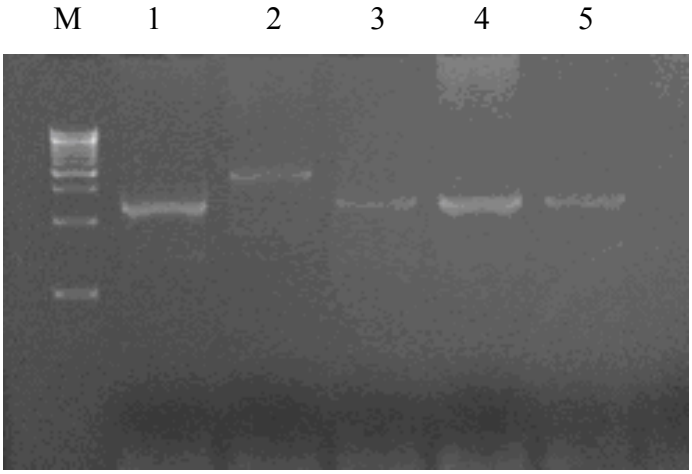


Figure 5.4. PCR amplification of int1h-2 region. M: 500 bp ladder; lane 2 indicates intron 1 inversion of 278HA1003; lane 1, 3, 4, 6 indicate hemophilia A patients without intron 1 inversion

5.1.3. Point Mutation Detection

The patients were then screened for point mutations by complete sequencing of the coding regions, intron/exon boundaries, promoter and 3' UTR regions of the *F8* gene. Pathological point mutations were identified in 8 HR and 3 LR patients, respectively. Five patients were suspected to have large deletions and one HR patient did not have any point mutation or inversion mutations in the F8 gene.

The most prevalent point mutation in this study was nonsense mutations and small deletions detected in 3 and 6 patients, respectively. One of the 3 patients, 264HA989 had a c.1812 G→A change in exon 12 resulting in a novel nonsense mutation (W585X) in the A2 domain and hence, the heavy chain of FVIII protein (Figure 5.5). Three novel small deletions 1631delAT, 2182delT, and 6602delG resulting in frameshift and terminations (N525-X535, S709-X731 and S2182-X2216) in A2 and C2 domains occurred in patients 275HA1000, 87HA344 and 126HA604, respectively (Figure 5.6, 5.7 and 5.8).

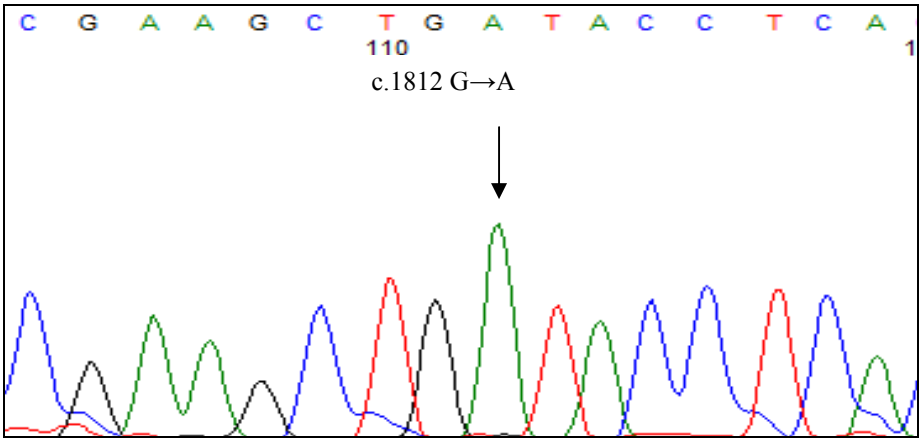


Figure 5.5. c.1812 G→A mutation in exon 12 of patient 264HA989

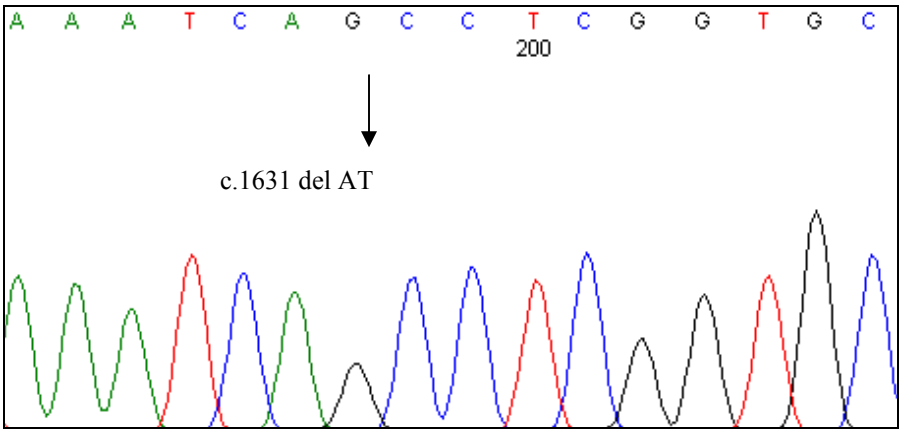


Figure 5.6. c.1631delAT mutation in exon 11 of patient 275HA1000

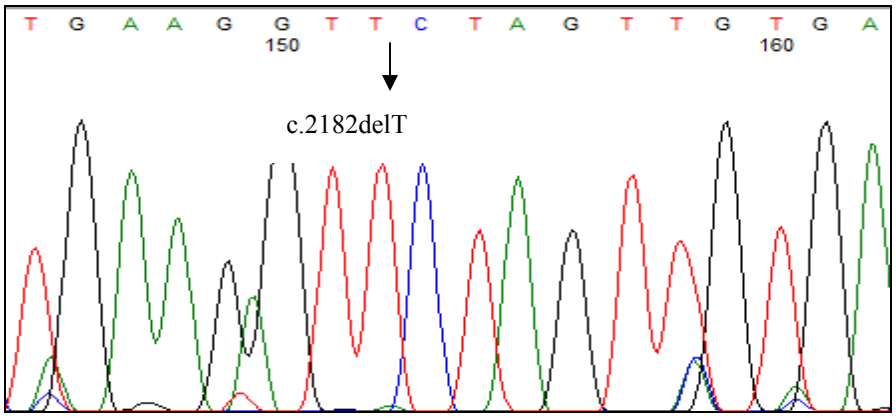


Figure5.7. c.2182delT mutation in exon 14 of patient 87HA344

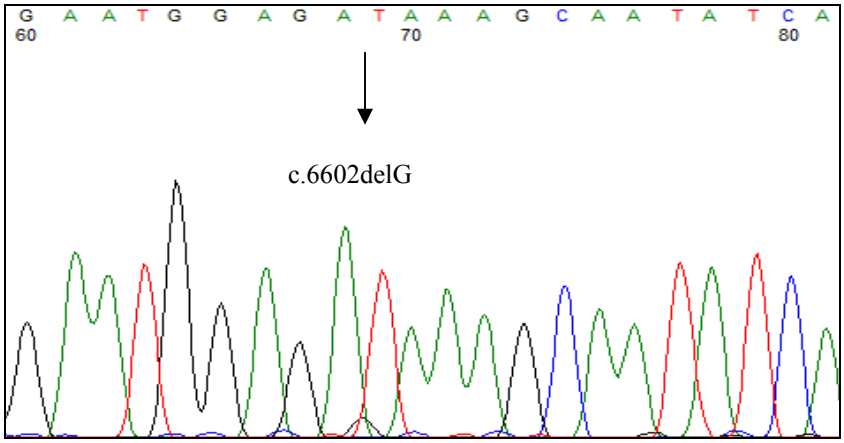


Figure 5.8. c.6602delG mutation in exon 24 of patient 126HA604

The recurrent point mutations c.5953 C→T and c.6682 C→T found in patients 274HA1004 and 280HA1005 resulted in nonsense mutations (R1966X and R2209X) in exons 18 and 24 corresponding to A3 and C2 domains and hence, to the light chain of FVIII, respectively (Figure 5.9 and 5.10). Two identical G deletions (c.6049delG) in patients 282HA1007 and 284HA 1010 at V1998 resulted in a frameshift and truncation at codon 2011 corresponding to the A3 domain (Figure 5.11). The *Bsl* I (exon 14) and *Bcl* I (intron 18) genotypes of these two patients suggested that the mutations arose on different haplotypes.

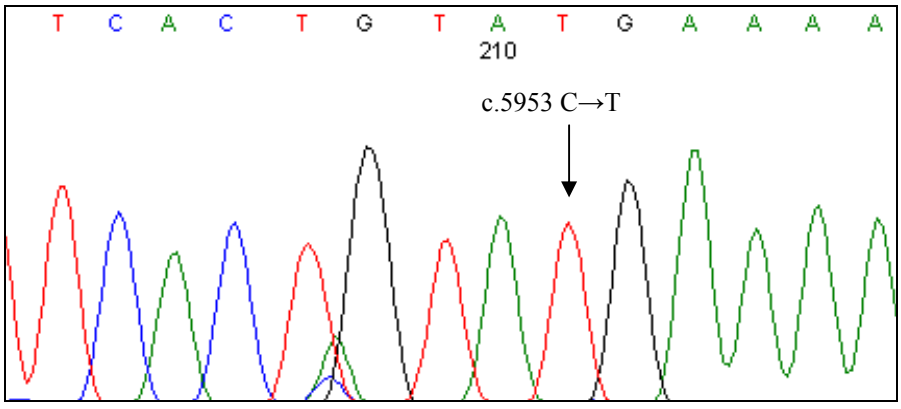


Figure 5.9. c.5953 C→T mutation in exon 18 of patient 279HA1004

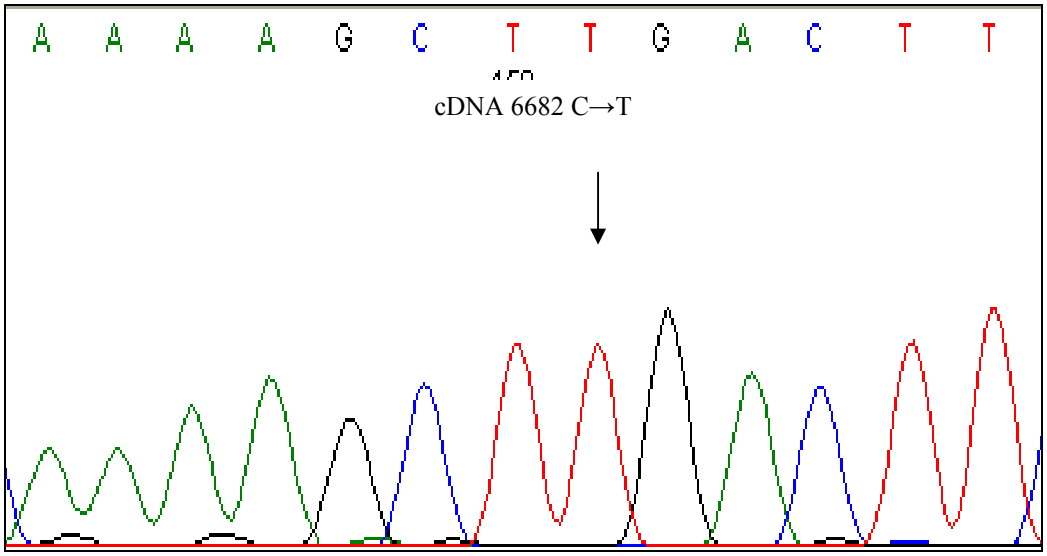


Figure 5.10. c.6682 C→T mutation in exon 24 of patient 280HA1005

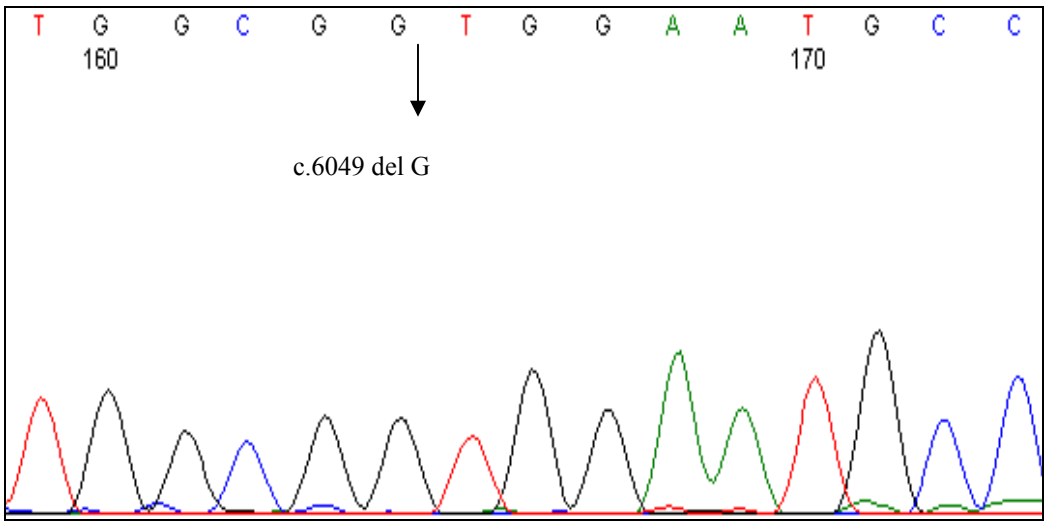


Figure 5.11. c.6049delG mutation in exon 19 of patients 282HA1007 and 284HA 1010

Three novel missense mutations (K1732E, R405S and L377M) have been detected in patients 103HA388, 264HA989 and 273HA998, respectively. c.5251A→G transition in exon 15 that resulted in K1732Q substitution in the A3 domain is the only change detected in the severely affected patient 103HA388 and is most probably the causative mutation since it affected an evolutionary conserved amino acid (Figure 5.12). According to prediction of functional effects of human tool (PolyPhen-2) data, this amino acid change is predicted to be possibly damaging in FVIII protein structure (http://genetics.bwh.harvard.edu/pph2/index.shtml).

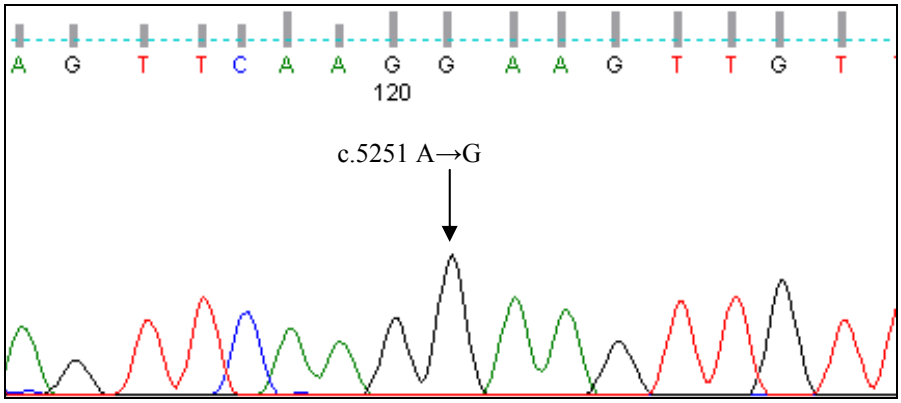


Figure 5.12. c.5251 A→G mutation in exon 15 of patient 103HA388

Two patients had double mutations. In addition to a novel nonsense mutation (W585X), patient 264HA989 had a novel c.1271 A→C transversion that resulted in a missense mutation (R405S substitution) (Figure 5.13). R405S is not conserved in the murine but changes the SR protein-binding site (http://rulai.cshl.edu/tools/ESE/). Patient 273HA998 had also a c.1187 A→T transition resulting in L377M missense mutation (Figure 5.14) in addition to a proximal inversion. L377M is evolutionarily conserved and its possibility of being a polymorphic change was excluded when 117 alleles in 70 apparently healthy individuals were genotyped by *Nsi* I restriction analysis, suggesting that patient 273HA998 had double pathological mutations. On the other hand, R405S, may be a polymorphism, however, both L377M and R405S was not listed as polymorphisms in HAMSTeRS database and they had not been detected in previous point mutation analyses through complete sequencing of 46 Turkish hemophilia A patients (El-Maarri *et al*., 1999) (Timur *et al*., 2001).

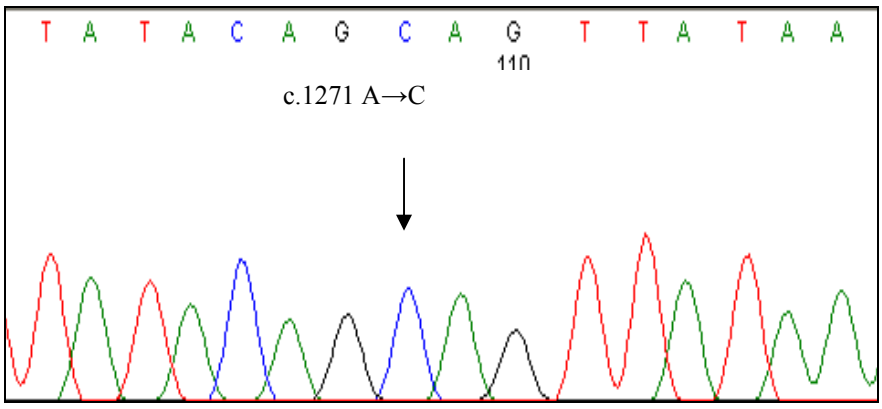


Figure 5.13. c.1271 A→C mutation in exon 9 of patient 264HA989

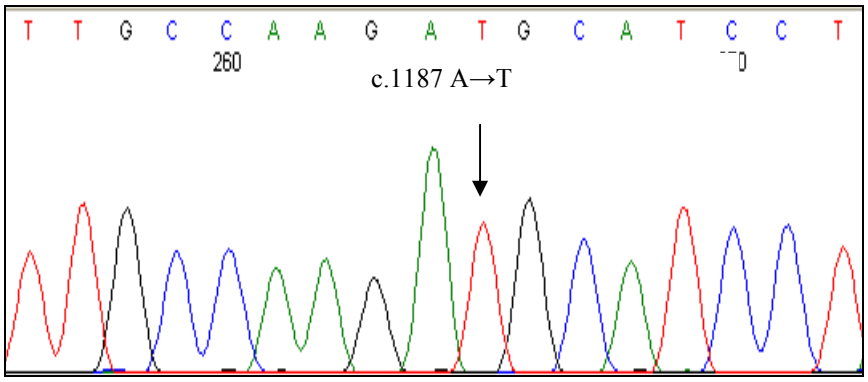


Figure 5.14. c.1187 A→T mutation in exon 9 of patient 273HA998

Only one patient 208HA798 had a splice site mutation. This novel mutation (-2A>T) that occurred in the acceptor site junction of intron 16 (Figure 5.15). This A→T substitution is calculated for the splicing sites scores. If this transition occurs, the acceptor site is fully changed (http://fruitfly.org/seq_tools/splice.html).

One HR patient (162HA675) did not have a sequence change upon complete sequencing of the *F8* gene including the promoter and 3'UTR regions. Promoter and 3' UTR specific primers were given in Table 3.1.

Table 5.1 and 5.2 lists the phenotype and genotype of 30 HR and 4 LR patients, respectively.

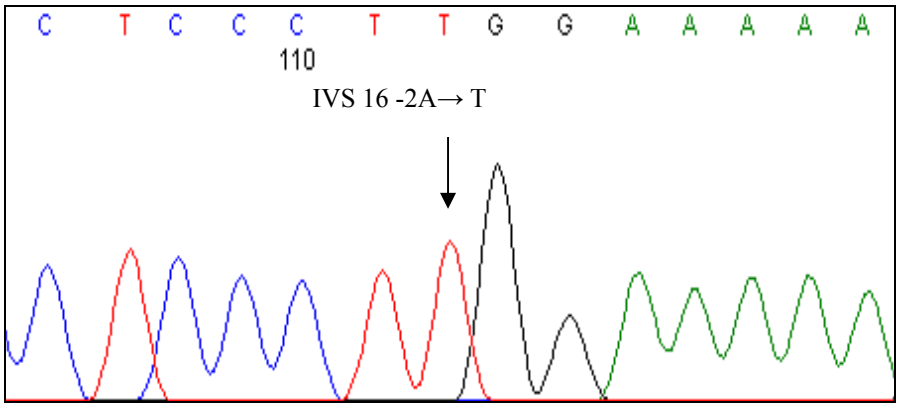


Figure 5.15. IVS16-2AT mutation in intron 16 of patient 208HA798

Table 5.1. Phenotype and genotype of 30 HR patients

No	Patient ID	Intron22 Inversion	Intron1 Inversion	Protein Domain	Exon (E) Intron (I)	cDNA	Amino Acid	Severity	FVIII:C %	Inhibitor Titer (Bu/ml)	Comment
1	85HA 342	Distal	-					S	1	HR	
2	80HA 337	Distal	-					S*	1	7.5	
3	124HA 469	Distal	-					S	2	32	
4	197HA 786	Distal	-					M-S^{f}	3	20	
5	265HA 990	Distal	-					S	1	16-250	
6	266HA 991	Distal	-					S	1	400	
7	269HA 994	Distal	-					S	0.4	176	
8	270HA 995	Distal	-					S	1	250	
9	271HA 996	Distal	-					S	1-5	60	
10	272HA 997	Distal	-					S	1-4	5	
11	273HA 998	Proximal	-	A2	E8	1187A>T	L377M[1]	S	1	12	Double mutation Inversion-missense
12	276HA 1001	Proximal	-					S	1.7	10	
13	281HA 1006	Distal	-					S	0.5	32	
14	287HA 1013	Proximal	-					S	1	19	
15	289HA 1015	Distal	-					S	0.3	6.6	

[1] indicates a novel mutation *S refers to severe
#M refers to moderate ^{f}M-S refers to moderate-severe
HA refers to hemophilia A

Table 5.1. Phenotype and genotype of 30 HR patients (continued)

No	Patient ID	Intron22 Inversion	Intron1 Inversion	Protein Domain	Exon (E)/ Intron (I)	cDNA	Amino Acid	Severity	FVIII:C %	Inhibitor Titer (Bu/ml)	Comment
16	278HA 1003	-	Yes					M	1	16	
17	264HA 989	-	-	A2	E12 E9	1812 G>A 1272 A>C	W585X[1] R405S[1]	S	1	400	Nonsense Missense Changes the SR protein-Binding Site
18	279HA 1004	-	-	A3	E18	5953C> T	R1966X	S	1	88	Nonsense
19	280HA 1005	-	-	C2	E24	6682C>T	R2209X	S	1-4	5	Nonsense
20	275HA 1000	-	-	A2	E11	1631delAT	N525-X535[1]	S	0<1	10	Frameshift (Nonsense)
21	282HA 1007	-	-	A3	E19	6049delG	V1998-X2011	S	1	9	Frameshift (nonsense)
22	284HA 1010	-	-	A3	E19	6049delG	V1998-X2011	S	1	474	Frameshift (nonsense)
23	103HA 388	-	-	A3	E15	5251 A>G	K1732E[1]	S	1.2	15	Missense Possibly damaging in FVIII structure (polyphen -2)
24	208HA 798	-	-	A3	I16		-2A>T[1]	S	0.8	950	Splicing Error
25	162HA 675	-	-	-				S	1	16	No sequence change
26	268HA 993	-	-		I22h-2 and I22h-3 3'UTR	 8728A>G		S	1-3	14	Large deletion No change in exons Polymorphism

HA refers to hemophilia A [1] indicates a novel mutation *S refers to severe [#]M refers to moderate [/]M-S refers to moderate-severe

Table 5.1. Phenotype and genotype of 30 HR patients (continued)

No	Patient ID	Intron22 Inversion	Intron1 Inversion	Protein Domain	Exon (E)/ Intron (I)	cDNA	Amino Acid	Severity	FVIII:C %	Inhibitor Titer (Bu/ml)	Comment
27	267HA 992	-	-	C	E13-25			S	<1	26	Large Deletion
28	274HA 999	-	-	A1-A2	E2-8			M[#]	2	80	Large Deletion
29	283HA 1008	-	-	A2	E10			S	3	49	Large Deletion
30	288HA 1014	-	-	A3-C1	E19-22			S	<1	600	Large Deletion

[1] indicates a novel mutation *S refers to severe [#]M refers to moderate [/]M-S refers to moderate-severe HA refers to hemophilia A

Table 5.2. Phenotype and genotype of 4 LR patients

No	Patient ID	Intron22 Inversion	Intron1 Inversion	Protein Domain	Exon (E)/ Intron (I)	cDNA	Amino Acid	Severity	FVIII:C %	Inhibitor Titer (Bu/ml)	Comment
1	247HA 925	Distal								yes	
2	87HA 344			A2	14	2182delT	S709-X731[1]	M-S	2	1	Frameshift (nonsense)
3	126HA 604			C2	24	6602delG	S2182-X2216[1]	S	1	3	Frameshift (nonsense)
4	277HA 1002			B	14	3637delA	I1194-X1198	M-S.	1	0.8	Frameshift (nonsense)

[1] indicates a novel mutation *S refers to severe [#]M refers to moderate [/]M-S refers to moderate-severe HA refers to hemophilia A

Large deletions were suspected to occur in 5 HR patients as judged by the failure of PCR amplifications of the corresponding exons in patients 267HA992, 274HA999, 283HA1008 and 288HA1014 and southern blot regions in patients 268HA993 (Table 5.3). The deletions are suspected to span exons 14-25 in 267HA992 who also lacked int22h-1 region locating in intron 22 of the *F8* gene as judged by Southern blot analysis. Patient 268HA993 only lacked the extragenic copies of int22h-2 and int22h-3. Patients 274HA999, 283HA1008 and 288HA1014 lacked exons 2-8, exon 10, and exons 19-22, respectively.

Table 5.3. PCR results of 5 patients suspected with large deletions

Patient No	**267HA992**	**268HA993**	**274 HA 999**	**283HA1008**	**288HA 1014**
PCR Results					
Intron 1	No inversion	No inversion	No inversion	No inversion	No inversion
Intron 22	No band for intragenic Int22h–1	No bands for extragenic Int22h–2 and 3	No inversion	No inversion	No inversion
5' UTR	+	+	+	+	+
3' UTR	+	+	+	+	+
Exon 1	+	+	+	+	+
Exon 2	+	+	-	+	+
Exon 3	+	+	-	+	+
Exon 4	+	+	-	+	+
Exon 5	+	+	-	+	+
Exon 6	+	+	-	+	+
Exon 7	+	+	-	+	+
Exon 8	+	+	-	+	+
Exon 9	+	+	+	+	+
Exon 10	+	+	+	-	+
Exon 11	+	+	+	+	+
Exon 12	+	+	+	+	+
Exon 13	+	+	+	+	+
Exon 14A	-	+	+	+	+
Exon 14B	-	+	+	+	+
Exon 14C	-	+	+	+	+
Exon 14D	-	+	+	+	+
Exon 14E	-	+	+	+	+
Exon 14F	-	+	+	+	+
Exon 14G	-	+	+	+	+
Exon 14H	-	+	+	+	+
Exon 14J	-	+	+	+	+
Exon 14K	-	+	+	+	+
Exon 15	-	+	+	+	+
Exon 16	-	+	+	+	+
+ means regions were amplified and sequenced.- means regions could not be amplified.					

Table 5.3. PCR results of 5 patients suspected with large deletions (continued)

Patient No	267HA992	268HA993	274 HA 999	283HA1008	288HA 1014
PCR Results					
Exon 17	-	+	+	+	+
Exon 18	-	+	+	+	+
Exon 19	-	+	+	+	-
Exon 20	-	+	+	+	-
Exon 21	-	+	+	+	-
Exon 22	-	+	+	+	-
Exon 23	-	+	+	+	+
Exon 24	-	+	+	+	+
Exon 25	-	+	+	+	+
Exon 26	+	+	+	+	+
+ means regions were amplified and sequenced.- means regions could not be amplified.					

5.1.4. Absolute and Relative Quantification Analysis of Patients with Suspected Large Deletions

qPCR assay were conducted to confirm deletions and determine the heterozygosity of mothers by detecting the relative amount of template DNA. Absolute quantification analysis was used to quantify the target sequence and reference sequences. Relative quantification was used to compare the target and reference sequence concentrations. Target sequence was *F8* regions and reference sequence was exon 6 of SCN1A gene. Normal PCR primers (intronic) were used for target and reference regions. qPCRs were performed in real time for patients 267HA992, 274HA999 and 283HA1008 and their mothers. PCR amplifications of target sequences were optimized and run in patient, mother, normal male and normal female DNA samples. PCR amplifications of the reference sequence were also optimized for the same individuals. Concentrations of of target and reference sequences and their ratios were shown in Table 5.4 and Table 5.5. The normalized ratio for target sequence to reference sequence had a value 1 and 0.5 in a normal female and in a normal male, respectively. The ratio of 0.5 was interpreted as one copy of exons 2, 7, 8, and 10 confirming the carrier status of the mother of patients 274HA999 and 283HA1008. The ratio of 1 in the mother of patient 267HA992 was interpreted as two copies of exons between 13 and 25 and she was diagnosed as a non-carrier. Amplification curves were not observed in patients 267HA992, 274HA999 and 283HA1008 themselves for their target regions confirming the suspected deletions in the three patients.

Tabel 5.4. Concentrations and ratios of target and reference sequences of patients suspected to have large deletions

Standards	Standard1 5ng/ul	Standard2 10ng/ul	Standard3 20ng/ul						
Individuals				Normal Male	Normal Female	267HA992	Mother of 267HA992	274 HA 999	Mother 274HA999
Target Concentrations (F8 exon2) (E*)	4.89 E0	1.01 E1	1.97 E1	6.36 E0	2.06 E1			0	5.34E0
Target Concentrations (F8 exon7) (E)	4.92 E0	1.06 E1	2.03 E1	6.16 E0	1.90 E1			0	5.43E0
Target Concentrations (F8 exon8) (E)	5.04 E0	9.85 E0	2.01 E1	6.72 E0	2.06 E1			0	5,25E0
Target Concentrations (F8 exon10) (E)	4.83 E0	9.78 E0	2.06 E1	6.98 E0	2.09E1				
Target Concentrations (F8 exon13) (E)	5.04 E0	9.99 E0	1.99 E1	7.83 E0	1.79 E1	0	1.78 E1		
Target Concentrations (F8 exon25) (E)	5.00 E0	1.00 E1	2.02 E1	7.98 E0	2.06 E1	0	1.81 E1		
Reference Concentrations (SCN1A exon6) (E)	4.98 E0	1.02 E1	2.03 E1	1.58 E1	2.00 E1	2.23 E1	1.80 E1		1.33 E1
Ratio	0.99	0.98	0.99	0.45	0.99		0.99		0,51
E: means 10, E0 means 0, E1 means10									

Table 5.5. Concentrations and ratios of target and reference sequences of patients suspected with large deletions

Standards	Standard1 5ng/ul	Standard2 10ng/ul	Standard3 20ng/ul				
Individuals				Normal Male	Normal Female	283HA1008	Mother of 283HA1008
Target Concentrations (F8 exon2) (E*)	4.89 E0	1.01 E1	1.97 E1	6.36 E0	2.06 E1		
Target Concentrations (F8 exon8) (E)	5.04 E0	9.85 E1	2.01 E1	6.72 E0	2,06 E1		
Target Concentrations (F8 exon10) (E)	4.97 E0	1.05 E1	1.97 E1	4.99 E0	2.04E1	0	5.35E0
Reference Concentrations (SCN1A exon6) (E)	4.98 E0	9.95 E1	1.99 E1	1.47 E1	2.01 E1	1.14 E1	1.13 E1
Ratio	0.99	1.02	0.99				0,47

E: means 10, E0 means 0, E1 means10

5.1.5. RT-PCR Analysis

Analysis of cDNA by RT-PCR can be a powerful tool to detect causative splicing mutations. This technique was employed on patient 162HA675 who did not have mutations in the *F8* gene and also on patients with suspected large deletions.

RT-PCR amplification was performed in two rounds of PCR using a nested approach and the specific 12 primers pairs were used as described in Table 3.3 and Figure 4.2.

A total of eight overlapping regions were amplified in positive control (Figure 5.16) (M indicates 100 bp ladder, lanes 1-8 indicate eight overlapping regions amplified in a positive control. However, an abnormal size product was detected in patient 162HA675 in region 6 of the cDNA amplification (Figure 5.17) (M indicates 500 bp ladder, lanes 1-8 indicate eight overlapping regions amplified in patient162HA675). This region normally amplifies as 583 bp product, but the size was reduced in this patient. The sequencing of the product showed that exon 19 was missing (Figure 5.18). Apparently, it was not an aberrant splicing unique to the patient but was an alternative splicing mechanism shown to exist in the normal population (El-Maarri *et al*., 2005).

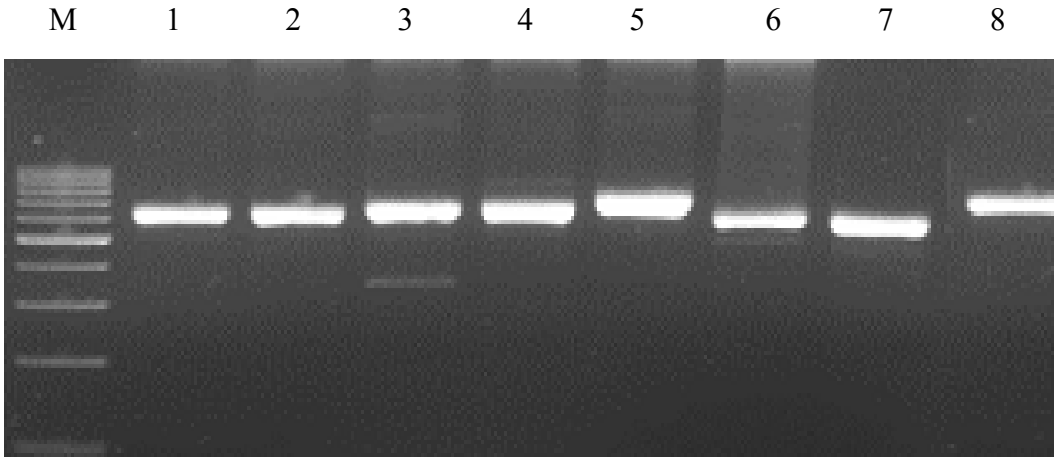


Figure 5.16. RT-PCR products in a positive control.

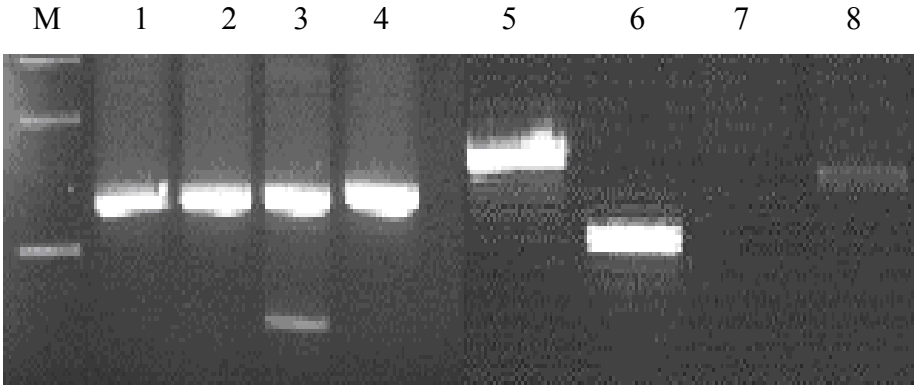


Figure 5.17. RT-PCR products of patient162HA675

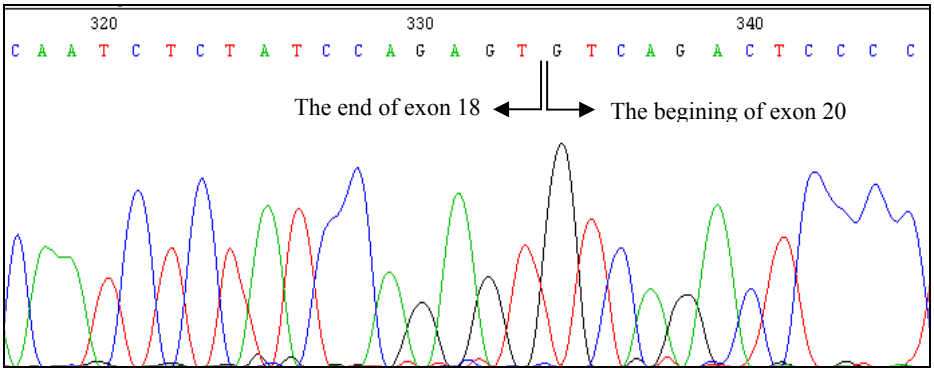


Figure 5.18. Sequencing result of region 6 in patient 162HA675 showing exon 19 deletion

Two of the patients suspected to have large deletions 267HA992 and 283HA1008 were also analyzed by RT-PCR to confirm the lack of normal *F8* mRNA. Abnormal size products were detected in patient 267HA992 suspected to have deletion of exons 14-25. Five regions (1-2, and 6-8) had PCR products with reduced size in regions 2 and 6 (Figure 5.19). The sequencing of these products showed that exons 5, 6 and 19 were skipped. In patient 283HA1008 (suspected to have a large deletion in exon 10), regions 6-8 were amplified (Figure 5.20). Exon 19 was missing in the cDNA. RT-PCR of mothers of the three patients was successful for all of the 8 regions. The results confirmed the lack of a complete *F8* mRNA in two of the patients shown to have large deletions. The RT-PCR regions amplified in the two patients and their corresponding exons are summarized in Table 5.6.

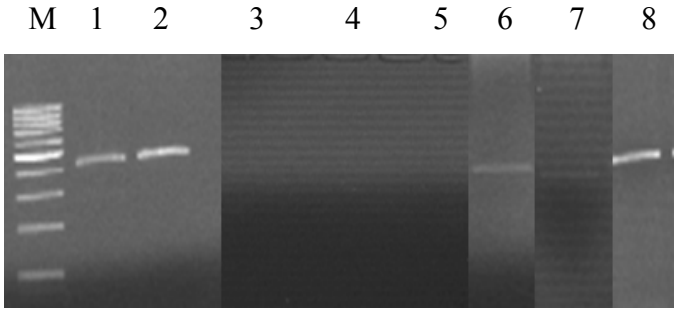


Figure 5.19. RT-PCR products of patient 267HA992

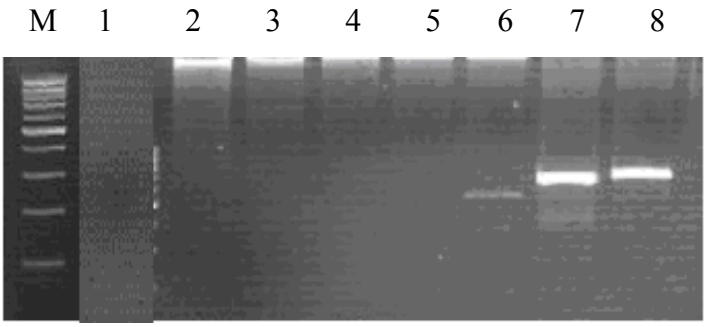


Figure 5.20. RT-PCR products of patient 283HA1008

Table 5.6. RT-PCR results of patients 267HA992 and 283HA1008

Patient No	**267HA992 Del exon14-25**	**283HA1008 Del exon10**
RT-PCR Results		
Exon 1–5 (region 1)	+	-
Exon 4–8 (region 2)	reduced size (lacks exon 5 and 6)	-
Exon 8–11 (region 3)	-	-
Exon 11–14 (region 4)	-	-
Exon 14–17 (region 5)	-	-
Exon 17–21(region 6)	reduced size (lacks exon 19)	reduced size (lacks exon 19)
Exon 19–24 (region 7)	+	+
Exon 24–26 (region 8)	+	+

5.1.6. Amplification and Direct DNA Sequencing of Intron 18 and Intron 19 of The *F8* Gene

In order to see possible sequence alterations affecting alternative splicing of exon 19, intron 18 and intron 19 of *F8* gene were sequenced in patients 162HA675, 267HA992 and 283HA1008. Intron 18 was amplified by five specific primers and intron 19 was amplified by two specific primers. Interestingly, no additional sequence changes except the well-known *Bcl*I polymorphism in intron 18 and *Hind*III polymorphism in intron 19 were found in the intronic regions of three patients suggesting that alternative splicing of exon 19 is controlled by factors (proteins) interacting with each other rather than changes with intronic sequences.

5.1.7. Long PCR Analysis of Three HR Patients Suspected to have Large Deletions

In order to detect deletion breakpoints, long-PCR analysis was also attempted in patients 267HA992, 274HA999, 283HA1008 and a healthy control. Primer pairs that cover the suspected large deletion were shown in Table 5.7. Long PCR of regions 1 and 2 were not successful in the control due to very large size of the involved region and they failed in patients as well. Region 3 amplification failed in patient 283HA1008 but successful in controls and patient's mother (Figure 5.21). (Lane M indicates *Hind*III-Lambda DNA Marker. Lane 1 and 2 indicate PCR products of male and female healthy controls. Lane 3 is lack of long PCR product in patient number 283HA1008. Lane 4 indicates long PCR product of mother of patient 283HA1008.

Table 5.7. Primer combinations used in long PCR

Patient No	Primer Pairs for Regions 1-3	Expected Size in Control
267HA992 (del exon14-25)	Region 1: New Forward E13-Reverse E26A	110 kb
274HA999 (del exon2-8)	Region 2: Forward E1-Reverse E9	56,8 kb
283HA1008 (del exon10)	Region 3:Forward E9-Reverse E11	9419bp

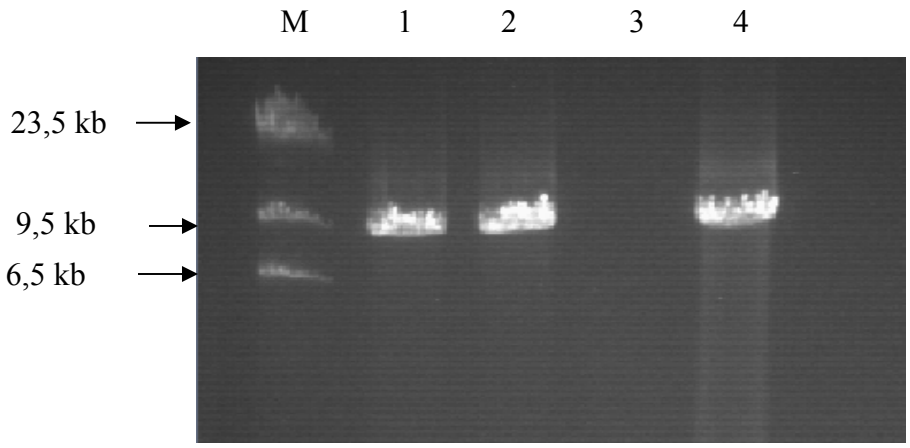


Figure5.21. Products of forward exon 9 and reverse exon 11 primer amplification

Summary of the studies for patients with suspected deletions and patient without any mutations in the *F8* gene are shown in Table 5.8

5.1.8. Karyotype Analysis of Two HR Patients

In order to see there was a gross genomic rearrangement, karyotype analyses were done on new blood samples for patients 267HA992 and 268HA993 by Premed Laboratory, Turkey (Appendix D) and a gross genomic rearrangement was excluded. Karyotype analysis was not possible on other patients with suspected deletions since new blood samples could not be obtained.

5.1.9. CGH Analysis of The *F8* Gene

CGH analysis was performed by using custom designed NimbleGen array 3X720K for six HR patients 5 of whom were suspected to have large deletion/rearrangements and one without any *F8* gene mutations. Six other individuals without hemophilia A phenotype were also included in the CGH analysis. The array was designed such that it included probes from intronic and exonic regions of the *F8* gene and were able to detect deletions/duplications >300 bp. These patients and other individuals were scanned for the *F8* gene to detect large deletions or rearrangements. Scores out of the range 0-0.25 and 0-(-0.25) were accepted as significant. Exon 14-25 deletion in patient 267HA992, exon 10 deletion in patient 283HA1008, exon 2-8 deletion in patient 274HA999, exon 19-22 deletion in patient 288HA 1014 were confirmed by CGH analysis. Probe regions, positions

of deletions were given in Figure 5.22-5.25 and scores were given in Table 5.8. In patient 268HA993, deleted region in *F8*A2 and *F8*A3 genes positions that detected with southern blotting was also confirmed with CGH analysis (Figure 5.26). and Table 5.9 shows the CGH scores in the *F8* gene in 5 hemophilia A patients and controls.

Patient 162HA675 without any *F8* gene mutations was also analyzed by CGH method. No significant score related to duplications or deletions was detected in the intronic regions. In six other individuals who did not have hemophilia A phenotype no changes were observed in their *F8* gene (Table 5.9). In addition to *F8* gene region, other chromosomal regions were scanned for these six patients. Their CGH data showing significant scores corresponding to common CNVs or new deletions and duplications were given in Appendix E. Overall CGH data analysis on the custom designed array is given in Appendix E.

The CGH analysis firmly confirming the large deletions in 5 hemophilia A patients with inhibitors completes part I of this thesis work and reveals that inhibitor development is observed in severe hemophilia A patients with major mutations in the *F8* gene that have large effects on gene function namely, inversion mutations (17/34, 50 per cent), nonsense mutations (9/34, 26 percent) and large deletions (5/34, 15 per cent).

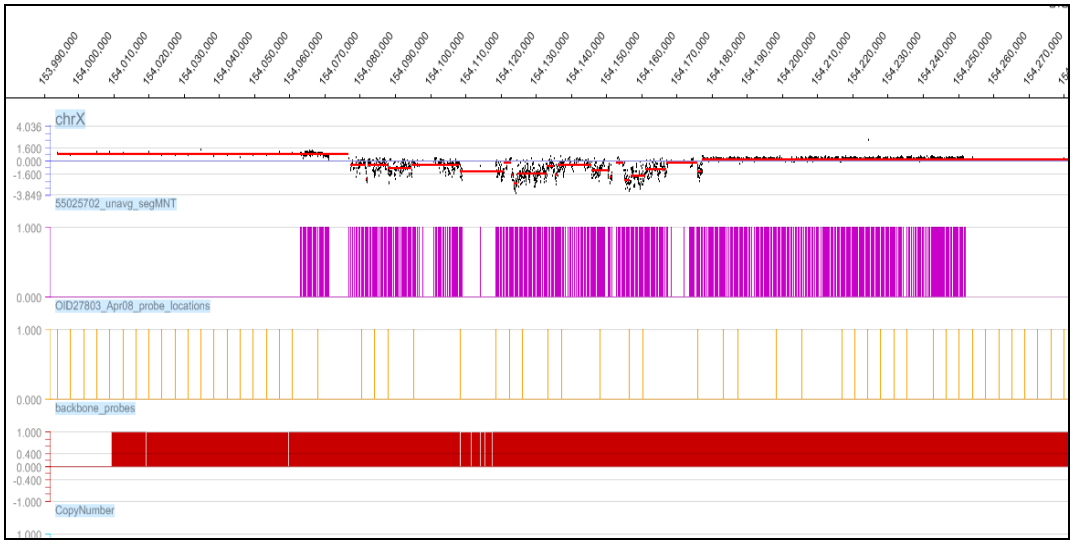


Figure 5.22. Results of CGH analysis of patient 267HA992 (scattered black points below score -0.25 show the deleted regions (exon 14-25), purple bars show designed *F8* probe locations, red bars show CNVs in these regions)

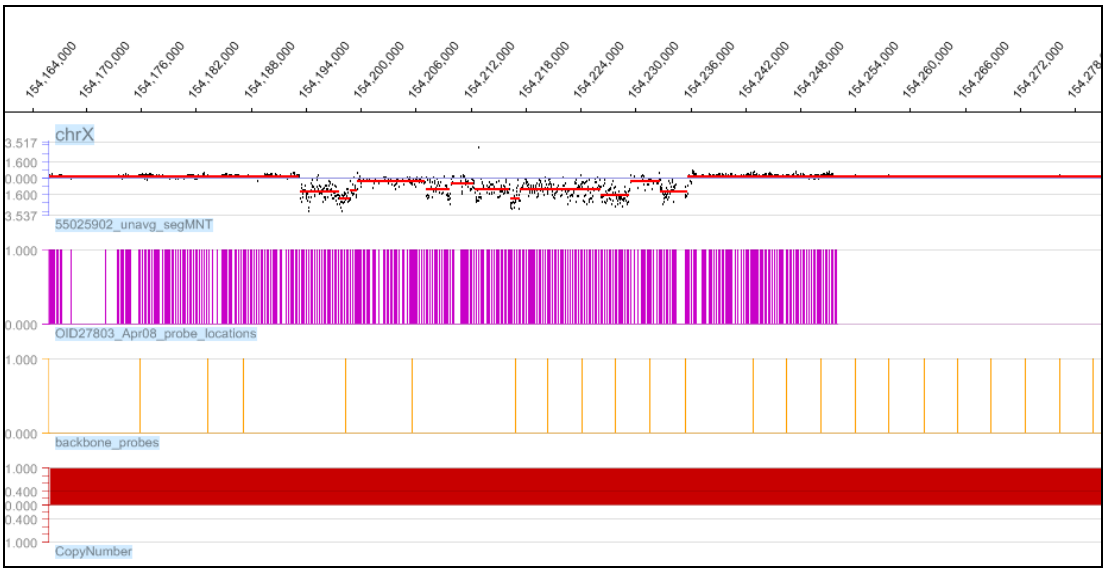


Figure 5.23. Results of CGH analysis of patient 274HA999 (scattered black points below score -0.25 show the deleted regions (exon 2-8), purple bars show designed *F8* probe locations, red bars show CNVs in these regions)

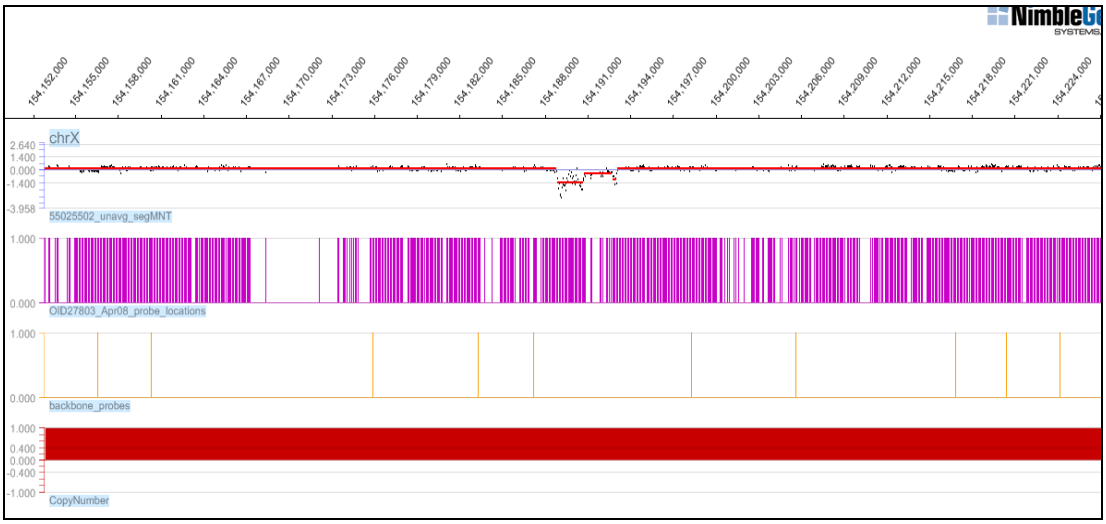


Figure 5.24. Results of CGH analysis of patient 283HA1008 (scattered black points below score -0.25 show the deleted region (exon 10), purple bars show designed *F8* probe locations, red bars show CNVs in these regions)

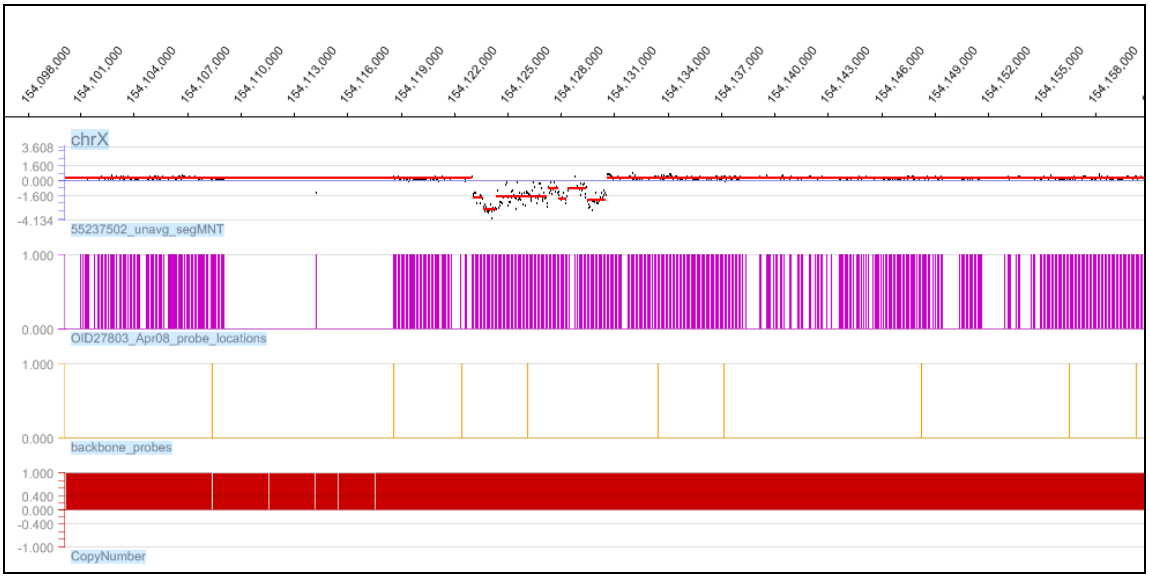


Figure 5.25. Results of CGH analysis of patient 288HA1014 (scattered black points below score -0.25 show the deleted regions (exon 19-22), purple bars show designed *F8* probe locations, red bars show CNVs in these regions)

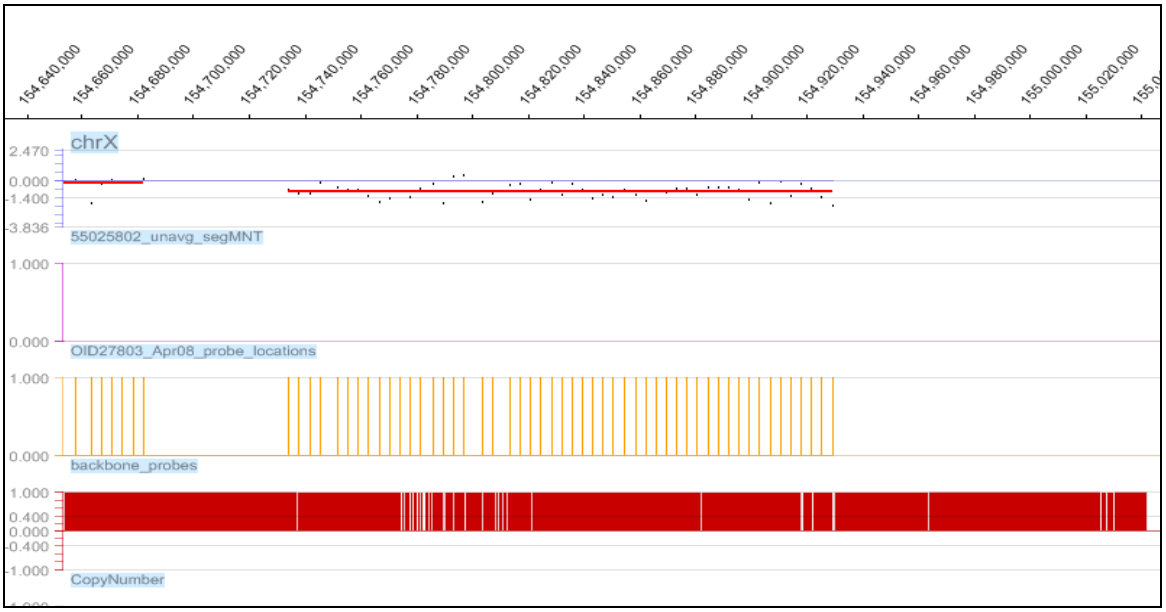


Figure 5.26. Results of CGH analysis of patient 268HA993 (scattered black points below score -0.25 show the deleted region (int22h-3), orange bars show default backbone probes, red bars show CNVs in these regions)

Table 5.8. PCR studies applied to HR patients

Patients	Lack of Exon PCRs	Southern Blot	qPCR	RT-PCR	Long PCR	CGH Analysis
162HA675	No	Done	Not done	IVS19 alternative splicing	Not Done	Done
267HA992	14-25	Deletion confirmed	Deletion confirmed	Lack of complete mRNA	No amplification	Deletion confirmed
274HA999	2-8	Not done	Deletion confirmed	Not Done	No amplification	Deletion confirmed
283HA1008	10	Not done	Deletion confirmed	Lack of complete mRNA	Amplification in control but not in patient	Deletion confirmed
268HA993	No Lack of extragenic copies	Deletion confirmed	Not done	Not done	Not done	Deletion confirmed
288HA1014	19-22	Not done	Not done	Not done	Not done	Deletion confirmed

Table 5.9. CGH analysis results showing approximate deletion breakpoints of 5 HR patients and 6 individuals for *F8* gene

Patient No	Gene	Exon	Start	Stop	Score
267HA992	*F8*	14	154175812	154177208	-1,4003
267HA992	*F8*	14	154156837	154160989	-1,92358
267HA992	*F8*	15-16	154133040	154135247	-0,81581

Table 5.9. CGH analysis results showing approximate deletion breakpoints of 5 HR patients and 6 individuals for *F8* gene (continued)

Patient No	Gene	Exon	Start	Stop	Score
267HA992	*F8*	17-18-19-20-21	154124871	154132988	-1,65371
267HA992	*F8*	22	154123629	154124829	-2,75079
267HA992	*F8*	23-24-25	154087871	154094667	-1,09428
268HA993	*F8A1-F8A2*	-	154579999	154924490	-0.94828
274HA999	*F8*	2	154226193	154229345	1,87255
274HA999	*F8*	3-4	154217389	154226149	-1,25346
274HA999	*F8*	5	154212427	154216237	-1,28276
274HA999	*F8*	6-7	154193390	154197628	-1,54137
274HA999	*F8*	8	154199659	154207019	-0,52134
274HA999	*F8*	8	154198880	154199571	-1,33134
274HA999	*F8*	8	154226193	154229345	-2,17575
283HA1008	*F8*	10	154188880	154190710	-1,50547
288HA1014	*F8*	19-20	154129460	154130514	-2,23799
288HA1014	*F8*	21	154127879	154128323	-2,06696
288HA1014	*F8*	22	154124373	154127223	-1,88158
12AE37	*F8*	-	-	-	-
24AE79	*F8*	-	-	-	-
25AE82	*F8*	-	-	-	-
28AE93	*F8*	-	-	-	-
31AE99	*F8*	-	-	-	-
52AE165	*F8*	-	-	-	-

5.2. Assessment of Genetic Factors Other than *F8* Gene Mutations Involved in Inhibitor Development

Inhibitor development may be present or absent in severe hemophilia A patients with null *F8* gene mutations (mutations that result in the lack of a functional mRNA) such as

intron 22 inversions, nonsense mutations and large deletions. Several studies have shown that immune response genes constitute decisive risk factors for the development of inhibitors. In order to answer the decisive genetic risk factors of inhibitor development in severe hemophilia A patients, a case-control study was aimed targeting some SNPs of genes like IL4, IL5, IL10, TGFB1 and IFNG that are involved in the regulation of B-cell development. One hundred and seventy three of 256 unrelated severe hemophilia A patients with and without inhibitors provided by various hematology clinics were scanned for the intron 22 inversion mutation. The intron 22 inversion mutation positive patients were grouped as patients with and without inhibitors and constituted the case-control groups.

5.2.1. Screening for Intron 22 Inversion Mutation by using inverse PCR and subcycling long PCR

Since the southern blot technique was laborious and time consuming, intron 22 inversion was detected by inverse PCR (Rosetti *et al*., 2005) and long PCR (Liu *et al*., 1998) techniques. Primers for inverse PCR and subsycling long PCR were given in Table 3.6. In inverse PCR, intron 22 inversion was detected according to the size of PCR products. A 487 bp product was detected in patients without intron 22 inversion and a 559 bp product was detected in patients with intron 22 inversions (Figure 5.27). In subcycling long PCR, intron 22 inversion was detected according to the size of PCR products as well. Using PQ and PB primer pairs in a single reaction 12 kb product was detected in patients without inversion and a 11 kb product was detected in patients with inversion respectively (Figure 5.28).

Ninety five patients (95/173) had intron 22 inversions. Thirty four patients constituted the group of patients with inhibitors and 61 patients constituted the group patients without inhibitors. In order to increase the size of the group with inhibitors, 8 inhibitors (+) patients with *F8* gene mutations (nonsense and large deletion mutations) were added to inhibitor (+) patients group. The inhibitor (+) patient group was then 42.

5.2.2. Optimization of PCR Amplification of SNPs in Immune Response Genes

Cytokine and interleukin genes involved in B cell activation like IL2, IFNG, IL4, IL5, IL10 and TGFB genes were examined for the presence of SNPs especially in their promoter and intronic regions in the taken from HapMap and NCBI. These genes had SNPs with an average heterozygosity near to 50 percent in different populations (Asian, European and African-American). Nine SNPs and one VNTR in promoter and intronic regions of these genes were chosen, PCR amplification were optimized and the regions were sequenced for 10 healthy Turkish individuals to reveal whether they are polymorphic in the Turkish population (Table 5.10 and Table 5.11).

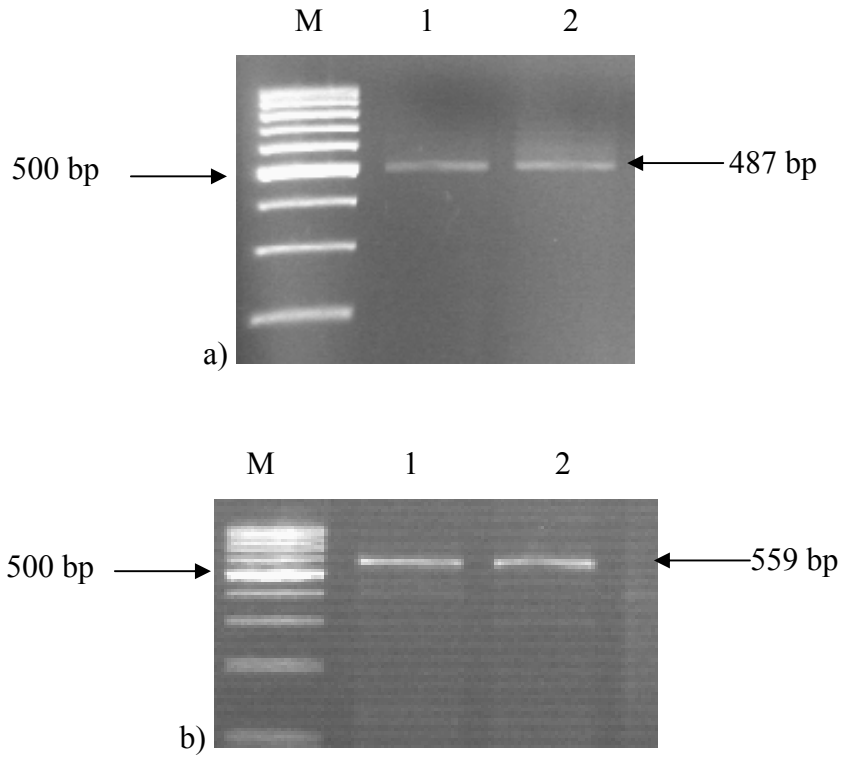


Figure 5.27. PCR products of inverse PCR a) M indicates 500 bp DNA ladder, 1 and 2 indicate inverse PCR products of patients without intron 22 inversion b) M indicates 500 bp DNA ladder, 1 and 2 indicate inverse PCR products of patients with intron 22 inversion

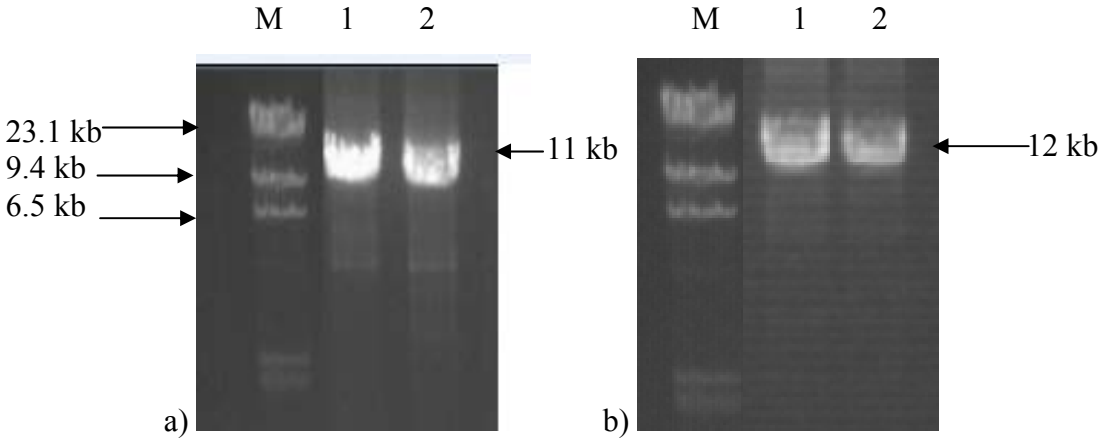


Figure 5.28. PCR products of subcycling long PCR a) M indicates Lambda DNA/HindIII marker, 1 and 2 indicate subcycling long PCR products of patients with inversion b) M indicates Lambda DNA/HindIII marker, 1 and 2 indicate subcycling long PCR products of patients without inversion

Table 5.10. Optimized PCR conditions, products of 9 SNPs and VNTR

SNP Name	Mg Concentration (mM)	Primer Concentration (mM)	Annealing Tm (^{o}C)	Product Size (bp)
Rs2241715	2	0.2	54.7	367
Rs1800871	2	0.2	54.7	347
Rs1554286	2	0.2	56.2	342
Rs3024496	2	0.2	54.7	365
Rs2069812	2	0.2	54.7	385
Rs2069705	1.5	0.2	59	381
Rs1861494	2	0.2	54.7	334
Rs2243267	2	0.2	54.7	390
Rs2243282	2	0.2	65	383
VNTR	2	0.2	65	383
Mg: means magnesium, Tm means melting temperature				

Table 5.11. Heterozygosity rate in 10 healthy Turkish individuals for 9 SNPs and VNTR

SNP Name	Heteozygosity (%)
Rs2241715	30
Rs1800871	25
Rs1554286	20
Rs3024496	25
Rs2069812	25
Rs2069705	25
Rs1861494	25
Rs2243267	15
Rs2243282	-
VNTR	15

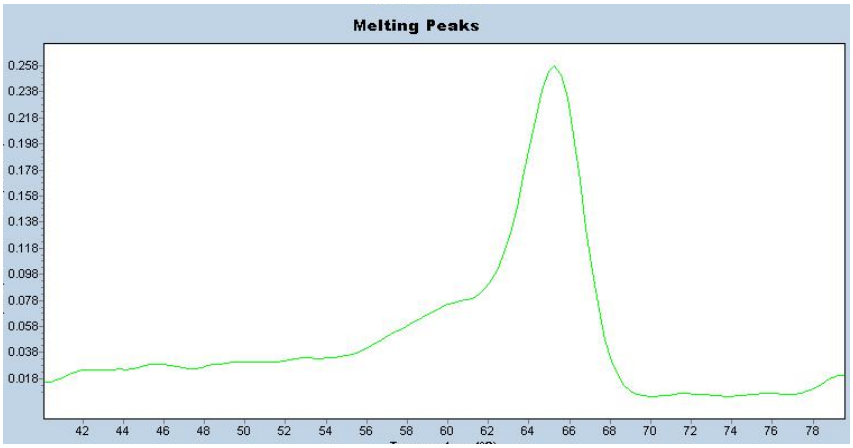
5.2.3. Design and Optimization of SNP Primers and Hybrobe Probes

For genotyping case-control groups, Hybrobe probes designed by TIB-MOLBIOL were used for 9 SNPs. The designs were given in Figure 3.1. Genotyping assays were optimized and performed with melting curve analysis by using LC480 instrument (Table 5.12). In melting curve analysis, wild type and mutant alleles were melted at different temperatures thus the fluorescent peak for the wild type was observed at high Tm while mutant allele was at lower Tm (except rs2069705 and rs2241715) The melting curves for all three genotypes for rs2069812 is given in Figure 5.29. Tm for wild type and mutant alleles for other SNPs are listed in Table 5.13 and melting curves were given in Appendix F.

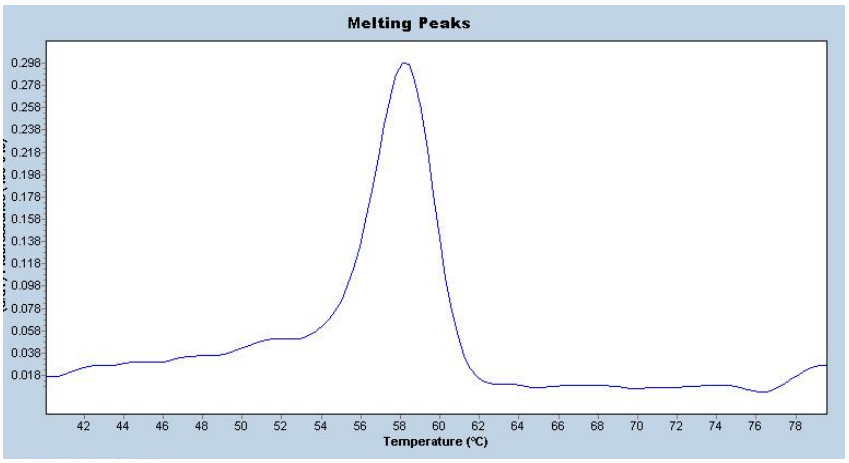
Table 5.12. Optimized conditions for 9 SNPs in LC480

SNP name	Primer Concentration (mM)	Sensor Concentration (mM)	Anchor Concentration (mM)	Annealing Tm (^{0}C)
Rs2241715	Rs2241715S: 0.5 Rs2241715A:1.0	Rs2241715T 0.2	Anc Rs2241715 0.2	55
Rs1800871	0819F: 0.1 0819R:0.5	0819C 0.2	0819Anc 0.2	55
Rs1554286	10F: 0.5 10R:0.1	Sensor mut 0.2	Anc mut 0.2	55
Rs3024496	4496F: 0.1 4496R:0.5	4496C 0.2	4496Anc 0.2	55
Rs2069812	IL5S:0.5 IL5mis:0.1	Sensor C 0.2	Il5 mis 0.2	55
Rs2069705	Primer S:0.5 Primer A:0.1	Anc.Rs2069705 0.2	Rs2069705 wt 0.2	55
Rs1861494	Rs1861494F: 0.5 Rs1861494R:0.1	SensorC 0.2	Anc 0.2	55
Rs2243267	Rs2243267S: 0.5 Rs2243267A:0.5	SensorC 0.2	Anc 0.2	55
Rs2243282	Rs2243282F: 0.5 Rs2243282R:0.1 SLC4A3 R: 0.05	SensorC 0.2	Anc 0.2	55

a)



b)



c)

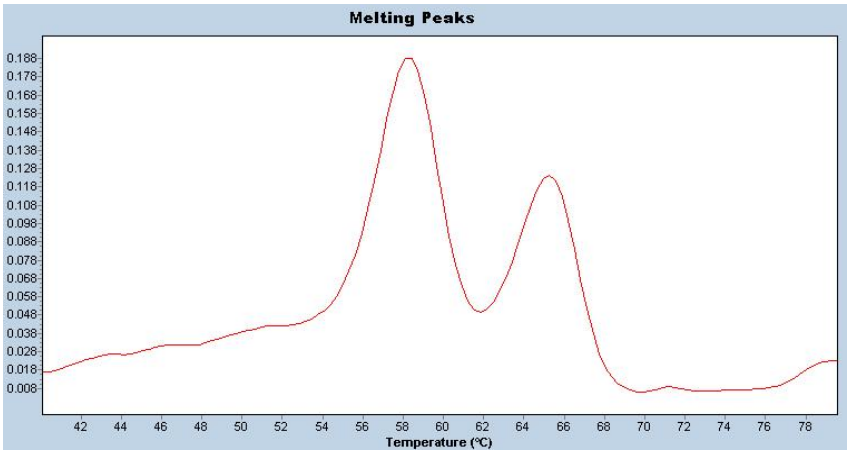


Figure 5.29. Melting peaks observed for rs2069812 a) Homozygous wild type genotype T-T (66 ^{0}C), b) homozygous mutant genotype C-C (58 ^{0}C) and c) heterozygous genotype T-C (58 ^{0}C and 66 ^{0}C).

Table 5.13. Tm for melting peaks of wild type and mutant allele of 9 SNPs

SNP name	Tm for wild type (^{o}C)	Tm for mutant type (^{o}C)
Rs2241715	62	70
Rs1800871	66	60
Rs1554286	68	62
Rs3024496	62	56
Rs2069812	66	58
Rs2069705	64	58
Rs1861494	66	58
Rs2243267	66	58
Rs2243282	64	58

Eight SNPs and one VNTR region were scanned in 100 healthy Turkish individuals by Hybrobe probes. SNPs were found to be in Hardy-Weinberg equilibrium and also had a minor allele frequency higher than 0.120 for each (Table 5.14). Genotyping of one of these SNPs failed for healthy controls due to DNA quality.

Table 5.14. SNP marker and VNTR analysis for 100 healthy control samples

SNP name	Gene	Position	ObsHET	PredHET	HWpval	MAF	Alleles
Rs1800871	IL10	Promoter	0.406	0.405	1	0.282	C:T
Rs1554286	IL10	IVS3	0.366	0.346	0.829	0.223	C:T
Rs3024496	IL10	E5	0.462	0.417	0.483	0.297	T:C
Rs2069812	IL5	Promoter	0.515	0.478	0.612	0.396	C:T
Rs2069705	IFNG	Promoter	0.468	0.380	0.043	0.255	T:C
Rs1861494	IFNG	IVS3	0.250	0.219	0.359	0.125	T:C
Rs2243267	IL4	IVS2	0.260	0.295	0.356	0.180	G:C
Rs2243282	IL4	IVS3	0.310	0.302	0.0012	0.185	C:A
VNTR	IL4	IVS2	0.242	0.284	0.242	0.172	1:2

ObsHet means observed heterozygozity, PredHet means predicted heterozygozity

HW means Hardy-Weinberg equilibrium, MAF means minor allele frequency

IVS means intervening sequence variation

E means exon

5.2.4. Case-Control Association Analysis

Fourty two inhibitor (+) and 61 inhibitor (-) severe hemophilia A patients were genotyped for 9 SNPs in realtime. Seventy bp repeat region (VNTR) located in the IVS2 region of IL4 gene was amplified as a 383 bp product (allele 2) if it was in 3 tandem copies and a 313 bp product was amplified if it was in 2 tandem copies (allele 1) by PCR. The results were analyzed by using Haploview 4 programme. Two patient groups were in Hardy Weinberg equilibrium for 9 SNPs and VNTR region initially (Table 5.15 and Table 5.16). They had a minor allele frequency higher than 0.19 for inhibitor (-) patients group, and 0,13 for inhibitor (+) patients group.

Table 5.15. Genotyping results of 42 inhibitor (+) hemophilia A patients

SNP name	Gene	Position	ObsHET	PredHET	HWpval	MAF	Alleles
Rs2241715	TGFB1	IVS1	0.48	0.48	1	0.400	G:T
Rs1800871	IL10	Promoter	0.444	0.417	1	0.296	C:T
Rs1554286	IL10	IVS3	0.357	0.375	1	0.250	C:T
Rs3024496	IL10	E5	0.500	0.477	1	0.393	T:C
Rs2069812	IL5	Promoter	0.381	0.482	0.261	0.405	C:T
Rs2069705	IFNG	Promoter	0.393	0.423	0.959	0.304	T:C
Rs1861494	IFNG	IVS3	0.393	0.316	0.590	0.196	T:C
Rs2243267	IL4	IVS2	0.357	0.337	1	0.214	G:C
Rs2243282	IL4	IVS3	0.357	0.337	1	0.214	C:A
VNTR	IL4	IVS2	0.357	0.337	1	0.214	1:2

ObsHet means observed heterozygozity, PredHet means predicted heterozygozity
HW means Hardy-Weinberg equilibrium, MAF means minor allele frequency
IVS means intervening sequence variation
E means exon

Table 5.16. Genotying results of 61 inhibitor (-) hemophilia A patients

SNP name	Gene	Position	ObsHET	PredHET	HWpval	MAF	Alleles
Rs2241715	TGFB1	IVS1	0.333	0.499	0.028	0.480	G:T
Rs1800871	IL10	Promoter	0.283	0.340	0.366	0.217	C:T
Rs1554286	IL10	IVS3	0.164	0.236	0.093	0.136	C:T
Rs3024496	IL10	E5	0.473	0.492	0.932	0.436	T:C
Rs2069812	IL5	Promoter	0.492	0.416	0.288	0.295	C:T
Rs2069705	IFNG	Promoter	0.434	0.414	1	0.292	T:C
Rs1861494	IFNG	IVS3	0.273	0.331	0.327	0.209	T:C
Rs2243267	IL4	IVS2	0.218	0.249	0.603	0.145	G:C
Rs2243282	IL4	IVS3	0.200	0.236	0.487	0.136	C:A
VNTR	IL4	IVS2	0.218	0.249	0.603	0.145	1:2

ObsHet means observed heterozygozity, PredHet means predicted heterozygozity
HW means Hardy-Weinberg equilibrium, MAF means minor allele frequency
IVS means intervening sequence variation
E means exon

These two intron 22 inversion (+) subgroups constituted the cases and controls and the association analysis was carried out using the Haploview 4 programme. The associated alleles and *p*-values were given in Table 5.17. Rs 2069812 showed a significant association with a *p*-value of 0,0251 and the T-allele was found to be associated with inhibitor (+) patients. Multiple test correction was done by 100K permutations (*p*-value of 0.0294).

Table 5.17. Association analysis of inhibitor (+) and inhibitor (–) patient subgroups

SNP Name	Associated Allele	X^2	*p*- value
Rs2069705	C	0,022	0,8828
Rs2241715	T	0,875	0,3496
Rs3024496	C	0,288	0,5915
Rs1800871	T	1,221	0,2692
Rs1554286	T	4,016	0,1342
Rs2069812	T	5,019	0,0251
Rs1861494	T	0,037	0,8484
Rs2243267	C	1,254	0,2628
Rs2243282	A	1,654	0,1984
VNTR	Allele 1	1,254	0,2628

X^2 means chi-square

This association analysis was repeated using all patients (inhibitor + and inhibitor -) against healthy individuals and no significant associations were detected that supported the association of this SNP with the inhibitor formation (Table 5.18). Genotype frequencies were calculated in two patient groups for 9 SNP and VNTR region by using chi-square test in webpage of University of Kansas (http://people.ku.edu/~preacher/chisq/chisq.htm). *P*-value of rs2069812 was found to be 0.0082 and TT genotype was found to be associated with inhibitor (+) patients. These results were also confirmed the results of Haploview 4 programme case-control association analysis (Table 5.19). The pattern of inheritance of rs2069812 indicated a similar and reduced risk of CT and CC genotypes in inhibitor (+) patients in the crude genetic model (Table 5.20) (Lunetta, 2009). In the model where T-allele was recessive, TT genotype carried a risk of 6.86 fold compared to those with CT or CC genotypes indicating that the T-allele was the susceptibility allele. On the other hand,

considering C-allele has a dominant inheritance, CT or CC genotypes reduced the disease risk by the rate of 0.02 per cent (odds ratio=0.14). Therefore, C-allele could be considered to have a dominant protective effect.

Table 5.18. **Association analysis of all patients versus controls**

Case Control Groups	**Associated Allele**	**X^2**	***p*- value**
Inhibitor (+) vs Healthy Individuals	T	0,019	0,8909
Inhibitor (-) vs Healthy Individuals	C	3,373	0,0773
All Patients vs Healthy Individuals	C	1,387	0,2389

Table 5.19. Genotype and allele frequencies of patients for 9 SNPs and VNTR region

Rs2069812	**Allele Frequency (%)**		**Genotype Frequency (%)**						
	C	**T**	**CC**	**CT**	**TT**	**Missing Data**	**Total**	**X^2**	***p*-value**
Inhibitor(+) patients	30(53)	26(46)	16(38)	15(35)	11(27)	-	42		
Inhibitor(-) patients	87 (71)	35(29)	29(47)	29(47)	3(5)	-	61		
								9,603	**0.0082**
Rs1554286	**Allele Frequency (%)**		**Genotype Frequency (%)**						
	C	**T**	**CC**	**CT**	**TT**	**Missing Data**	**Total**	**X^2**	***p*-value**
Inhibitor(+) patients	42(75)	24(32)	16 (56)	10(35)	2(7)	14	42		
Inhibitor(-) patients	93(84)	17(15)	42(70)	9(16)	4(7)	6	61		
								4,016	0,1342
Rs 2241715	Allele Frequency (%)		Genotype Frequency (%)						
	T	**G**	**GG**	**TG**	**TT**	**Missing Data**	**Total**	**X^2**	***p*-value**
Inhibitor(+) patients	20(40)	30 (60)	9 (36)	12 (48)	4 (16)	17	42		
Inhibitor(-) patients	49(48)	53(52)	18 (35)	17 (33)	16 (31)	10	61		
								2,455	0,2930

Table 5.19. Genotype and allele frequencies of patients for 9 SNPs and VNTR region (continued)

Rs3024496	**Allele Frequency (%)**		**Genotype Frequency (%)**						
	T	**C**	**TT**	**TC**	**CC**	**Missing Data**	**Total**	**X^2**	***p*-value**
Inhibitor (+) patients	34 (60)	22(40)	10 (36)	14 (50)	4 (14)	14	42		
Inhibitor (-) patients	63 (57)	47 (43)	18 (33)	27 (49)	10 (18)	6	61		
								0,219	0,8962
Rs 1800871	**Allele Frequency (%)**		**Genotype Frequency (%)**						
	C	**T**	**CC**	**CT**	**TT**	**Missing Data**	**Total**	**X^2**	***p*-value**
Inhibitor (+) patients	38(70)	16 (30)	13 (48)	12 (44)	2 (8)	15	42		
Inhibitor (-) patients	82 (77)	24 (23)	33 (62)	16 (30)	4 (8)	8	61		
								1,659	0,4362
Rs2069705	**Allele Frequency (%)**		**Genotype Frequency (%)**						
	T	**C**	**TT**	**CT**	**CC**	**Missing Data**	**Total**	**X^2**	***p*-value**
Inhibitor (+) patients	39 (70)	17 (30)	14(50)	11 (39)	3 (11)	14	42		
Inhibitor (-) patients	76 (72)	30 (28)	27 (51)	22 (42)	4 (8)	9	61		
								0,238	0,8878

Table 5.19. Genotype and allele frequencies of patients for 9 SNPs and VNTR region (continued)

Rs1861494	**Allele Frequency (%)**		**Genotype Frequency (%)**						
	C	**T**	**TT**	**CT**	**CC**	**Missing Data**	**Total**	**X^2**	***p*-value**
Inhibitor (+) patients	11 (20)	45 (80)	17 (61)	11 (39)	0 (0)	14	42		
Inhibitor (-) patients	24 (22)	86 (78)	35 (64)	16 (29)	4 (7)	6	61	1,659	0,4362
Rs 2243282									
	Allele Frequency (%)		**Genotype Frequency (%)**						
	C	**A**	**CC**	**CA**	**CC**	**Missing Data**	**Total**	**X^2**	***p*-value**
Inhibitor (+) patients	35 (62)	21 (38)	17 (61)	1 (4)	10 (36)	14	42		
Inhibitor (-) patients	86 (78)	24 (22)	42 (76)	2 (4)	11 (20)	6	61		
Rs 2243267								2,45	0,2937
	Allele Frequency (%)		**Genotype Frequency (%)**						
	G	**C**	**GG**	**GC**	**CC**	**Missing Data**	**Total**	**X^2**	***p*-value**
Inhibitor (+) patients	44 (79)	12 (21)	17 (61)	10 (36)	1 (4)	14	42		
Inhibitor (-) patients	95 (86)	15 (14)	42 (76)	11 (20)	2 (4)	6	61		
								2,45	0,2937

Table 5.19. Genotype and allele frequencies of patients for 9 SNPs and VNTR region (continued)

VNTR	Allele Frequency (%)		Genotype Frequency (%)						
	Allele 1	Allele 2	Allele 22	Allele 12	Allele 11	Missing Data	Total	X^2	*p*-value
Inhibitor (+) patients	13 (23)	43 (77)	16 (57)	11 (39)	1 (4)	14	42		
Inhibitor (-) patients	17 (15)	93 (85)	40 (72)	13 (26)	2 (3)	6	61	2,24	0,3262

Table 5.20. Test of association between rs2069812 genotypes and inhibitor development

Genetic Model	Genotypes			df	X^2	*p*-Value
	CC	CT	TT			
Crude OR (vs TT)	0.15	0.14	1	2	9.603	0.0082
Dominant T allele OR (vs CT+CC)	0.68	1	1	1	0.902	0.3422
Recessive T allele OR (vs CT+CC)	1	1	6.86	1	9.584	0.0019
Dominant C allele OR (vs TT)	0.14	0.14	1	1	9.584	0.0019
X^2 means chi-square OR means odds ratio, df means degree of freedom						

5.2.5. CNV Analysis of Rs2069812 Region

In order to investigate CNVs in the associated SNP region, qPCR analysis was applied to patients who had homozygote and heterozygote genotype for the rs2069812 SNP. Absolute quantification analysis was used to quantify the target sequence and reference sequences. Relative quantification was used to compare these targets and reference sequences' concentrations. qPCRs were performed in real time. Target sequence was IL5 promoter rs2069812 region and the reference sequence was exon 6 of SCN1A gene. qPCR assay was performed for 28 homozygous inhibitor (+) patients and 30 homozygous inhibitor (-) patients in two groups. Concentrations of individuals for target and reference sequence their ratios were shown in Table 5.21 and Table 5.22. The normalized ratio for target sequences to reference sequence had a value near to 1. qPCR assay was also performed for 14 heterozygous inhibitor (+) patients and 31 heterozygous inhibitor (-) patients in two groups (Table 5.23 and Table 5.24). The normalized ratio for target sequences to reference sequence had a value near to 1.

Table 5.21. Concentrations of 28 homozygous inhibitor (+) patients for target and reference sequence and their ratios

	Target Concentration (Rs2069812) (E)	**Reference Concentration (SCN1A exon6) (E)**	**Ratio**
Standard 1	5,12 E0	5,00 E0	1,02
Standard 2	9,70 E0	1,01 E1	0,98
Standard 3	2,00 E1	2,02 E1	0,99
Case			
1HAI1	1,99 E1	2,05 E1	0,97
2HAI2	1,43 E1	1,74 E1	0,82
3HAI3	1,82 E1	2,29 E1	0,80
4HAI4	1,66 E1	1,53 E1	1,08
5HAI5	1,14 E1	1,31 E1	0,87
9HAI9	1,70 E1	1,45E1	1,12
11HAI11	1,88 E1	1,65E1	1,13
19HAI19	2,03 E1	1,99 E1	1,02
21HAI21	1,52 E1	1,28 E1	1,12
E means 10, E0 means 0, E1 means10			

Table 5.21. Concentrations of 28 homozygous inhibitor (+) patients for target and reference sequence and their ratios (continued)

	Target Concentration (Rs2069812) (E)	**Reference Concentration (SCN1A exon6) (E)**	**Ratio**
24HAI25	3,64 E0	4,39 E0	0,83
28HAI29	1.56 E1	1,43 E1	1,09
31HAI32	1,68 E1	1,62 E1	1,03
32HAI33	1,99 E1	1,74 E1	1,14
34HAI45	1,83 E1	2,10 E1	0,88
37HAI50	2,34 E1	1.94 E1	1,15
39HAI52	1,76 E1	2.07 E1	0,85
40HA54	1,80 E1	1,59 E1	1,13
43HAI63	1,57 E1	1,24 E0	1,21
47HAI73	1,73 E1	1,54 E0	1,12
49HAI75	1,90 E1	1,65 E1	1,15
51HAI78	1,58 E1	1,75 E1	0,90
52HAI79	2,07 E1	1,86 E0	1,10
54HAI85	1,90 E1	1,70 E1	1,12
56HAI91	1,30 E1	1,49 E1	0,88
61HAI98	9,16 E0	7,89 E0	1,16
62HAI100	2,01 E1	2,07 E1	0,97
66HAI106	1,81 E1	1,95 E1	0,92
67HAI108	1,91 E1	1,95 E1	0,97

E means 10, E0 means 0, E1 means10

Table 5.22. Concentrations of 30 homozygous inhibitor (-) patients for target and reference sequence and their ratios

	Target Concentration (Rs2069812) (E)	**Reference Concentration (SCN1A exon6) (E)**	**Ratio**
17HA58	1,98 E1	1,72 E1	1,15
52HA203	8,26 E0	9,57 E0	0,89
89HA346	1,65 E1	1,58 E1	1,04
138HA529	1,91 E1	1,72 E1	1,11
183HA754	1,45 E1	1,70 E1	0,85
243HA910	2,21 E1	2,07 E1	1,07
252HA949	2,14 E1	2,28 E1	0,94
260HA971	1,87 E1	1,92 E1	0,96
308HA1076	2,37 E1	2,44 E1	0,97
313HA1086	1,82 E1	2,03 E1	0,90
317HA1097	2,13 E1	2,09 E1	1,02
320HA1102	1,61 E1	1,54 E1	1,05
320HA1103	2,63 E1	2,36 E1	1,12
325HA1115	2,10 E1	1,96 E1	1,07
333HA1125	2,38 E1	2,11 E1	1,13
347HA1147	2,28 E1	2,02 E1	1,13
353HA1162	1,86 E1	1,62 E1	1,15
356HA1170	1,56 E1	1,60 E1	0,98
360HA1174	1,56 E1	1,80 E1	0,89
369HA1191	1,70 E1	2,15 E1	0,83
375HA1208	1,84 E1	1,74 E1	1,06
377HA1212	2,57 E1	2,55 E1	1,01
387HA1225	2,44 E1	2,39 E1	1,02
E means 10, E0 means 0, E1 means10			

Table 5.22. Concentrations of 30 homozygous inhibitor (-) patients for target and reference sequence and their ratios (continued)

	Target Concentration (Rs2069812) (E)	**Reference Concentration (SCN1A exon6) (E)**	**Ratio**
392HA1230	1,53 E1	1,27 E1	1,17
395HA1233	1,65 E1	1,96 E1	0,89
397HA1237	1,87 E1	2,17 E1	0,88
402HA1244	2,34 E1	1,97 E1	1,15
433HA1287	1,22 E1	1,19 E1	1,02
443HA1298	1,65 E1	1,57 E1	1,05
445HA1300	1,35 E1	1,27 E1	1,06
E means 10, E0 means 0, E1 means10			

Table 5.23. Concentrations of 14 heterozygous inhibitor (+) patients for target and reference sequence and their ratios

	Target Concentration (Rs2069812) (E)	**Reference Concentration (SCN1A exon6) (E)**	**Ratio**
7HAI7	1,72 E1	2,06 E1	0,83
8HAI8	1,98 E1	1,92 E1	1,03
10HAI10	1,56 E1	1,79 E1	0,87
12HAI12	1,24 E1	1,41 E1	0,87
17HAI17	1,14 E1	1,22 E1	0,93
18HAI18	1,85 E1	1,96 E1	0,94
20HAI20	4,15 E0	3,79 E0	1,09
28HAI29	1,36E1	1,31 E1	1,03
35HAI46	1,79 E1	1,74 E1	1,02
41HAI57	2,05 E1	1,84 E1	1,11
60HAI95	1,25 E1	1,31 E1	0,95
64HAI101	2,30 E1	2,40 E1	0,95
E: means 10, E0 means 0, E1 means10			

Table 5.24. Concentrations of 31 heterozygous inhibitor (-) patients for target and reference sequence and their ratios

	Target Concentration (Rs2069812) (E)	**Reference Concentration (SCN1A exon6) (E)**	**Ratio**
65HA270	1,27 E1	1,44 E1	0,90
158HA667	1,90 E1	1,75 E1	1,08
235HA877	1,32 E1	1,55 E1	0,85
258HA967	1,58 E1	1,40 E1	1,12
263HA986	1,93 E1	1,80 E1	1,07
294HA1037	2,15 E1	2,04 E1	1,05
311HA1084	1,98 E1	2,20 E1	0,90
312HA1085	1,65 E1	1,42 E1	1,16
314HA1088	1,23 E1	1,56 E1	0,79
318HA1100	1,77 E1	1,72 E1	1,02
328HA1119	1,77 E1	1,79 E1	0,98
330HA1121	2,11 E1	2,26 E1	0,93
331HA1122	7,76 E0	9,51 E0	0,81
334HA1127	1,30 E1	1,15 E1	1,13
337HA1130	4,98 E0	5,22 E0	0,95
339HA1132	1,92 E1	1,78 E1	1,07
342HA1137	1,44 E1	1,67 E1	0,86
348HA1149	1,45 E1	1,33 E1	1,09
349HA1151	1,50 E1	1,73 E1	0,86
358HA1172	1,30 E1	1,25 E1	1,04
371HA1195	1,49 E1	1,38 E1	1,07
378HA1216	1,43 E1	1,27 E1	1,12
381HA1219	1,30 E1	1,47 E1	0,88
384HA1222	1,22 E1	1,31 E1	0,91
400HA1242	1,01 E1	9,66 E0	1,04
E means 10, E0 means 0, E1 means10			

Table 5.24. Concentrations of 31 heterozygous inhibitor (-) patients for target and reference sequence and their ratios (continued)

	Target Concentration (Rs2069812) (E)	**Reference Concentration (SCN1A exon6) (E)**	**Ratio**
406HA1248	1,38 E1	1,25 E1	1,14
429HA1283	1,98 E1	2,08 E1	0,95
441HA1296	1,36 E1	1,57 E1	0,86
469HA1363	1,56 E1	1,34 E1	1,16
E means 10, E0 means 0, E1 means10			

5.2.6. HRM and DNA Sequence Analysis of the IL5 Gene

IL5 is an immune response gene whose product plays a role in B cell antibody synthesis. This gene is composed of 4 exons spanning 2078 bp coding region. In order to detect any pathological changes segregating with the SNP rs2069812, IL5 gene was divided into 7 regions for HRM and DNA sequencing. Promoter region containing rs2069812 SNP was divided into 3 regions. Two of them were amplified by PCR and sequenced for 42 inhibitor (+) and 61 inhibitor (-) patients. Promoter 1 region and exon 3 of IL5 gene were optimized and analyzed by HRM in real time for 42 inhibitor (+) patients and 61 inhibitor (-) patients. Remaining exon regions were amplified by PCR and sequenced for the two groups of patients (Table 5.25). Point mutations were not detected in the IL 5 gene.

In sequence analysis revealed the genotypes of other 14 SNPs located in IL5 gene beside rs206812 (Table 5.26 and Table 5.27). There were no haplotype associations.

In addition to sequencing and HRM analyses, a bioinformatic tools were used to detect any changes in transcription factor binding scores, because of the localization of SNP rs2069812. When a part of promoter region sequence of IL5 gene including SNP rs2069812 with two versions like T and C were studied, no changes were detected as

transcription factors binding site (http://alggen.lsi.upc.es/cgi-bin/promo_v3/) (Appendix G).

Table 5.25. Optimized conditions for amplification of the promoter and exons of IL5 gene

Region	Mg Concentration	Primer Concentration	Annealing Tm (^{0}C)	PCR Product	Analysis Type
Promoter 1	2.5mM	0.2mM	Touchdown 71→66	385	HRM
Promoter 2	2mM	0.2mM	55.2	345	Sequencing
Promoter 3	2mM	0.2mM	55.2	393	Sequencing
Exon 1	2.5mM	0.2mM	55.2	229	Sequencing
Exon 2	2mM	0.2mM	55.2	206	Sequencing
Exon 3	3mM	0.2mM	Touchdown 71→66	213	HRM
Exon 4	2mM	0.2mM	55.2	600	Sequencing

Table 5.26. SNP marker analysis in IL5 gene for 42 inhibitor (+) hemophilia A patients

SNP name	Gene	Position	ObsHET	PredHET	HWpval	MAF	Alleles
Rs2069812	IL5	299	0.357	0.497	0.222	0.464	C:T
Rs4986967	IL5	314	0.0	0.0	1	0.0	G:G
Rs2069813	IL5	331	0.0	0.0	1	0.0	C:C
Rs2069814	IL5	432	0.0	0.0	1	0.0	A:A
Rs3052201	IL5	616	0.0	0.0	1	0.0	A:A

ObsHet means observed heterozygozity, PredHet means predicted heterozygozity, HW means Hardy-Weinberg equilibrium, MAF means minor allele frequency, IVS means intervening sequence variation E means exon

Table 5.26. SNP marker analysis in IL5 gene for 42 inhibitor (+) hemophilia A patients (continued)

SNP name	Gene	Position (bp)	ObsHET	PredHET	HWpval	MAF	Alleles
Rs67023946	IL5	648	0.0	0.0	1	0.0	A:A
Rs6696885	IL5	827	0.0	0.0	1	0.0	A:A
Rs66653352	IL5	876	0.0	0.0	1	0.0	A:A
Rs1800474	IL5	1167	0.0	0.0	1	0.0	A:A
Rs2069815	IL5	1205	0.0	0.0	1	0.0	T:T
Rs34909832	IL5	1451	0.0	0.0	1	0.0	A:A
Rs34328342	IL5	1478	0.0	0.0	1	0.0	C:C
Rs2069823	IL5	2516	0.0	0.0	1	0.0	A.A
Rs2069818	IL5	2691	0.0	0.0	1	0.0	C:C
Rs56753728	IL5	2809	0.0	0.0	1	0.0	A:A

ObsHet means observed heterozygozity, PredHet means predicted heterozygozity

HW means Hardy-Weinberg equilibrium, MAF means minor allele frequency

IVS means intervening sequence variation

E means exon

Table 5.27. SNP marker analysis in IL5 gene for 61 inhibitor (-) hemophilia A patients

SNP Name	Gene	Position (bp)	ObsHET	PredHET	HWpval	MAF	Alleles
Rs2069812	IL5	299	0.492	0.416	0.288	0.295	C:T
Rs4986967	IL5	314	0.0	0.0	1	0.0	G:G
Rs2069813	IL5	331	0.0	0.0	1	0.0	C:C
Rs2069814	IL5	432	0.0	0.0	1	0.0	A:A
Rs3052201	IL5	616	0.0	0.0	1	0.0	A:A
Rs67023946	IL5	648	0.0	0.0	1	0.0	A:A
Rs6696885	IL5	827	0.0	0.0	1	0.0	A:A
Rs66653352	IL5	876	0.0	0.0	1	0.0	A:A
Rs1800474	IL5	1167	0.0	0.0	1	0.0	A:A
Rs2069815	IL5	1205	0.0	0.0	1	0.0	T:T
Rs34909832	IL5	1451	0.0	0.0	1	0.0	A:A
Rs34328342	IL5	1478	0.0	0.0	1	0.0	C:C
Rs2069823	IL5	2516	0.0	0.0	1	0.0	A.A
Rs2069818	IL5	2691	0.0	0.0	1	0.0	C:C
Rs56753728	IL5	2809	0.0	0.0	1	0.0	A:A

ObsHet means observed heterozygozity, PredHet means predicted heterozygozity

HW means Hardy-Weinberg equilibrium, MAF means minor allele frequency

IVS means intervening sequence variation

E means exon

6. DISCUSSION

6.1. *F8* Gene Mutation Profile of Severe Hemophilia A Patients with Inhibitors

F8 gene mutations were shown to be a decisive risk factor in inhibitor development. The present study was the first attempt to profile the *F8* gene mutations in Turkish patients who developed inhibitors to FVIII. The mutational analysis of the *F8* gene in 34 inhibitor patients revealed a total of 34 pathological changes including the double mutation increasing the total number of Turkish hemophilia A patients with known mutations to 231 and contributing 7 novel point mutations and 5 novel large deletions to the hemophilia A database. The cause of hemophilia A in the severely affected HR patient (162HA675) who lacked a change in the *F8* gene upon complete sequencing needs to be studied further since the defect can be in intronic regions of the *F8* gene or in one as yet unidentified modifier protein of FVIII function or even due to a novel genomic reorganization. This patient did not have a combined deficiency of FV and no mutation in vWF gene related to vWD Type 2N disease. RT-PCR showed lack of exon 19 which is known to be non-pathogenic. One can conclude that when the present and previous mutational analyses of the *F8* gene of Turkish hemophilia A patients are considered the mutation profile fits that of the HAMSTeRS database (http://hadb.org.uk/). The mutation subtypes in severe patients who develop inhibitors are also consistent with previous studies such that intron 22 inversions are the most prevalent followed by highly damaging mutations such as large deletions, small deletions, and nonsense mutations in the *F8* gene (Fidancı *et al*., 2008). Only one splicing error and one missense mutation were found among HR patients. All mutations were in agreement with the clinical severity and coagulant activity of FVIII:C. The most prevalent mutation in LR patients was small deletions.

The risk of inhibitor development among 231 Turkish hemophilia A patients with known mutations were in agreement with the risk factors calculated for different mutation types in other populations confirming that the risk is high in patients with major molecular defects in the *F8* gene (Table 6.1). These patients most probably produce alloantibodies since they lack endogenous FVIII. The risk of inhibitor development seemed to be twice as high in patients with nonsense mutations located in the light chain compared with that of

patients with mutations in the heavy chain (Oldenburg *et al*., 2004). In our study, we found three nonsense mutations two of which were in the light chain. The presence of only one patient with a missense mutation among the HR group confirmed the low risk of developing alloantibodies in patients with missense mutations.

Table 6.1. *F8* gene mutation profile of all Turkish hemophilia A patients examined

Mutation Type	**HR Patients No (%)**	**LR Patients No (%)**	**Patients Studied Previously No (%)**	**Total Patients No (%)**
Inversion	16 (53)	1 (25)	123 (62)	140 (60)
Large Deletion	5 (17)	-	1 (1)	6 (2.5)
Small del/ins	3 (10)	3 (75)	10 (5)	16 (8.5)
Nonsense	3 (10)	-	10 (5)	13 (7)
Splicing Error	1 (3.3)	-	4 (2)	5 (2)
Missense	1 (3.3)		46 (23.3)	47 (20)
No Mutation	1 (3.3)		3 (1.7)	4 (1)
Total	**30 (100)**	**4 (100)**	**197 (100)**	**231 (100)**

6.1.1. Large Deletions in Five HR Patients

In order to confirm or support the failure of exonic amplifications in four HR patients and determine the heterozygosity of mothers for deletions, qPCR assay was used to detect the relative amount of template DNA (Higuchi *et al*., 1993). Absolute quantification using the "Fit Points Method" is an analysis used to quantify the target sequence and reference sequence and gives a concentration value. Relative quantification compares target and reference sequence concentrations and gives a ratio (Rasmussen, 2001).

The normalized ratio for target sequences to reference sequence had a value 1 and 0.5 in a normal female and in a normal male, respectively. The ratio of 1 in the mother of patient 267HA992 was interpreted as two copies of exons between 13 and 25 and she was diagnosed as a non-carrier (Table 5.5).

In patient 268HA993 the lack of int22h-2 and int22h-3 repeat regions, initially observed by Southern Blot analysis was evident in CGH analysis, and there was no gross chromosomal anomalies as judged by the karyotype analysis. Whether this large deletion

outside F8 gene is responsible for the hemophilia A phenotype needs to be investigated further.

The carrier status of the mother of patient 274HA999 was confirmed with the ratio 0.42 for exons 2-8 10 deletion in qPCR (Table 5.6). RT-PCR analysis could not be done for this patient.

The ratio of 0.41 in the mother of patient 283HA1008 was interpreted as one copy of exon 10 and she was diagnosed as a carrier. In addition, when the forward exon 9 and reverse exon 11 primers were combined, a 9419 bp PCR product could be amplified in normal female, male and the mother of patient but not in patient.

6.1.2. CGH Analysis of *F8* Gene in Five HR Patients Suspected with Large Deletion

In CGH analysis where DNA of patients and controls labeled with different fluorescent dyes are co-hybridized in an array containing known DNA sequences, ratios of fluorescent intensities show the different copy numbers between DNAs of patients and controls. With this technique, duplications, deletions, insertions or genomic rearrangements that can cause genetic diseases beyond copy number variations may be detected. Many genetic disorders like sarcoglycanopathies, cystic fibrosis, Duchenne and Becker muscular dystrophies caused by duplication or deletions in related genes (Saillour *et al*., 2008) and also recurrent rearrangements in 1q21.1 and deletions in 16p11.2 and 16p12.2 have been detected by CGH analysis (Mefford *et al*., 2008) (Ballif *et al*., 2007).

A custom designed array was used to detect deletions as small as 300bp in five HR patients and six other individuals. Scores out of the range 0-0.25 and 0-(-0.25) were accepted as significant. Exon 14-25 deletion in patient 267HA992, exon 10 deletion in patient 283HA1008, exon 2-8 deletion in patient 274HA999, exon 19-22 deletion in patient 288HA 1014 were confirmed by CGH analysis and shown in Figures 5.22-5.25.

Additional information was extracted from the CGH analysis regarding the whole genome as judged by the background probes and also the *F8* gene of 6 hemophilia A patients. A CNV numbered as 23331 is seen to be localized in chromosome X in positions

153,662,541 to 154,582,606 (153.7 Mb) including many genes along with the *F8* gene (http://projects.tcag.ca/variation/). The significant scores of novel deletions/duplications and their positions were given in Appendix E.

Patient 267HA992 had a significant negative score showing a deletion in chromosome 10 not located within a known CNV in 40.000 bp deletion range. This deletion includes partial end of macrophage mannose receptor 1 lectin domain family 1 gene (MRC1L1) gene that has a role in the immune system. This gene is in close proximity to MRC1 gene with homology suggesting that they represent a segmental duplication (www.ncbi.nlm.nih.gov/sites/entrez?db=gene&cmd). This deletion can be interpreted as a deletion of a copy of this duplication.

Patient 283HA1008 had a significant positive score showing duplications on chromosome 17, 19 and a negative score showing deletion on chromosome 21. Duplications are in 40.000 bp length and deletion in chromosome 21 is in 160.000 bp length. Duplication in chromosome 17 includes forkhead box protein N1 (FOXN1) gene. Mutations in this gene were found to be correlated with compromised immune system associated with the skin disorder congenital alopecia (Adriani *et al*., 2004). Duplication in chromosome 19 includes genes cartilage intermediate layer protein 2 gene (CILP2) and NADH dehydrogenase ubiquinone alpha 13 complex (NUDFA13) genes. These duplications and deletion are not in the CNV database. They may represent new CNVs in these chromosomal regions.

Patient 288HA1014 had significant positive score on chromosome 21 respectively that is not in the CNV database. Duplication is in 120.000 bp length and contains no gene. Patient 162HA675 without any *F8* gene mutatios had three significant scores on chromosome 1, 10 and 21 respectively. These duplications and deletion regions were not localized in a known CNVs or genes. They are in 320.000, 22187 and 40.000 bp length respectively. These new variants were not observed in other six individuals. In order to confirm these new variants are CNVs or pathological changes more patients and healthy controls need to be searched. Interestingly, these new changes are localized in some genes related with immune system like FOXN1 and MRC1L1. This may cause patients be immunodeficient and develop inhibitors.

The significance of all of these additional information from array CGH should be investigated further.

In overall, this study was the first attempt to profile the *F8* gene mutations in Turkish patients who developed inhibitors to FVIII. It also suggested that genetic risk factors other than *F8* gene mutations are involved in the development of inhibitors in the high risk group of patients who presumably do not have functional *F8* mRNAs. It was of interest to see whether about 60 per cent of Turkish inhibitor patients who carry a homogenous mutation, namely intron 22 inversion indeed carry the risk of inhibitor development due to polymorphic alleles in their immune response genes associated with inhibitor formation.

6.2. Investigation of an Association between the Inhibitor Formation and Some Interleukin/Cytokine Gene Polymorphisms in Hemophilia A Patients

The *F8* gene is prone to rearrangements caused by intrachromosomal homologous recombination between sequences within introns and homologous copies oriented in opposite directions out of the *F8* gene leading to inversions and causing severe hemophilia A. Intron 22 inversion occurs between int22h-1 region in intron 22 and one of its two homologous copies (int22h-2, int22h-3) that are telomeric to and 400 kb away from the *F8* gene.

6.2.1. Analysis of Intron 22 Inversion Mutation in Severe Hemophilia A Patients with or without Inhibitors

Intron 22 inversion is one of the null mutations of *F8* gene. This mutation does not produce any FVIII protein. Patients with this type of mutations develop inhibitors with greater than 30 per cent prevalence. Intron 22 inversion mutation was the most prevalent mutation type in Turkish HR patients with 60 per cent ratio. It may be proposed that immune response can be upregulated in most of patients with null mutations like intron 22 inversion (Oldenburg *et al.,* 2002). In order to avoid the influence of the heterogeneous *F8* gene mutations, the study should have been done with patient groups having a homogenous *F8* gene mutation with high prevalence like intron 22 inversion.

In this study, intron 22 inversion was selected as a homogeneous mutation type to constitute two patient groups for the association study.

6.2.2. Association Study

F8 gene mutations were shown to be a decisive risk factor in inhibitor development (Oldenburg, 2001). However, the same type of *F8* gene mutation can be seen in hemophilia A patients both with and without inhibitors. The observation that African-American patients with severe hemophilia have two fold increased risk of inhibitor formation compared with a white population group indicates that genetic risk factors other than *F8* gene mutations exist for inhibitor development (Key, 2004).

MHC class II genes DQ, DR, and DP play important role in the presentation of extracellular antigens, such as exogenous FVIII to the patient's immune system. it was concluded that polymorphisms, especially in HLA DQ and DR loci do not make a strong contribution to the risk of inhibitor formation. Other genetic risk factors include some immune response genes like interleukin and cytokine genes.

In studies of patients with autoimmune disease, polymorphisms in immune response genes have been found to be associated with the antibody formation. Up to date more than 10 million SNPs have been described. In order to understand the role of these SNPs in clinics of patient, DNA analyses of populations from Africa, Asia and Europe were occurred in Hapmap project to identify common haplotypes. With the help of these Hapmap data, candidate regions have been determined to understand the influence of immune response to FVIII in international multi-center Haemophilia Inhibitor Genetics Study (HIGS).

It was found that there was a strong association between an allele with 134 bp in one of the CA repeat microsatellites (IL10.G) located in the promoter region of the IL10 gene, and development of inhibitor. Initially, 77 inhibitor (+) patients and 87 inhibitor (-) patients were analyzed in this study. First association study was performed regardless of clinical severity or *F8* gene mutation type. When a significant association was obtained, subgroups were constituted according to *F8* gene mutation type. The association was consistent in the

subgroup of families in severe hemophilia A with inversions ($p<0.0001$) which means that constitution of isolated subgroups will give the exact association results statistically. IL10 is the first gene located outside the causative *F8* gene mutation to be associated with inhibitor development. IL10 is secreted by Th2 cells and promotes B cells differentiate and produce antibodies. It is proposed that the allele 134 is associated with enhanced secretions of IL10 in patients. The SNP in the promoter region of TNF-α has a strong link between inhibitor formations in hemophilia A siblings in the MIBS study ($p=0.008$) (Astermark *et al*., 2006). A C/T SNP in the promoter region of the gene CTLA-4 was found to be associated with inhibitor formation with 31.2 per cent of T-allele- carriers ($p=0.012$). The same strategy for association studies were performed with that of IL10G. According to these results, constituting of subgroups will support the significancy and reliability of statistical results.

In this study, cytokine and interleukin genes in B cell activation like IL2, IFNG, IL4, IL5, IL10 and TGFB1 genes, especially in their promoter and intronic regions were scanned for the SNPs. Only the SNPs with an average heterozygosity frequency near to 0.5 in different populations (Asian, European and African-American) were considered. SNP data were taken from HapMap and NCBI. Due to possible regulatory roles, nine SNPs and one VNTR especially in promoter and intronic regions of these genes were chosen. Eight SNPs and one VNTR region were scanned in 100 healthy Turkish individuals by Hybrobe probes. SNPs were found to be in Hardy-Weinberg equilibrium.

Up to date 256 unrelated severe hemophilia A patients with inhibitors and without inhibitors were collected in the various hematology clinics within the country. One hundred and seventy three of these patients were scanned for the intron 22 inversion mutation and two groups were constituted. Thirty four patients constituted the group patients with inhibitors and 61 patients constituted the group patients without inhibitors. In order to balance the size of two groups, 8 inhibitors (+) patients with F8 gene nonsense and large deletion mutations were added to inhibitor (+) patients group.

These two severe hemophilia A patient groups were scanned for 9 SNPs and 1VNTR region. Inhibitor (+) group and inhibitor (-) group were accepted as case and control groups in the Haploview programme respectively. Rs 2069812 showed a significant association

with a 0.0251 *p*-value and T-allele was found to be associated with inhibitor (+) patients (Table 5.15). False positive association was excluded with permutation test. Genotype frequencies were calculated in two patient groups for 9 SNP and VNTR region by using chi-square test in webpage of University of Kansas (http://people.ku.edu/~preacher/chisq/chisq.htm). *P*-value of rs2069812 was found to be 0.0082 and TT genotype was found to be associated with inhibitor (+) patients. These results were also confirmed the results of Haploview 4 programme case-control association analysis (Table 5.19). In order to support this association, inhibitor (+) patients and healthy individuals were also analyzed case and control groups respectively but no significant value was detected. The analysis for the inheritance pattern revealed that carrying TT genotype for rs2069812 meant a 6.86 times more probability to develop inhibitors. On the other hand, patients carrying CT or CC had the risk at rate 0.02 per cent (OR=0.14) compared to TT genotype (Table 5.20). Therefore, T-allele was considered as a recessive susceptibility allele and C allele as a dominant protective allele.

In order to support the effect of this SNP, mutation analysis of IL5 gene in two patient groups were performed. However, no causative mutation or variant segregating with this SNP was detected. In addition, no copy number variation was found to be co-localized with this SNP.

Rs2069812 is located in the promoter region (C-703T) of the IL5 gene. This gene expresses the IL5 glycoprotein which plays a pleiotropic role in the immune system and inflammation. It supports the growth and the differentiation of B cells and it has a key mediator role in eosinophil activation. It is produced by Th2 cells and mast cells. IL-5 cytokines are the key molecules for the disease as allergy and eosinophilic inflammation (Takatsu, 1998). In previous studies, rs2069812 was found to be associated with diseases like atopic bronchial asthma (Freidin *et al*., 2003), gastric cancer risk (Mahajan *et al*., 2008) and atopic dermatitis (Yamamoto *et al*., 2003). Because of the localization this SNP, it may be suggested that it could play a role in the up regulation or the down regulation of the IL5 gene and influence the level of IL5 protein. This could be assumed that T variant in inhibitor (+) patients of this gene was expressed in different level in comparison with that of the C allele and caused increased or decreased production of IL5 protein causing inhibitor formation. IL5 gene is expressed in CD4+ Tcells, mast cells and eosinophils, and

in any allergical reactions expression level of IL5 gene can be varied (Takatsu, 1998). In order to see the specific IL5 gene expression against recombinant FVIII protein, CD4+ T cells responding to FVIII antigens need to be isolated from peripheral blood and treated with recombinant FVIII protein in cell culture studies.

Despite the lack of transcription factor binding sites (Appendix G), it may be worth to examine the role of the SNP in epigenetic regulation since some SNPs and CpG sites show significant *cis*- or trans-associations. It was hypothesized that a considerable proportion of CpG sites may be quantitative traits with regulation by specific genetic variants (Zhang *et al*., 2010). With help of sensitive array based techniques SNP genotyping and methylation patterns of CpG sites and CpG islands could be analysed.

Approximately 5000 bases of 5' region of IL5 gene was scanned for CpG islands bioinformaticaly (http://www.ualberta.ca/~stothard/javascript/cpg_islands.html). However no CpG islands were detected in this region. On the other hand, this gene, together with genes IL4, IL13 and colony stimulating factor 2 (CSF2), form a cytokine gene cluster on chromosome 5q31. This cytokine, IL4, and IL13 are regulated coordinately by long-range regulatory elements 120 kilobases in length on chromosome 5q31. When this region was scanned for CpG islands approximately 70 CpG islands were found. This SNP does not need to be segregated with a pathological mutation to lead inhibitor formation, but it could probably be in cis or trans-association with CpG island in further distances even 1Mb. Further studies need to be performed to find any association with gene regulation and this SNP.

7. CONCLUSION

During the treatment of hemorrhages in patients with severe hemophilia A, a major complication as an inhibitor development against recombinant FVIII protein can occur. Genetic variants including SNPs, CNVs or mutations in immune response genes other than *F8* gene may affect inhibitor development. Because of limited polymorphism observed in immune response genes only 9 SNPs and a VNTR region could be selected to be tested for associations with inhibitor development in patients with known and relatively homogenous *F8* gene mutations. A preliminary study was conducted to reveal the mutation profile in HA patients with inhibitors and intron 22 was found to be 50 per cent followed by nonsense mutations, and large deletions. A designed array CGH was employed for the first time to confirm large deletions in Turkish hemophilia A patients. A homogenous group was constructed by screening severe hemophilia A patients who have intron 22 inversion mutations. Intron 22 inversion mutation positive patients were grouped in two, those with and without inhibitors and associations with SNP alleles in 5 interleukin/ cytokine genes were carried our revealing a significant association with T-allele in IL5 gene and inhibitor development and TT genotype was found to be associated with inhibitor (+) patients. In the genetic model where T-allele was recessive, TT genotype carried a risk of 6.86 fold compared to those with CT or CC genotypes indicating that the T-allele was the susceptibility allele. This is the first and informative study to detect genetic variants in IL5 gene that possibly play a role in inhibitor formation in Turkish severe hemophilia A patients.

The specific design of array CGH revealed the following additional information: Genes that cover these SNPs and VNTR region were also included for CGH analysis to see any variants (Table 4.6). When six HR patients were analyzed for these regions no variants as deletions/duplications were detected. However, in patients 267HA992 and 283HA1008 new genetic variants were detected in other genes related to immunodeficency that confirms the findings of gene ontology studies that CNVs are associated with immune response genes more frequently. It would be of interest to design a new array CGH to detect CNVs that covers all of the immune response genes included in B cell activation for inhibitor formation against FVIII (Key, 2004).

APPENDIX A: CLINICAL FORM FOR SEVERE HEMOPHILIA PATIENTS

AĞIR HEMOFİLİ A HASTALARI İÇİN HASTA FORMU

ÖNEMLİ NOT:

- ❖ Bu çalışmaya absans nöbeti olan hastaların bulunduğu aileler dahil edilecektir.
- ❖ Çalışma için gerekli örnekler (önem sırasına göre):
  1- anne-baba- hasta çocuk üçlüsü (ve mümkünse sağlıklı kardeş)
  2- sağlıklı veya hasta diğer aile bireyleri
  3- anne- baba ve aile bireylerine ulaşılamaması durumunda sadece hasta bireyin örneği

Adı Soyadı: Doğum Tarihi: Doğum yeri: Cinsiyet: Tel: Adres:	Gönderen Doktor: Çalıştığı Kurum: Tarih: Tel: Adres: Eposta:

AİLE BİLGİLERİ

Anne Adı/Doğum Yeri:		Anne Tarafının Kökeni (Yaşadığı Yöre, Göçmenlik Durmu vs.) :
Baba Adı/Doğum Yeri:		Baba Tarafının Kökeni (Yaşadığı Yöre, Göçmenlik Durmu vs.) :
Akraba Evliliği:	Var: ☐	Akrabalık Derecesi:
	Yok: ☐	

HASTALIK BİLGİLERİ	
Hastalığın Derecesi	
FVIII:C Düzeyi	
FVIII:Ag Düzeyi	
Tedavi Şekli (Replacement/On demand)	
Inhibitor Düzeyi	
Tedavinin Başlama Yaşı:	
Kanama Sıklığı	
e Belirtilmesi Gereken Diğer İlgili Özellikler:	
Alınan Örneklerin Gönderileceği Adres ve Ulaştırma Yöntemi	Prof. Dr. S Hande Çağlayan Moleküler Biyoloji ve Genetik Bölümü Boğaziçi Üniversitesi Etiler, İstanbul Tel: 0212 359 6881 Faks:0212 287 2468 Eposta:hande@boun.edu.tr GSM: 0 532 652 04 61 (Hande Çağlayan) 0532 788 39 49 (Inanç Değer Fidancı) Ulaşım ödemeli olarak yurtiçi kargo ile yapılabilir. Yurtiçi Kargo çağrı merkezi : 444 9999

APPENDIX B: CLINICAL DATA OF HEMOPHILIA A PATIENTS WITH INHIBITORS

Table B.1. Clinical Data of patients with inhibitors

Patient No	Name of Doctor	FVII:C	BU
80HA 337	Prof.Dr Kaan Kavaklı	5	7.5
85HA 342	Prof. Dr. Kaan Kavaklı	5	37.5
103HA 388	Prof. Dr. Kaan Kavaklı	1.2	15
124HA 469	Prof. Dr. Kaan Kavaklı	2	32
162HA 675	Prof. Dr. Kaan Kavaklı	1	16
197HA 786	Dr. Gülersu İrken	3	20
264HA 989	Prof. Dr. Kaan Kavaklı	1	400
265HA 990	Prof. Dr. Kaan Kavaklı	1	16
266HA 991	Prof. Dr. Kaan Kavaklı	1	408
267HA 992	Dr. Hülya Sayılan	1	250
268HA 993	Dr. Hülya Sayılan	1-3	14
269HA 994	Prof. Dr. Yurdanur Kılınç	0.4	176
270HA 995	Prof. Dr. Kaan Kavaklı	1	250
271HA 996	Doç. Dr. Canan Uçar	5	60
272HA 997	Doç Dr. Canan Uçar	4-10	5
273HA 998	Doç. Dr. Canan Uçar	1.1	12
274HA 999	Doç. Dr. Adalet Meral	1.5	80
275HA 1000	Doç. Dr. Adalet Meral	0	10
276HA 1001	Doç. Dr. Adalet Meral	1.7	10
278HA 1003	Doç. Dr Canan Uçar	1	16
279HA 1004	Prof. Dr. Yurdanur Kılınç	1	88.2
280HA 1005	Dr Çetin Timur	1-4	58
281HA 1006	Dr Çetin Timur	0.5	32

Table B.1. Clinical Data of patients with inhibitors (continued)

Patient No	Name of Doctor	FVIII:C	BU/ml
282HA 1007	Dr Çetin Timur	1	8.96
283HA 1008	Dr Çetin Timur	3	49.2
284HA 1010	Doç. Dr. Alphan Küpesiz	1	474
287HA 1013	Dr. Berna Atabay	0.6	19
208HA 798	Dr. Canan Vergin	0.84	950
288HA 1014	Prof. Dr. Yüksel Pekçelen	1	600
289HA 1015	Dr. Elif kazancı	0.31	6.60
87HA 344	Prof. Dr. Kaan Kavaklı	2	1
126HA 604	Dr. Ziya Ekrem Öktel	0-1	2.9
247HA 925	Mersin Üniversitesi	1	5
277HA 1002	Doç. Dr. Canan Uçar	1	5

APPENDIX C: CLINICAL DATA OF 256 SEVERE HEMOPHILIA A PATIENTS

Table C.1. Clinical Data of 256 severe hemophilia A patients

DNA Number	Doctor Name	F VIII:C	BU/ml
17 HA 58	Dr. Cem Ar	1	No
44 HA 171	Prof.Dr. Bülent Zülfikar	1	No
52 HA 203	Prof.Dr. Bülent Zülfikar	0.5	No
59 HA 233I-101	Dr. Cem Ar	0	HR
62 HA 253	Prof.Dr. Bülent Zülfikar	0.2	No
64 HA 260	Dr. Cem Ar	1	No
65 HA 270	Dr. Cem Ar	0.1	No
80 HA 337I-45	Prof. Dr. Kaan Kavaklı	5	7.5
82 HA 339	Prof. Dr. Kaan Kavaklı	4.5	No
85 HA 342I-46	Prof. Dr. Kaan Kavaklı	5	HR
89 HA 346	Prof. Dr. Kaan Kavaklı	2.1	No
124 HA 469I-48	Prof. Dr. Kaan Kavaklı	2	32
138 HA 529	Prof. Dr. Kaan Kavaklı	2	No
148 HA 592	Prof.Dr. Bülent Zülfikar	2	No
126 HA 604I-30	Prof.Dr. Bülent Zülfikar	1	3
158 HA 667	Prof. Dr. Kaan Kavaklı	1	No
177 HA 725	Prof.Dr. Bülent Zülfikar	1	No
183 HA 754	Prof.Dr. Bülent Zülfikar	0	No
208HA798 I-31	Prof. Dr. Yurdanur Kılınç	0.8	950
223 HA 817I-94	Prof.Dr. Bülent Zülfikar	0.6	Yes
231 HA 859I-50	Prof. Dr. Kaan Kavaklı	1	65
235 HA 877	Dr. Cem Ar	0.1	No
243HA910	Pretam		No
252 HA 949	Dr. Cem Ar	0.2	No
257HA964I-82	Prof. Dr. Kaan Kavaklı	1	No
258HA967	Prof. Dr. Kaan Kavaklı	<1	No
260HA971	Dr. Cem Ar		No
260HA972I-63	Dr. Cem Ar	0.3	1.7
260HA973	Prof.Dr. Bülent Zülfikar	0	No
263HA986	Prof. Dr. Kaan Kavaklı	2	No
265HA990I-2	Prof. Dr. Kaan Kavaklı	1	16-250
266HA991I-3	Prof. Dr. Kaan Kavaklı	1	400
267HA992I-4	Prof.Dr. Bülent Zülfikar	0.1	26
270HA995I-7	Prof. Dr. Kaan Kavaklı	1	250
269HA994I-8	Prof. Dr. Yurdanur Kılınç	0.4	176
271HA996I-9	Dr. Canan Uçar	5	60
272HA997I-10	Dr. Canan Uçar	4	5
273HA998I-11	Dr. Canan Uçar	1	12

Table C.1. Clinical Data of 256 severe hemophilia A patients (continued)

DNA Number	Doctor Name	F VIII:C	BU/ml
276HA1001I-14	Prof. Dr. Yurdanur Kılınç	1.7	10
279HA1004I-17	Prof. Dr. Yurdanur Kılınç	1	88
281HA1006I-19	Dr. Çetin. Timur	0.5	32
197HA786I-25	Dr. Gülersu erken	1	20
283HA1009I-23	Dr. Cem Ar	0.9	No
287HA1013I-29	Dr. Berna. Atabey	1	19
289HA1015I-33	Dr. Elif Kazancı	0.3	6.6
291HA1024	Dr. Aysegül Tecer	1.3	No
294HA1037	?	1	No
303HA1065I-52	Prof. Dr. Kaan Kavaklı	1	Yes
304HA1067I-54	Prof. Dr. Tiraje Celkan	0.3	43.5
305HA1071I-57	Prof. Dr. Tiraje Celkan	0.2	6
308HA1076	Prof. Dr. Kaan Kavaklı	0	No
309HA1082	Dr. Cem Ar	0.5	No
310HA1083	Dr. Cem Ar	0.3	No
311HA1084	Dr. Cem Ar	1	No
312HA1085	Dr. Cem Ar	0.3	No
313HA1086	Prof. Dr. Yurdanur Kılınç	1	No
314HA1088	Prof. Dr. Yurdanur Kılınç	0.8	No
315HA1091	Prof. Dr. Yurdanur Kılınç	0.6	No
316HA1094I-60	Prof. Dr. Yurdanur Kılınç	0.6	1.1
317HA1097	Prof. Dr. Yurdanur Kılınç	1.3	No
318HA1100	Prof. Dr. Yurdanur Kılınç	0.8	No
319HA1101	Prof. Dr. Kaan Kavaklı	1	No
320HA1102	Prof. Dr. Kaan Kavaklı	1	No
320HA1103	Prof. Dr. Kaan Kavaklı	1	No
321HA1106	Prof. Dr. Kaan Kavaklı	1	No
321HA1107	Prof. Dr. Kaan Kavaklı	1	No
322HA1110	Prof. Dr. Kaan Kavaklı	1	No
323HA1113	Prof. Dr. Kaan Kavaklı	1	No
324HA1114	Prof. Dr. Kaan Kavaklı		No
325HA1115	Prof. Dr. Kaan Kavaklı	1	No
326HA1116I-78	Prof. Dr. Kaan Kavaklı	1	3.5
327HA1118	Prof. Dr. Kaan Kavaklı	1	No
328HA1119	Dr. Cem Ar	1	No
329HA1120	Dr. Cem Ar	0.2	No
330HA1121	Dr. Cem Ar	0.4	No
331HA1122	Prof. Dr. Kaan Kavaklı	1	No
332HA1123	Prof. Dr. Kaan Kavaklı	1	No
333HA1125	Prof. Dr. Kaan Kavaklı	2	No
334HA1127	Dr. Cem Ar	0.1	No
335HA1128	Dr. Cem Ar	0.1	No
336HA1129	Dr. Cem Ar	0.1	No
337HA1130	Dr. Cem Ar	1	No

Table C.1. Clinical Data of 256 severe hemophilia A patients (continued)

DNA Number	Doctor Name	F VIII:C	BU/ml
338HA1131	Prof. Dr. Kaan Kavaklı	1	No
339HA1132	Prof. Dr. Kaan Kavaklı	1	No
339HA1132	Prof. Dr. Kaan Kavaklı	1	No
340HA1133	Prof. Dr. Kaan Kavaklı	1	No
341HA1136	Prof. Dr. Kaan Kavaklı	1	No
342HA1137	Prof. Dr. Kaan Kavaklı	1	No
343HA1139I-85	Prof. Dr. Kaan Kavaklı	1	Yes
344HA1141	Prof. Dr. Kaan Kavaklı	1	No
345HA1142	Prof. Dr. Kaan Kavaklı	1	No
346HA1143	Prof. Dr. Kaan Kavaklı	1	No
347HA1147	Prof. Dr. Yurdanur Kılınç	1	No
348HA1149	Prof. Dr. Yurdanur Kılınç	1	No
349HA1151	Prof. Dr. Yurdanur Kılınç	1	No
350HA1154I-65	Prof. Dr. Yurdanur Kılınç	1	31.74
351HA1157I-68	Prof. Dr. Yurdanur Kılınç	0.77	7.68
352HA1160	Prof. Dr. Yurdanur Kılınç	3.6	No
353HA1162	Prof. Dr. Yurdanur Kılınç	0.2	No
354HA1165I-78	Prof. Dr. Yurdanur Kılınç	0.6	0.85
355HA1167	Prof. Dr. Yurdanur Kılınç	1	No
356HA1169I-73	Dr. Cem Ar	0.3	39.6
356HA1170	Dr. Cem Ar	0.3	No
357HA1171	Dr. Cem Ar	1	No
358HA1172	Dr. Cem Ar	0.4	No
359HA1173I-74	Dr. Cem Ar	0.3	14
360HA1174	Dr. Cem Ar	0.2	No
329HA1175	Dr. Cem Ar	1	No
363HA1181I-75	Dr. Cem Ar	0.3	12.5
362HA1180	Dr. Cem Ar	0.3	No
363HA1181I-75	Dr. Cem Ar	0.3	12.5
364HA1182	Dr. Cem Ar	0.4	No
365HA1183I-76	Dr. Cem Ar	0.3	1
366HA1184	Dr. Cem Ar	1	No
367HA1185	Prof. Dr. Kaan Kavaklı	1	No
368HA1188I-79	Prof. Dr. Kaan Kavaklı	1	1.5
369HA1191	Dr. Cem Ar	0.2	No
357HA1192	Dr. Cem Ar	0.3	No
357HA1193	Dr. Cem Ar	0.3	No
370HA1194	Dr. Cem Ar	0.2	No
371HA1195	Dr. Cem Ar	0.1	No
372HA1196	Prof. Dr. Yurdanur Kılınç	1	No
373HA1202	Prof. Dr. Kaan Kavaklı	1	No
374HA1204I-87	Dr. Yusuf Z. Aral	<1	3.6
375HA1208	Prof. Dr. Kaan Kavaklı	1	No

Table C.1. Clinical Data of 256 severe hemophilia A patients (continued)

DNA Number	Doctor Name	F VIII:C	BU/ml
376HA1210	Prof. Dr. Kaan Kavaklı	1	No
377HA1212	Dr. Canan Vergin	0	No
378HA1216	Prof.Dr. Bülent Zülfikar	0.1	No
379HA1217	Prof.Dr. Bülent Zülfikar	0.2	No
380HA1218I-91	Prof.Dr. Bülent Zülfikar	0.1	800
380HA1218I-91	Prof.Dr. Bülent Zülfikar	0.1	800
381HA1219	Prof.Dr. Bülent Zülfikar	0.1	No
382HA1220	Prof.Dr. Bülent Zülfikar	3	No
383HA1221	Prof.Dr. Bülent Zülfikar	2.5	No
384HA1222	Prof.Dr. Bülent Zülfikar	0.1	No
385HA1223I-92	Prof.Dr. Bülent Zülfikar	0.4	5.94
386HA1224	Prof.Dr. Bülent Zülfikar	1	No
387HA1225	Prof.Dr. Bülent Zülfikar	1	No
388HA1226	Prof.Dr. Bülent Zülfikar	1	No
389HA1227	Prof.Dr. Bülent Zülfikar	1	No
390HA1228	Prof.Dr. Bülent Zülfikar	0	No
391HA1229	Prof.Dr. Bülent Zülfikar	1	No
392HA1230	Prof.Dr. Bülent Zülfikar	0.4	No
393HA1231	Prof.Dr. Bülent Zülfikar	0.01	No
394HA1232	Prof.Dr. Bülent Zülfikar	1.5	No
395HA1233	Prof.Dr. Bülent Zülfikar	0	No
396HA1234	Prof. Dr. Kaan Kavaklı	1	No
397HA1237	Prof. Dr. Kaan Kavaklı	1	No
398HA1240	Prof.Dr. Bülent Zülfikar	0.1	No
399HA1241	Prof.Dr. Bülent Zülfikar	0	No
400HA1242	Prof.Dr. Bülent Zülfikar	0.2	No
401HA1243	Prof.Dr. Bülent Zülfikar	0.5	No
402HA1244	Prof.Dr. Bülent Zülfikar	0.5	No
403HA1245	Prof.Dr. Bülent Zülfikar	0.5	No
404HA1246	Prof.Dr. Bülent Zülfikar	0.6	No
405HA1247	Prof.Dr. Bülent Zülfikar	1	No
406HA1248	Prof.Dr. Bülent Zülfikar	1.2	No
407HA1249I-93	Prof.Dr. Bülent Zülfikar	0	63.4
408HA1250	Prof.Dr. Bülent Zülfikar	0.8	No
409HA1251	Prof.Dr. Bülent Zülfikar	1.88	No
410HA1252	Prof.Dr. Bülent Zülfikar	0.8	No
411HA1253	Prof.Dr. Bülent Zülfikar	0.4	No
412HA1254	Prof.Dr. Bülent Zülfikar	0.02	No
413HA1255	Prof.Dr. Bülent Zülfikar	3.04	No
371HA1256	Prof.Dr. Bülent Zülfikar	1	No
414HA1257	Prof.Dr. Bülent Zülfikar	0	No
414HA1258	Prof.Dr. Bülent Zülfikar	0	No
415HA1259	Prof.Dr. Bülent Zülfikar	0.6	No
416HA1260	Prof.Dr. Bülent Zülfikar	0.2	No

Table C.1. Clinical Data of 256 severe hemophilia A patients (continued)

DNA Number	Doctor Name	F VIII:C	BU/ml
417HA1262I-95	Prof. Dr. Yurdanur Kılınç	0	2.56
418HA1266	Prof.Dr. Bülent Zülfikar	0.5	No
419HA1267	Prof.Dr. Bülent Zülfikar	0.5	No
420HA1268	Prof.Dr. Bülent Zülfikar	0	No
421HA1269	Prof.Dr. Bülent Zülfikar	0.8	No
422HA1270	Dr. Cem Ar	0.6	No
422HA1271	Dr. Cem Ar	0.2	No
423HA1272	Dr. Cem Ar	0.2	No
424HA1273	Dr. Cem Ar	0.4	No
424HA1274	Dr. Cem Ar	0.3	No
425HA1275	Dr. Cem Ar	0.5	No
426HA1276	Prof. Dr. Yurdanur Kılınç	0.2	No
426HA1277	Prof. Dr. Yurdanur Kılınç	0.2	No
427HA1279	Prof. Dr. Yurdanur Kılınç	0.1	No
428HA1281-98	Prof. Dr. Yurdanur Kılınç	1	6
429HA1283	Prof.Dr. Bülent Zülfikar	0.9	No
430HA1284	Prof.Dr. Bülent Zülfikar	1	No
431HA1285	Prof.Dr. Bülent Zülfikar	1	No
432HA1286	Prof.Dr. Bülent Zülfikar	0	No
433HA1287	Prof.Dr. Bülent Zülfikar	0	No
434HA1288	Prof.Dr. Bülent Zülfikar	0	No
435HA1289	Prof.Dr. Bülent Zülfikar	0	No
435HA1290	Prof.Dr. Bülent Zülfikar	0	No
436HA1291	Prof.Dr. Bülent Zülfikar	0.5	No
437HA1292	Prof.Dr. Bülent Zülfikar	0.4	No
438HA1293	Prof.Dr. Bülent Zülfikar	1	No
439HA1294-100	Prof.Dr. Bülent Zülfikar	0.8	2.57
440HA1295	Prof.Dr. Bülent Zülfikar	0.2	No
441HA1296	Dr. Cem Ar	0.3	No
442HA1297	Dr. Cem Ar	0.9	No
443HA1298	Dr. Cem Ar	1	No
444HA1299	Dr. Cem Ar	1	No
445HA1300	Prof. Dr. Yurdanur Kılınç	0.8	No
446HA1302	Prof.Dr. Bülent Zülfikar	0	No
447HA1303	Prof.Dr. Bülent Zülfikar	0	No
448HA1304	Dr. Cem Ar	0.3	No
450HA1317	Dr. Cem Ar	0.1	No
451HA1318	Dr. Cem Ar	1	No
452HA1320	Prof. Dr. Yurdanur Kılınç	1	No
453HA1322	Prof. Dr. Yurdanur Kılınç	0.3	No
455HA1324I-102	Prof. Dr. Yurdanur Kılınç	1	9
456HA1325	Dr. Cem Ar	1	No
457HA1326.I106	Prof. Dr. Yurdanur Kılınç	0.74	<1
458HA1328	Prof. Dr. Yurdanur Kılınç	0.5	No

Table C.1. Clinical Data of 256 severe hemophilia A patients (continued)

DNA Number	Doctor Name	F VIII:C	BU/ml
459HA1330	Prof. Dr. Yurdanur Kılınç	1	No
460HA1334.I-103	Prof. Dr. Yurdanur Kılınç	1	1.45
461HA1337	Prof. Dr. Yurdanur Kılınç	<1	No
462HA1339	Prof. Dr. Yurdanur Kılınç	1	No
463HA1341	Prof. Dr. Yurdanur Kılınç	0.67	No
464HA1344	Prof. Dr. Yurdanur Kılınç	1	No
465HA1358	Prof. Dr. Yurdanur Kılınç	<1	No
466HA1360	Prof. Dr. Kaan Kavaklı	1	No
467HA1361	Prof. Dr. Kaan Kavaklı	1	No
468HA1362	Prof. Dr. Kaan Kavaklı	1	No
469HA1363	Prof. Dr. Kaan Kavaklı	1	No
470HA1364	Prof. Dr. Kaan Kavaklı	1	No
471HA1365	Prof. Dr. Kaan Kavaklı	1	No
472HA1366	Prof. Dr. Kaan Kavaklı	1	No
473HA1367	Prof. Dr. Kaan Kavaklı	1	No
474HA1368	Prof. Dr. Kaan Kavaklı	1	No
475HA1369	Prof. Dr. Yurdanur Kılınç	1	No
476HA1371.I-108	Prof. Dr. Kaan Kavaklı		HR
477HA1373.I-110	Prof. Dr. Yurdanur Kılınç	1	1.45
478HA1374	Prof. Dr. Yurdanur Kılınç	0.67	No
479HA1377	Prof. Dr. Yurdanur Kılınç	1	No
480HA1379	Prof. Dr. Yurdanur Kılınç	0.27	No
481HA1381	Prof. Dr. Yurdanur Kılınç	0.17	No
124HA1384.I-111	Prof. Dr. Kaan Kavaklı	1	6.6
264HA989I-1	Prof. Dr. Kaan Kavaklı	0	400
267HA992I-4	Dr. Hülya Sayılan	3	26
268HA993I-5	Dr. Hülya Sayılan	2	14
274HA999I-12	Prof. Dr. Adalet Meral	1	80
279HA1004I-17	Prof. Dr. Yurdanur Kılınç	4	88
280HA1005I-18	Dr. Çetin Timur	3	5
283HA1008I-21	Dr. Çetin Timur	1	49
288HA1014I-32	Prof. Dr. Yüksel Pekçelen	1	600
482HA1385	Prof.Dr. Bülent Zülfikar	1	No
483HA1387	Prof.Dr. Bülent Zülfikar	1	No
484HA1388	Prof.Dr. Bülent Zülfikar	1	No
485HA1389	Prof.Dr. Bülent Zülfikar	1	No
486HA1390	Prof.Dr. Bülent Zülfikar	1	No
487HA1391	Prof. Dr. Kaan Kavaklı	1	No
488HA1395	Prof. Dr. Yurdanur Kılınç	1	No
489HA1396	Prof. Dr. Yurdanur Kılınç	0.5	No
490HA1397	Prof. Dr. Yurdanur Kılınç	1	No
491HA1401	Prof. Dr. Kaan Kavaklı	1	No
492HA1405	Prof. Dr. Kaan Kavaklı	1	No
493HA1406I-117	Prof. Dr. Kaan Kavaklı	1	Yes

APPENDIX D: KARYOTYPE ANALYSIS

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PRENATAL TANI VE GENETİK HİZMETLERİ LTD. ŞTİ.

SİTOGENETİK ANALİZ RAPORU

Protokol No : 115/10
Laboratuvar no : K20 /10
Adı Soyadı [redacted]
Gönderen Doktor : Prof.Dr. Hülya ÇAĞLAYAN
Endikasyon : Hemofili A

Materyal Geliş Tarihi : 01.03.2010
Raporun Veriliş Tarihi : 04.03.2010
Doğum Tarihi :

İncelenen Materyal : Periferik kan lenfositleri
Analiz Yöntemi ve Bantlama : High Resolution Banding
Bant Düzeyi : 600-650
Metafaz-Sayısı : 20

KARYOTİP: 46,XY

YORUM:

High resolution banding tekniği ile yapılan analizde herhangi bir sayısal yada gros bir yapısal kromozom anomalisi saptanmadı.

Bu test ile tek gen hastalıkları, konjenital malformasyonlar, mikrodelesyon ve mikroduplikasyonlar ile ancak özel incelemeler ile gösterilebilecek (Örn:frajil X sendromu) kromozom anomalileri ve düşük oranlı mozaisizm dışlanamaz.

Saygılarımızla,

Uz.Dr.Tahir DEHGAN

Prof.Dr. Seher BAŞARAN

"Merkezimiz Ulusal Sitogenetik Kalite Değerlendirme Programı' nda yer almaktadır."

Hüsrev Gerede cad.No.68 Kat 1 Teşvikiye-İstanbul-TÜRKİYE Tel/Fax:90 212 260 22 83- 0 212 259 26 23 Direkt: 90 212 259 04 09
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PRENATAL TANI VE GENETİK HİZMETLERİ LTD. ŞTİ.

SİTOGENETİK ANALİZ RAPORU

Protokol No : 804/09
Laboratuvar no : K160 / 09
Adı Soyadı : [redacted]
Gönderen Doktor : Prof.Dr. Hande ÇAĞLAYAN
Endikasyon : Hemofili A

Materyal Geliş Tarihi : 26.12.2009
Raporun Veriliş Tarihi : 30.12.2009
Doğum Tarihi : 14.06.1994

İncelenen Materyal : Periferik kan lenfositleri
Analiz Yöntemi ve Bantlama : Kısa Süreli Hücre Kültürü/ GTG /HRBT
Bant Düzeyi : 600-650
Metafaz Sayısı : 20

KARYOTİP: 46,XY

YORUM:
Herhangi bir sayısal yada gros bir yapısal kromozom anomalisi saptanmadı.

Bu test ile tek gen hastalıkları, konjenital maiformasyonlar, mikrodelesyon ve mikroduplikasyonlar ile ancak özel incelemeler ile gösterilebilecek (Örn:frajil X sendromu) kromozom anomalileri ve düşük oranlı mozaisizm dışlanamaz.

Saygılarımızla,

Uz.Dr.Tahir DEHGAN

Prof.Dr. Seher BAŞARAN

"Merkezimiz Ulusal Sitogenetik Kalite De€erlendirme Programı' nda yer almaktadır."
Hüsrev Gerede cad.No.68 Kat 1 Teşvikiye-İstanbul-TÜRKİYE Tel/Fax:90 212 260 22 83- 0 212 259 26 23 Direkt: 90 212 259 04 09
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APPENDIX E: CGH DATA OF SIX HR PATIENTS AND SIX INDIVIDUALS

Table E.1. CGH analysis results of patient 162HA675,

Patient No	Chromosome	Start	Stop	Score	CNV	Gene
162HA675	chr1	16939999	17299999	0.29424	CNV 97301	NBPF1 CROCCL2
	chr1	21539999	21619999	0.33869	CNV 5475	
	chr1	72019999	72339999	-0.61996	NO CNV	
	chr1	142779999	142899999	0.41042	CNV 69772	
	chr1	196739999	196779999	-0.35535	CNV 0686	CFHR1
	chr2	87379999	87979999	-0.28605	CNV 30960	
	chr3	162579999	162619999	0.51509	CNV 23278	
	chr4	3459999	3499999	0.27669	CNV 30195	
	chr4	131979999	132339999	0.35635	CNV 51238	
	chr6	4259999	4459999	-0.58501	CNV 3603	
	chr6	31259999	31299999	-0.49819	CNV 5387	HLA-B
	chr6	32419999	32459999	-0.52433	CNV 69434	
	chr9	45139999	45339999	0.50806	CNV 83010	
	chr10	38819999	38859999	-0.35480	CNV 2860	
	chr10	48099999	48179999	-0.26496	CNV 65661	
	chr10	135499999	135522186	-0.48433	NO CNV	
	chr12	9619999	9699999	-0.28234	CNV 3874	
	chr16	2659999	2739999	0.38153	CNV 66964 CNV 66965 CNV 66966 CNV 77242 CNV 66967 CNV 49681	IL4R
	chr17	34459999	34499999	-0.25786	CNV 88438	
	chr20	61859999	61899999	0.32704	CNV 30113	
	chr21	10179999	10219999	0.30049	NO CNV	
	chr22	24259999	24299999	0.30517	CNV 3238	DDT
	chrX	3739999	3899999	0.42361	CNV 0820	
	chrX	72019999	72059999	-0.32421	CNV 37387 CNV 96755	DMRTC1

Table E.2. CGH analysis results of patient 267HA992

Patient No	Chromosome	Start	Stop	Score	CNV	Gene
267HA992	chr3	162539999	162619999	-0.81081	CNV 4360 CNV 59901	
	chr10	17939999	17979999	-0.33710	NO CNV	MRC1L1
	chr10	46979999	47099999	0.25025	CNV 65654	
	chr16	32179999	32339999	0.31607	CNV 4944 CNV 37179	HERC2P4
	chr16	33219999	33339999	0.43828	CNV 72564	TP53TG3
	chr16	70179999	70219999	0.31042	CNV 88194	
	chr17	44419999	44619999	0.47376	CNV 0502 CNV 37194	ARL17B
	chr19	43699999	43739999	-0.16171	CNV 1521	PSG4
	chr22	18699999	18819999	0.31954	CNV 8900	GGT3P
	chr22	20259999	20619999	0.31463	CNV 90891	RTN4R CR603232
	chr22	24339999	24379999	0.34545	CNV 32452	GSTT1

Table E.3. CGH analysis results of patient 288HA1014

Patient No	Chromosome	Start	Stop	Score	CNV	Gene
288HA1014	chr15	102459999	102520437	-0.28413	CNV 72386	OR4F17
	chr21	9899999	10019999	0.32177	NO CNV	
	chr22	24339999	24379999	0.27454	CNV 32452	GSTT1

Table E.4. CGH analysis results of patient 268HA993

Patient No	Chromosome	Start	Stop	Score	CNV	Gene
268HA993	chr1	223699999	223779999	0.28558	CNV 84863	CAPN
	chr2	96699999	96819999	0.26490	CNV 74170	
	chr4	9179999	9259999	-0.43340	CNV 2497 CNV 0347	
	chr5	69059999	69219999	0.26633	CNV 69133	
	chr5	69699999	69939999	0.26126	CNV59615	SMA4
	chr6	32419999	32539999	-0.51039	CNV 69434	
	chr6	78979999	79019999	-0.99557	CNV 51971	
	chr8	7779999	7899999	-0.25809	CNV 70272	
	chr9	38899999	38979999	0.32611	CNV 31470	
	chr15	77859999	78139999	0.25223	NO CNV	
	chr15	84859999	84939999	0.26039	CNV 72334	ADAMTSL3
	chr16	28699999	28739999	0.31923	CNV 66966 CNV 88025 CNV 66967 CNV 35372 CNV 8821 CNV 88035 CNV 77251 CNV 2208 CNV 3999	JMJD5 NSMJ1 IL4R IL21R GTF3C1 SBK1
	chr16	70739999	70779999	0.32846	CNV 49743	
	chr17	34539999	34619999	-0.55250	CNV 8840	TBC1D3B CCL3L3 CCL4L1
	chr17	44019999	44059999	0.29374	CNV 8850 CNV 37194	
	chr19	55259999	55339999	0.30394	CNV 73427	KIR-K65
	chr20	25779999	25819999	-0.25534	CNV 4098	FAM182B
	chr22	24339999	24379999	0.34530	CNV 32452	GSTT1
	chrX	154579999	154924490	-0.94828	CNV 23331 CNV 37367	F8A1 F8A2 H2AFB3

Table E.5. CGH analysis results of patient 274HA999

Patient No	Chromosome	Start	Stop	Score	CNV	Gene
274HA999	chr1	196739999	196779999	-0.44299	CNV O686	CFHR1
	chr2	87379999	87979999	0.26883	CNV 30960	
	chr2	97819999	97939999	-0.27088	CNV 9958	
	chr5	69059999	69219999	0.27602	CNV 69133	
	chr5	69779999	69939999	0.50477	CNV 69129 CNV 37263	
	chr5	98779999	99179999	-0.48921	CNV 51765	
	chr6	29699999	29939999	0.25670	CNV 31268	
	chr6	32419999	32499999	-0.40695	CNV 69434	
	chr8	7139999	7899999	-0.26323	CNV 72045 CNV 37303 CNV 0348	FAM90A5, FAM66B,
	chr8	12219999	12259999	-0.25508	CNV 70303 CNV 37319	
	chr8	39259999	39379999	-0.83084	CNV 95380	
	chr9	42139999	42219999	-0.38358	CNV 4637	
	chr9	45459999	45539999	-0.28398	CNV 4631	
	chr9	67059999	67219999	-0.25982	CNV 3745	
	chr15	20219999	20339999	0.31950	CNV 76814	
	chr16	32939999	32979999	0.28032	CNV 32120	
	chr17	34419999	34619999	-0.31773	CNV 8840	TBC1 CCL3L3 CCL4L1
	chr17	44139999	44179999	-0.28036	CNV 2225 CNV 37194	KIAA1267
	chr1	196739999	196779999	-0.44299	CNV O686	CFHR1

Table E.6. CGH analysis results of patient 283HA1008

Patient No	Chromosome	Start	Stop	Score	CNV	Gene
283HA1008	chr1	16819999	17299999	0.39648	CNV 74362	CROCCL2
	chr1	200939999	200979999	0.26469	CNV 6805	KIF21B
	chr2	96699999	96779999	0.33165	CNV 74170	
	chr2	111019999	111099999	0.40601	CNV 30991	
	chr3	299999	379999	0.34170	CNV 3412	CHL1
	chr3	52819999	52859999	0.32015	CNV 4339	ITIH1
	chr5	69779999	70059999	0.46366	CNV 69129 CNV 37263	SMA3
	chr5	180659999	180858122	0.27934	CNV 51657	GNB2L1
	chr6	29579999	30059999	-0.26096	CNV 64460	HLA-G
	chr6	31259999	31299999	-0.25554	CNV 5387	HLA-B
	chr6	41659999	41699999	0.26043	CNV 3609	TFEB
	chr6	168459999	168579999	0.26866	CNV 2667	FERMD1
	chr8	7259999	7779999	-0.31131	CNV 8593 CNV 37303 CNV 0348	
	chr8	11979999	12019999	-0.43162	CNV 70286 CNV 37319	
	chr8	39259999	39379999	-0.28863	CNV 95380	
	chr9	43659999	43899999	-0.26240	CNV 82989	
	chr10	72939999	73099999	0.25671	CNV 22672	
	chr10	88419999	88459999	0.30130	CNV 53451	
	chr10	121179999	121219999	0.26651	CNV 101058	GRK5
	chr15	77859999	78219999	0.29092	CNV 72294	
	chr16	4379999	4419999	0.40997	CNV 72438	VASN
	chr16	16419999	16499999	0.42172	CNV 72486	
	chr16	32939999	32979999	0.26187	CNV 32120	
	chr16	67219999	67259999	0.30353	CNV 77385	E2F4 ELMO3
	chr17	18019999	18419999	0.25448	CNV 30808	SHMT1 LGALS9C
	chr17	26819999	26859999	0.31725	NO CNV	FOXN1
	chr17	48139999	48259999	0.28452	CNV 7756	PDK2
	chr17	77099999	77139999	0.37713	CNV 5034	C1QTNF1 HRNBP3
	chr19	19619999	19659999	0.31362	NO CNV	NUDFA13 CILP2
	chr19	33899999	33939999	0.28999	CNV 4076	PEPD
	chr20	62939999	62962064	-0.35152	CNV 73588	
	chr21	9419999	9579999	-0.27858	NO CNV	
	chr21	46339999	46419999	0.30713	CNV 03111	C21

Table E.7. CGH analysis results of individual 12AE37

Patient No	Chromosome	Start	Stop	Score	CNV	Gene
12AE37	chr3	162539999	162619999	0.31832	CNV 4360 AND 59901	
	chr5	17539999	17619999	-0.38822	CNV 80842	
	chr5	68899999	69739999	-0.25203	CNV31238	
	chr9	38899999	38979999	0.25841	CNV 31470	
	chr9	65779999	65819999	0.46604	CNV96072	
	chr9	69379999	69459999	0.29269	CNV 96094	
	chr10	48099999	48139999	-0.34583	CNV65661	
	chr10	89019999	89139999	0.25021	CNV75328	
	chr17	36299999	36379999	0.39497	CNV 72903	
	chr17	44419999	44619999	-0.29557	CNV 0502 37194	TBC1D3
	chr22	20299999	20499999	0.27743	CNV 90891	ARL17B
	chr3	162539999	162619999	0.31832	CNV 4360-9901	RTNR4

Table E.8. CGH analysis results of individual 24AE79

Patient No	Chromosome	Start	Stop	Score	CNV	Gene
24AE79	chr5	109379999	109419999	-0.71829	NO CNV	
	chr10	135419999	135522186	-0.43672	CNV9171	
	chr15	20019999	22579999	0.29510	CNV 72093	
	chr17	18339999	18419999	-0.30723	CNV 30808	LGALS9C
	chr17	44419999	44619999	-0.27441	CNV 0502 37194	ARL17B
	chr22	18699999	18859999	0.42311	CNV 8900	GGT3P
	chr22	20379999	20459999	-0.32306	CNV 73722	RIMBP3
	chr22	20499999	20659999	0.32802	CNV 90891	
	chr22	21499999	21619999	0.32448	CNV 5170 79461	

Table E.9. CGH analysis results of individual 25AE82

Patient No	Chromosome	Start	Stop	Score	CNV	Gene
25AE82	chr15	20579999	20659999	-0.36794	CNV 72243	BCL8
	chr15	20859999	20979999	-0.36430	CNV 4867	BCL8 OR4M2
	chr15	21099999	22579999	-0.25354	CNV 76814	
	chr15	102339999	102379999	0.31878	CNV 8809	
	chr22	18699999	18819999	0.33386	CNV 8900	GGT3P
	chr22	20339999	20619999	0.29799	CNV 90891	DGCR6L RIMBP3
	chr22	24339999	24379999	0.30494	CNV 32452	GSTT1

Table E.10. CGH analysis results of individual 28AE93

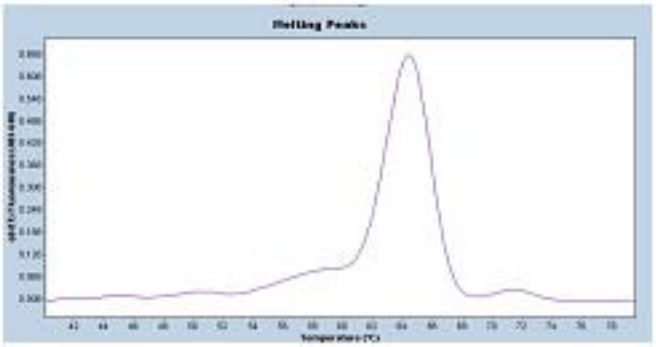
Patient No	Chromosome	Start	Stop	Score	CNV	Gene
28AE93	chr8	7659999	7859999	-0.29404	CNV 70274	
	chr8	39259999	39379999	0.47705	CNV 95380	
	chr15	22659999	22699999	-0.28736	CNV 31891	
	chr16	6899999	7019999	-0.55594	CNV 34611	A2BP1
	chr17	18339999	18379999	0.37833	CNV 30808	
	chr17	20379999	20419999	0.26622	CNV 30809	CK16
	chr22	18739999	18859999	0.25345	CNV 34485	PRODH1 DGCR6L
	chr22	20499999	20699999	0.28112	CNV 90891	
	chr22	25699999	25899999	0.29216	CNV 67861	

Table E.11. CGH analysis results of patient 31AE99

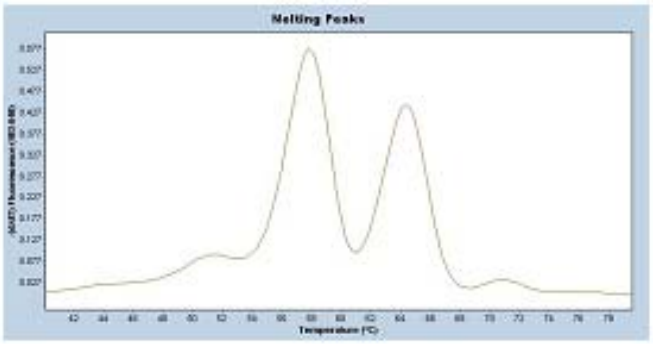
Patient No	Chromosome	Start	Stop	Score	CNV	Gene
31AE99	chr8	7859999	7899999	-0.26129	CNV 70262	
	chr8	39259999	39379999	0.52491	CNV 95380	
	chr9	38899999	38979999	0.31946	CNV 31470	
	chr9	40019999	40139999	0.35705	CNV82970	
	chr9	65779999	65819999	0.54625	CNV96072	
	chr12	9659999	9699999	0.67232	CNV3874	
	chr12	131739999	131779999	-0.60489	NO CNV	
	chr15	20579999	20619999	0.51518	CNV 76804	
	chr15	20859999	20899999	0.42363	CNV 4867	
	chr15	21059999	21179999	0.40245	CNV 76814	
	chr15	22179999	22579999	0.43366	CNV 72815	
	chr15	22659999	22699999	-0.29280	CNV 31891	
	chr15	23459999	23499999	0.25454	CNV66728	
	chr15	34699999	34859999	-0.30317	CNV 87617	GOLGA8A
	chr22	18699999	18739999	0.44607	CNV 8900	GGT3P
	chr22	20339999	20619999	0.25088	CNV 90891	DGCR6L RIMBP3
	chr22	25739999	25979999	0.27775	CNV 29717	ADRBK2

APPENDIX F: PROBE OPTIMIZATIONS

a) Homozygous wild type



b) Heterozygous



c) Homozygous mutant

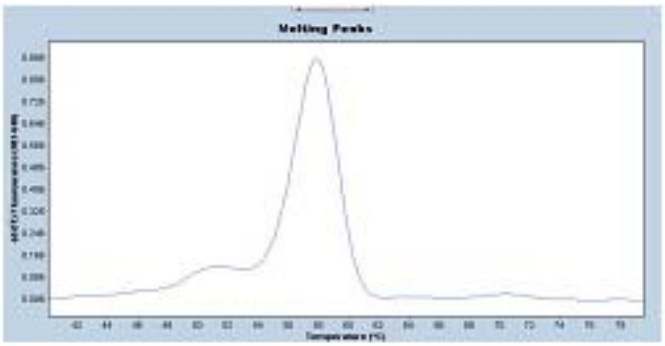
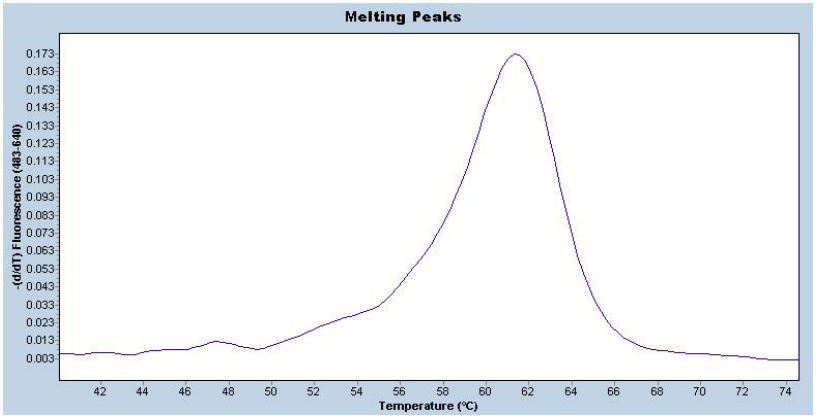
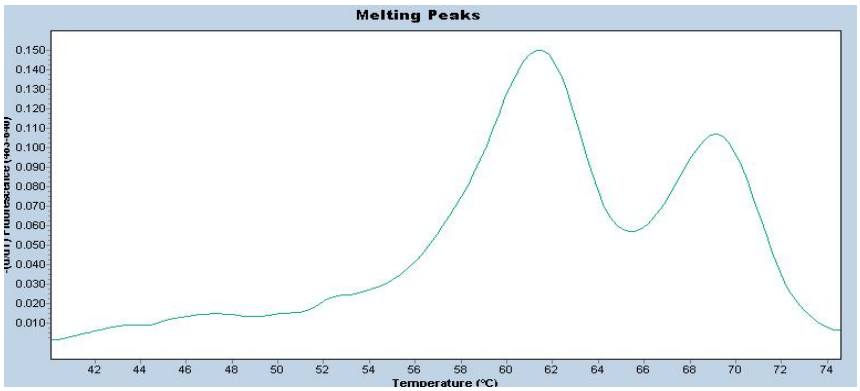


Figure F.1. Melting peaks observed for rs2069705 a) Homozygous wild type genotype (64^0C), b) heterozygous genotype (64^0C and 58^0C) and c) homozygous mutant genotype (58^0C).

a) Homozygous wild type



b) Heterozygous



c) Homozygous mutant

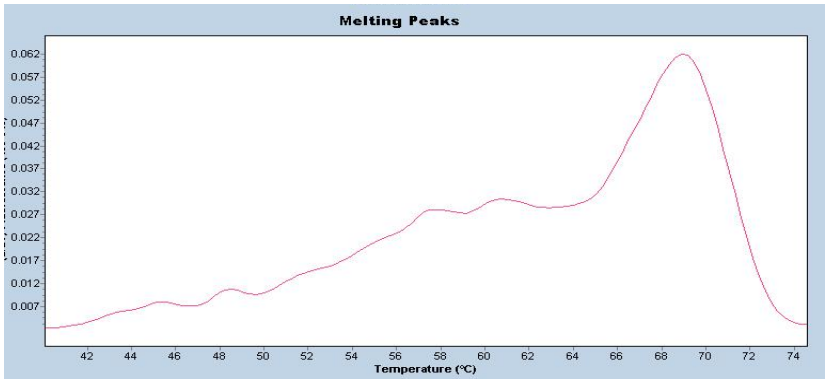
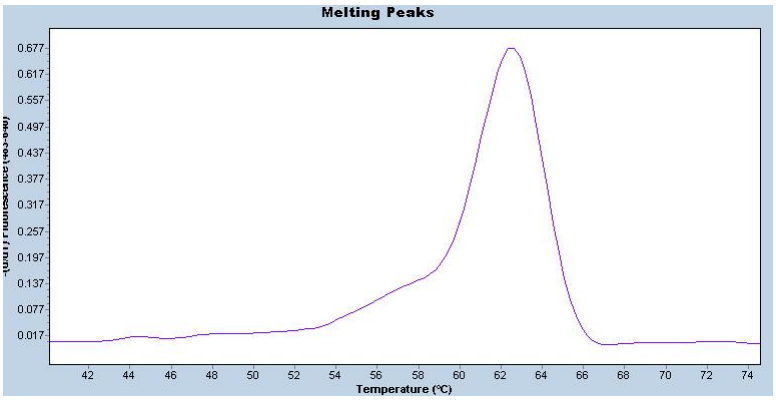
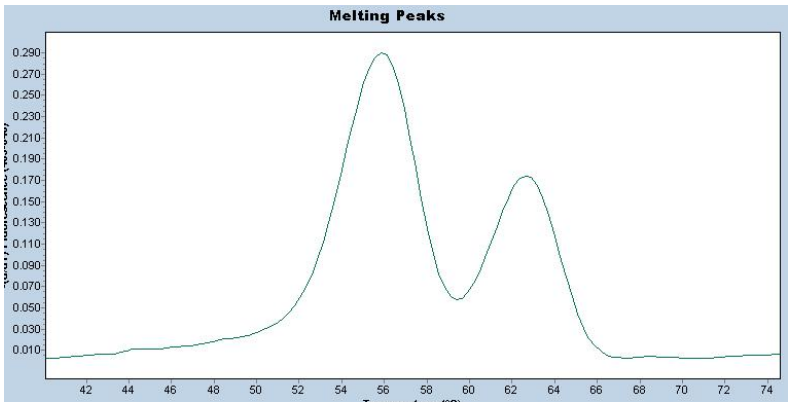


Figure F.2. Melting peaks observed for rs2241715 a) Homozygous wild type genotype (62^0C), b) heterozygous genotype (62^0C and 70^0C) and c) homozygous mutant genotype (70^0C).

a) Homozygous wild type



b) Heterozygous



c) Homozygous mutant

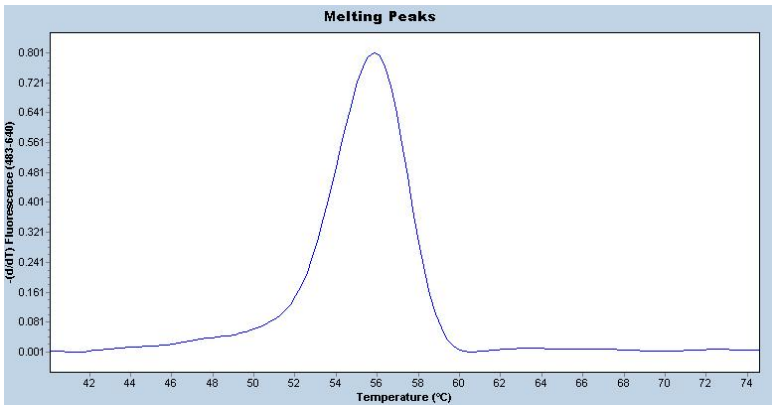
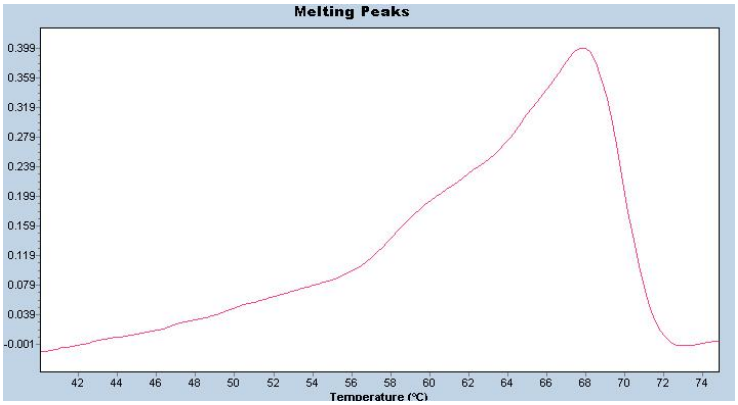
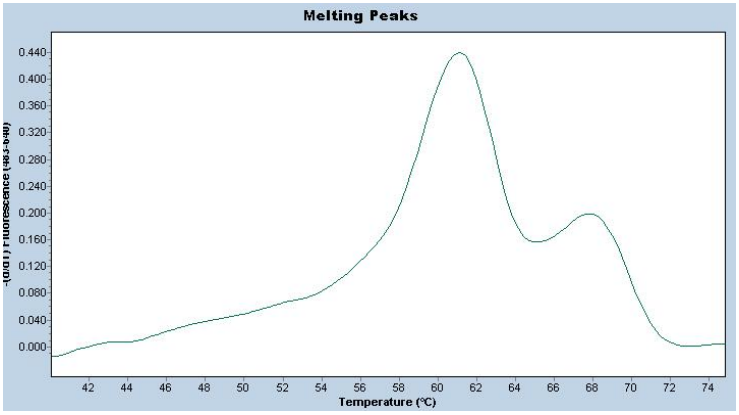


Figure F.3. Melting peaks observed for rs3024496 a) Homozygous wild type genotype (62^0C), b) heterozygous genotype (62^0C and 56^0C) and c) homozygous mutant genotype (52^0C).

a) Homozygous wild type



b) Heterozygous



c) Homozygous mutant

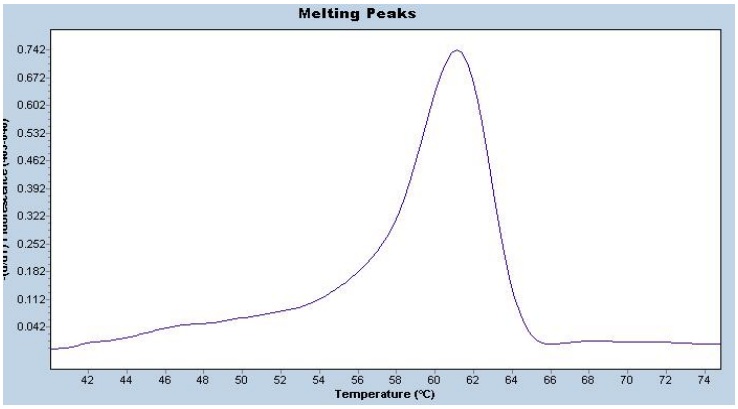
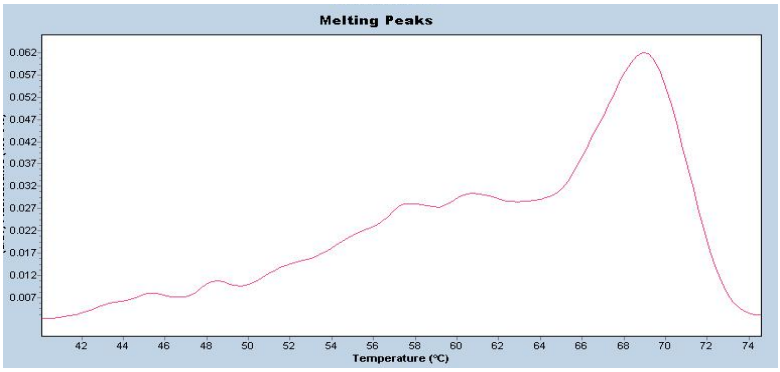
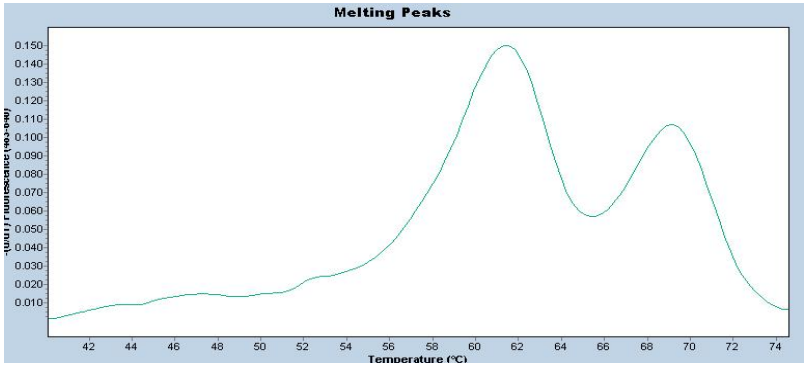


Figure F.4. Melting peaks observed for rs1800871 a) Homozygous wild type genotype (66^0C), b) heterozygous genotype (66^0C and 60^0C) and c) homozygous mutant genotype (60^0C).

a) Homozygous wild type



b) Heterozygous



c) Homozygous mutant

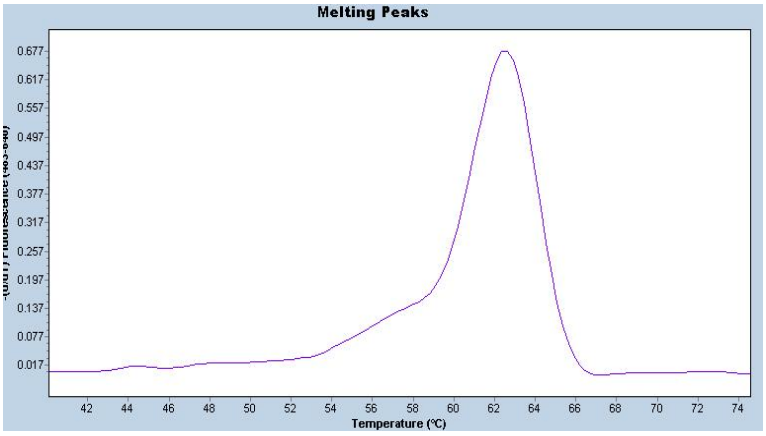
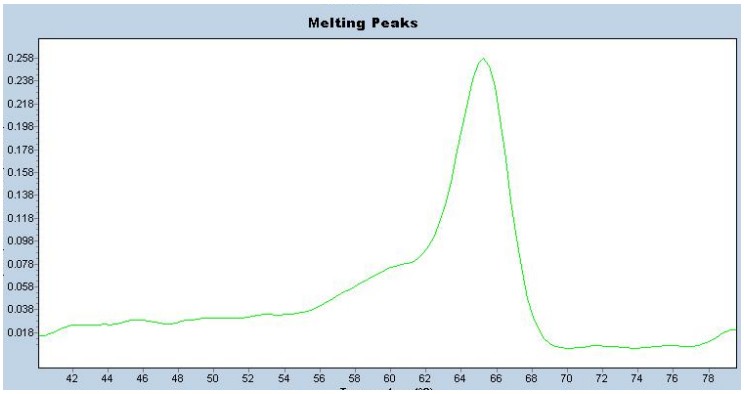
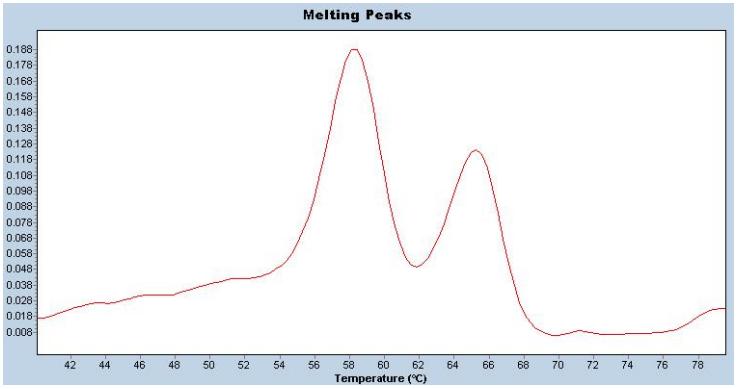


Figure F.5. Melting peaks observed for rs1554286 a) Homozygous wild type genotype (68^0C), b) heterozygous genotype (68^0C and 62^0C) and c) homozygous mutant genotype (62^0C).

a) Homozygous wild type



b) Heterozygous



c) Homozygous mutant

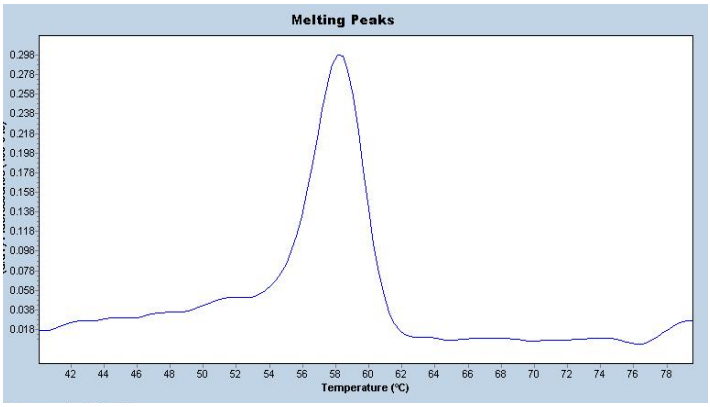
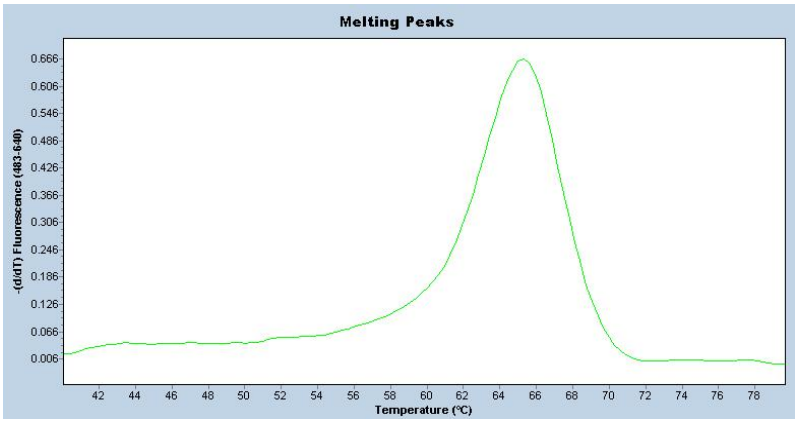
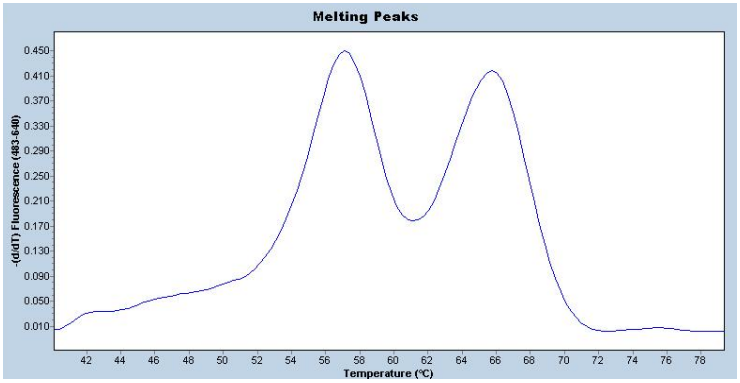


Figure F.6. Melting peaks observed for rs2069812 a) Homozygous wild type genotype T-T (66 ^{0}C), b) homozygous mutant genotype C-C (58 ^{0}C) and c) heterozygous genotype T-C (58 ^{0}C and 66 ^{0}C).

a) Homozygous wild type



b) Heterozygous



c) Homozygous mutant

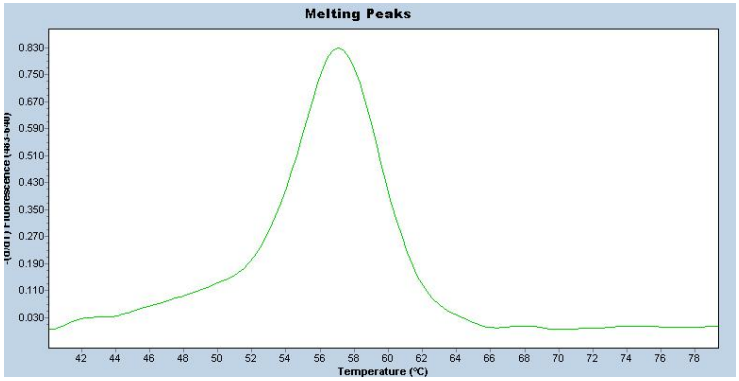
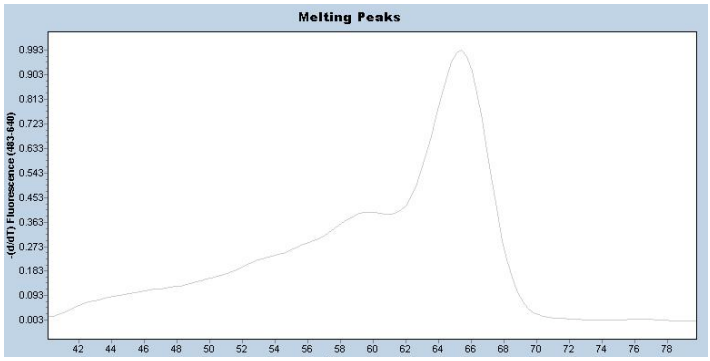
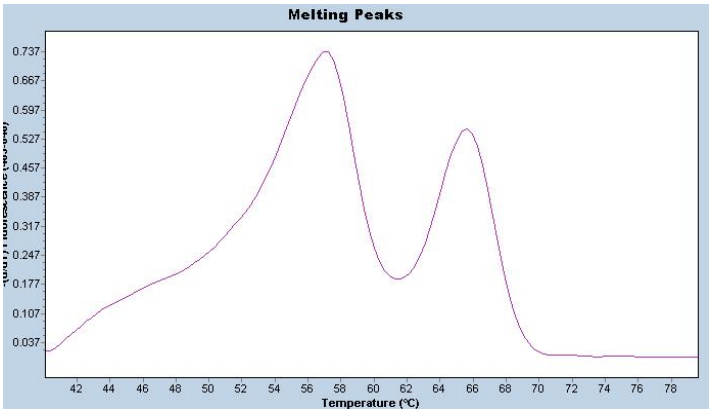


Figure F.7. Melting peaks observed for rs1861494 a) Homozygous wild type genotype (66^0C), b) heterozygous genotype (66^0C and 58^0C) and c) homozygous mutant genotype (58^0C).

a) Homozygous wild type



b) Heterozygous



c) Homozygous mutant

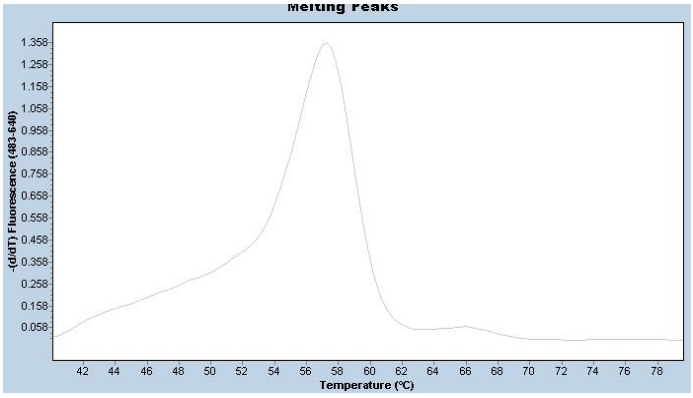
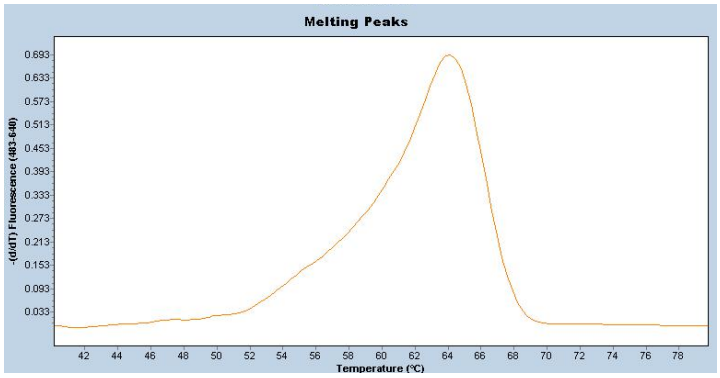
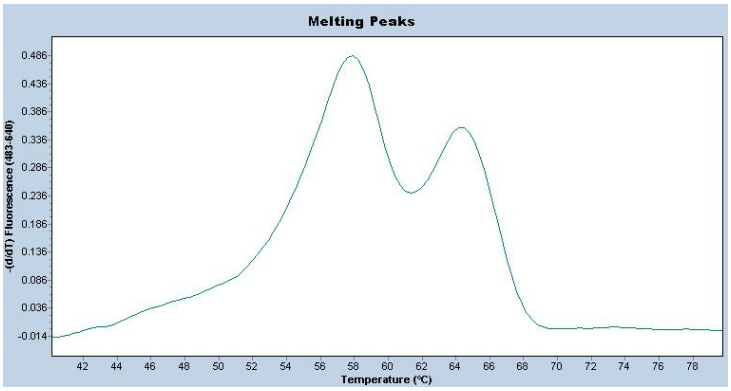


Figure F.8. Melting peaks observed for rs2243267 a) Homozygous wild type genotype (66^0C), b) heterozygous genotype (66^0C and 58^0C) and c) homozygous mutant genotype (52^0C).

a) Homozygous wild type



b) Heterozygous



c) Homozygous mutant

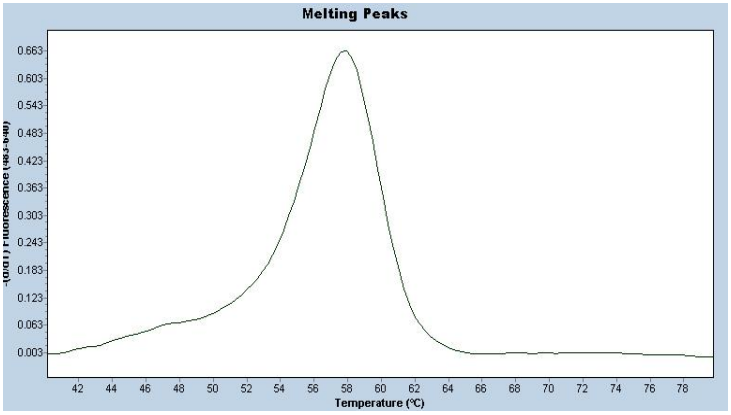
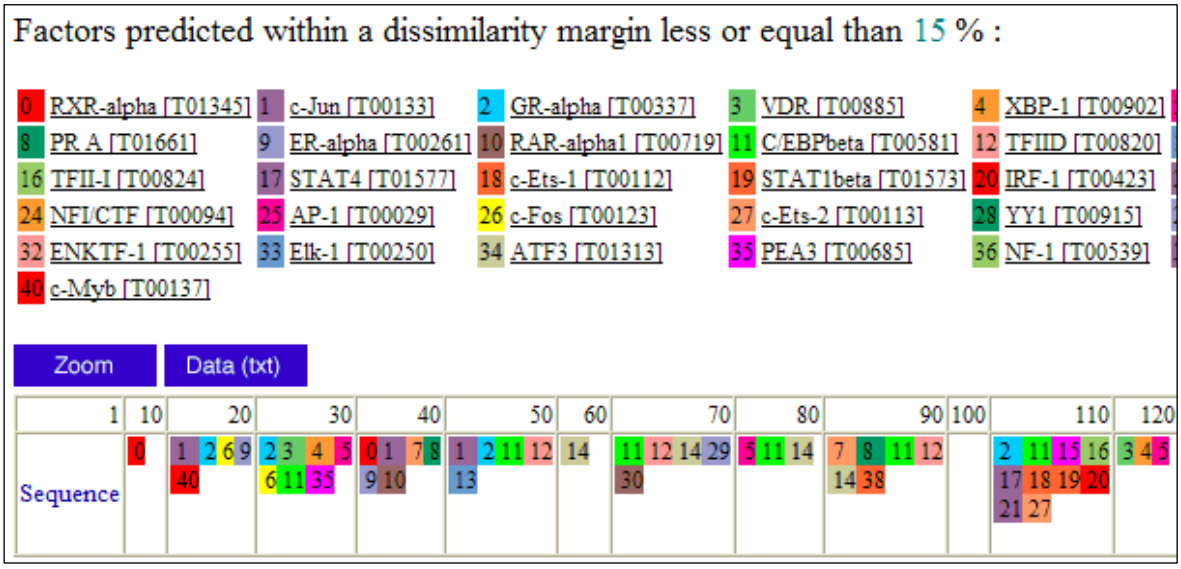
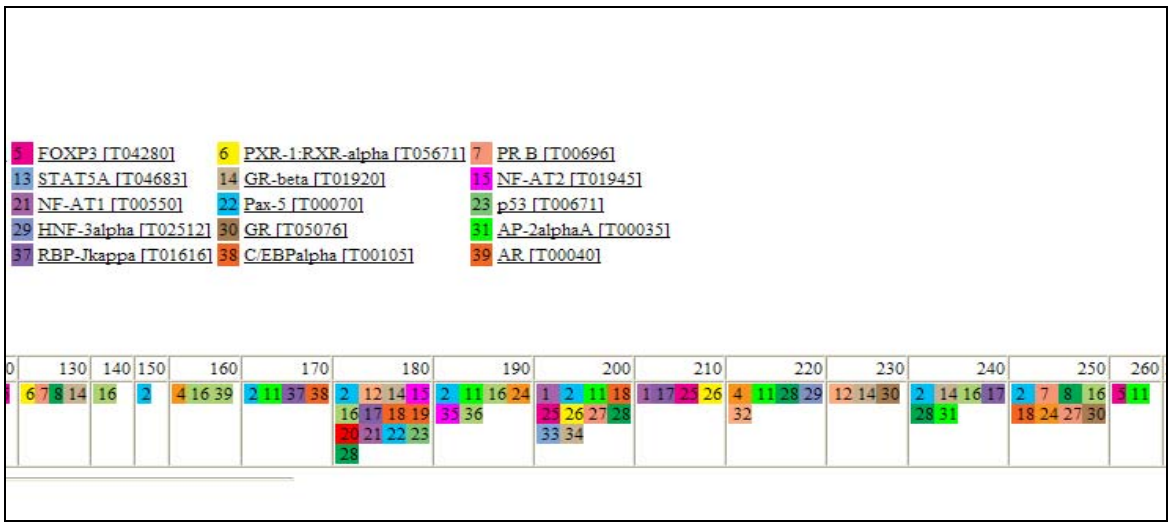


Figure F.9. Melting peaks observed for rs2243282 a) Homozygous wild type genotype (64^0C), b) heterozygous genotype (64^0C and 58^0C) and c) homozygous mutant genotype (58^0C).

APPENDIX G: RESULTS OF SEARCHING FOR TRANSCRIPTION BINDING SITES IN PROMOTER REGION OF IL5 GENE

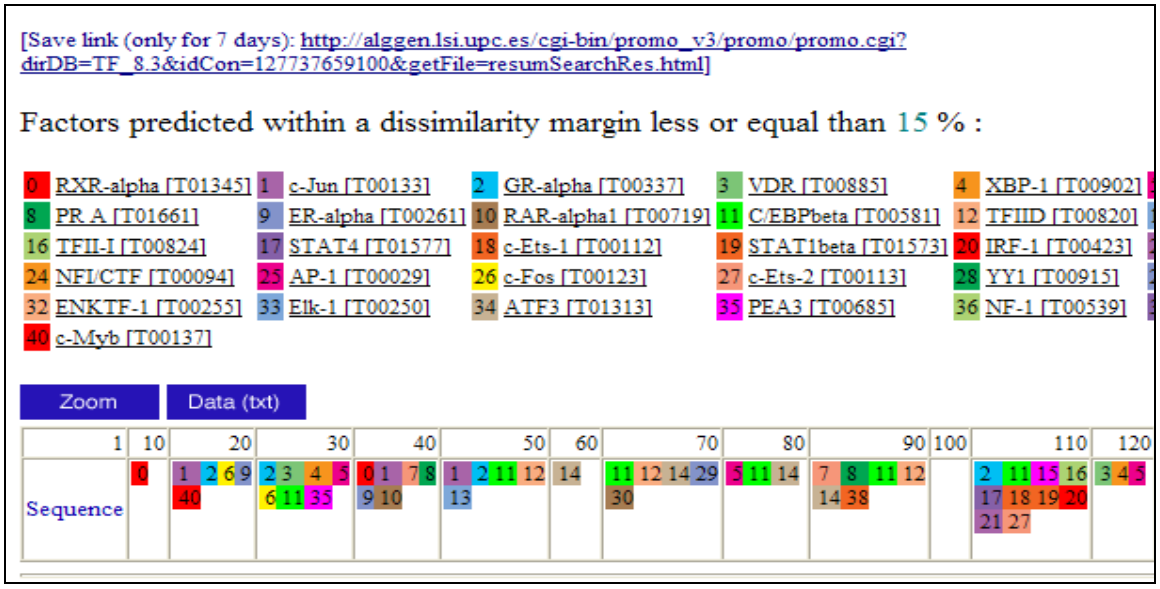


a)

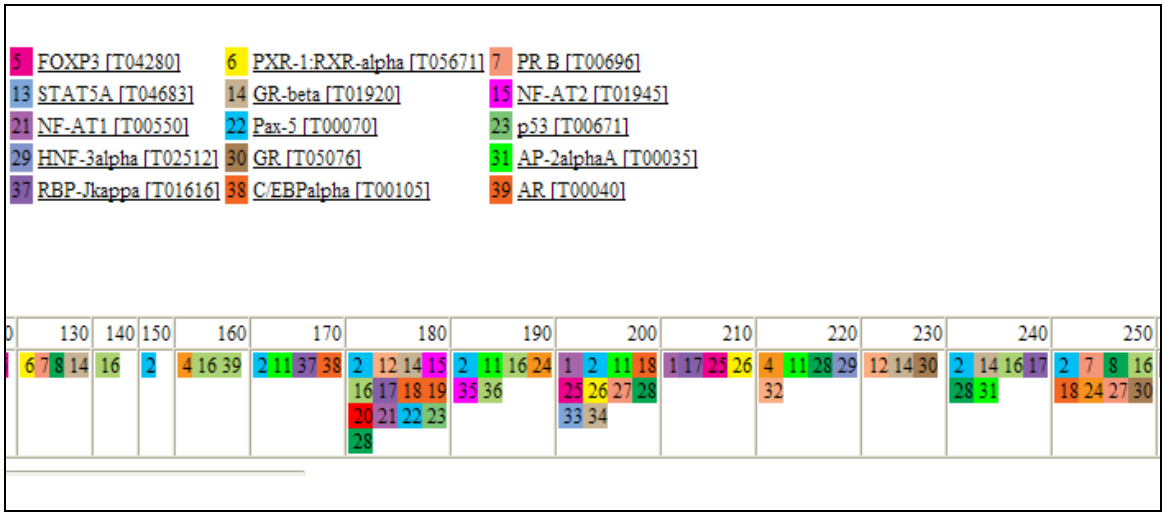


b)

Figure G.1. Results of the study for transcription factor binding sites of IL5 gene promoter region in 250 bp length a) the first part of 120 bp region of the promoter b) the rest part of 140 bp region of the promoter, C allele of rs2069812 is located in 136th bp



a)



b)

Figure G.2. Results of the study for transcription factor binding sites of IL5 gene promoter region in 250 bp length a) the first part of 120 bp region of the promoter b) the rest part of 140 bp region of the promoter, C allele of rs2069812 is located in 136th bp

APPENDIX H: PRODUCT OF THIS THESIS IN PROGRESS

A POLYMORPHISM IN THE IL5 GENE IS ASSOCIATED WITH INHIBITOR DEVELOPMENT IN SEVERE HEMOPHILIA A PATIENTS

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