MUTATIONAL ANALYSIS OF FACTOR XI AND VON WILLEBRAND FACTOR GENES BY HIGH RESOLUTION MELTING ANALYSIS AND DIRECT DNA SEQUENCING

Sunay Usluer

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by

Sunay Usluer

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APPROVED BY:

Prof. S. Hande Çağlayan……...
(Thesis Supervisor)

Prof. A. Nazlı Başak……..

Assis. Prof. Ergül Berber……...

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To my lovely son; İlhan

and

To my mother
with my gratitude for her endless support

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ABSTRACT

MUTATIONAL ANALYSIS OF FACTOR XI AND VON WILLEBRAND FACTOR GENES BY HIGH RESOLUTION MELTING ANALYSIS AND DIRECT DNA SEQUENCING

Continuous flow of blood is essential for life and its maintenance is an important task for the body. Hemostasis is the balance between the coagulants, factors that promote blood clot formation and anti-coagulants, factors that restricts the action of coagulants. As a result of their actions, abnormal blood loss after trauma is prevented as well as excessive clot formation in the vessels. Von Willebrand Factor (VWF) and Factor XI (FXI) are among several proteins maintaining this balance. The deficiency of vWF results in the most common congenital bleedings disorder; Von Willebrand Disease (vWD), though the estimation of prevalence is complicated due to the incomplete penetrance and extreme variability in clinical symptoms of mild Von Willebrand Disease (VWD). Disease manifests itself with mucosal and trauma related bleeding, and caused by the mutations in VWF gene.

Mutations in *Factor 11* (*F11*) gene results in Factor XI deficiency (Hemophilia C), symptoms of which are trauma or surgery related bleeding. The deficiency is common among Ashkenazi Jewish, but rare in other populations. Its diagnosis is also complicated due to incomplete penetrance and variable expressivity.

The identification of the underlying molecular pathology is important for the management of both diseases. 23 kb long F*actor 11* gene comprises 15 exons and molecular genetic analysis is feasible by direct DNA sequencing. Through such analysis, exons and the promoter region of the F*11* in two FXI deficiency patients and their relatives revealed one recurrent (c. 1556 G>A) and one novel (c. 151 A>C) mutation. The first mutation is in the catalytic domain and created a premature stop codon, the second is in the "Apple 1" domain, probably affecting the secretory pathway of the protein.

Molecular analysis of the VWF gene is complicated because of the length of the gene which comprises 52 exons and encodes 8,2 kb mRNA. Additionally, the gene, especially the longest exon, Exon 28, is highly polymorphic. Due to these facts, analysis of the gene by direct DNA sequencing is not manageable. Therefore it is important to employ a mutation detection system to quickly, efficiently and inexpensively identify all sequence variations in the VWF gene.

High Resolution Melting Analysis (HRMA) is a recently developed mutation screening system that detects nucleotide changes through differences in melting plots of amplicons. A light cycler platform combines PCR amplification with HRMA in a closed tube system and assays 96 samples in approximately 90 minutes. It also allows simultaneous analysis of more than one amplicon in one run so the whole gene analysis can potentially be screened in a few runs with appropriately optimized conditions for a single patient. The sequence alteration detected by HRMA is then identified by DNA sequencing.

For the molecular analysis of VWF gene in Turkish VWD patients, 38 amplicons of VWF gene out of 60 were optimized for HRMA. These 38 amplicons were grouped into seven amplification groups that have different thermal conditions. Among these, 24 amplicons were sequenced to confirm proper amplification of targeted regions. Patient screening was initiated in 10 VWD patients and an apparently healthy control for 12 amplicons. Although further investigations are required, the preliminary screening of VWD patients with HRMA is appropriate and promising.

ÖZET

FAKTÖR XI VE VON WILLEBRAND FAKTÖR GENLERİNİN YÜKSEK ÇÖZÜNÜRLÜKLÜ ERİME ANALİZİ VE DOĞRUDAN DNA DİZİLEMESİ İLE MUTASYON ANALİZİ

Düzenli kan akışı yaşam için esastır ve bunun sağlanması insan vücudu için büyük önem taşır. Hemostaz, koagulan- pıhtı oluşumunu destekleyen- ve antikoagulan- koagulanların etkisini sınırlayan- faktörler arasındaki dengedir. Bu denge ile, yaralanma sonrasında aşırı kan kaybının yanısıra, damarlarda fazla pıhtı oluşumu da engellenir. Von willebrand Faktör (VWF) ve Faktör XI (FXI) bu dengeyi sağlayan bir çok protein arasındadır. VWF eksikliği, en yaygın kalıtsal kanamalı hastalığa yol açar, fakat tam olmayan penetrans ve hafif Von Willebrand Hastalığının klinik belirtilerindeki aşırı değişkenlik kesin tahmini zorlaştırmaktadır. Hastalık kendini mukozal ve travmaya bağlı kanama ile gösterir ve VWF geni üzerindeki mutasyonlar nedeniyle ortaya çıkar.

Faktör 11 üzerindeki mutasyonlar, belirtileri, operasyon ve yaralanmaya bağlı kanamalar olan, FXI eksikliğine (Hemofili C) yol açar. Diğer topluluklar içinde nadir görülse de, hastalık Eşkenazi (Ashkenazi) yahudileri arasında yaygındır. Eksik penetrans ve değişken ekspressivite hastalığın kalıtımını karmaşıklaştırmaktadır.

Altta yatan moleküler patolojinin tanımlanması, her iki hastalığın da yönetimi için önemlidir. *Faktör 11* geni 15 exon içerir ve doğrudan DNA dizilemesi ile analiz etmek için uygundur. Bu nedenle, 15 exon ve bir promoter bölgesi için PZR çoğaltılması koşulları optimize edildi ve iki FXI eksikliği hastasında ve yakınlarında, *F11* geninde bir moleküler patolojinin olup olmadığı tarandı. Sonucunda, daha önceden rapor edilmiş (c. 1556 G>A, W 519 Stop) mutasyonu, ve henüz tanımlanmamış (c. 151 A>C,) mutasyon tyespit edildi. W 519 Stop mutasyonu katalitik alanda olup erken bir sonlandırma kodonu oluşturur. T 33 P mutasyon ise “Apple 1” bölgesinde olup, muhtemelen proteinin salınım yolunda bir bozukluğa yol açmaktadır.

VWF geninin moleküler analizi, 52 exondan oluşan ve 8.2 kb'lık bir mRNA kodlalyan genin uzunluğu nedeniyle karmaşıktır. İlaveten, gen, özellikle de en uzun ekzon olan Ekzon 28 oldukça polimorfiktir. Dolayısıyla, gen, doğrudan DNA dizilemesi ile analiz etmek için uygun değildir. Bu nedenle, VWF genindeki tüm dizi değişimlerini, daha hızlı, daha etkili ve ekonomik olarak tanımlayacak bir mutasyon belirleme yöntemi kullanmak önemlidir.

Yüksek Çözünürlüklü Erime Analizi (HRMA), mutasyonları amplikonların erime grafiklerindeki değişiklere göre belirleyen, yeni bir mutasyon tespit sistemidir. Bir light cycler platformu, PZR çoğaltılması ve HRM Analizini bir kapalı tüp sistemde birleştirir ve 96 örneği yaklaşık 90 dakikalık bir zaman diliminde, jele daylı metodlardan daha düşük bir maliyetle analiz eder. Ayrıca, birden fazla amplikonun aynı anda analiz edilmesine izin verir ve böylelikle, uygun şekilde optimize edilen koşullarda, potansiyel olarak bütün bir gen birkaç çalışmada analiz edilebilir. HRMA ile tespit edilen dizi değişiklikleri DNA dizilemesi ile doğrulanır.

Türk VWD hastalarında VWF geninin moleküler analizi için, VWF geninin 60 amplikonunun 38'i HRMA için optimize edildi. Sonucunda, bu 38 amplikon, farklı termal koşulları olan 7 çoğaltılma grubu halinde gruplandı. Bu amplikonlar arasından 24 tanesi dizilendi ve hedeflenen bölgenin çoğaltıldığı doğrulandı. Hasta taramaları 12 amplikon için, 10 hasta ve bir sağlıklı kontrol ile başlatıldı. Elde edilen ön bulgular, VWD hastalarında HRMA ile yapılan öncül taramaların sonuçları uygun ve umut vericidir.

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LIST OF SYMBOLS/ABBREVIATIONS

A	Adenine
C	Cytosine
G	Guanine
K'EDTA	Potassium Ethylene Diamine Tetraacetic Acid
KCl	Potassium Chloride
$KHCO_3$	Potassium Carbonate
$MgCl_2$	Magnesium Chloride
Na_2EDTA	Disodium Ethylene Diamine Tetraacetic Acid
NaCl	Sodium Chloride
NH_4Cl	Ammonium Chloride
ºC	Celsius Degrees
P	Proline
R	Arginine
T	Threonine
T	Thymine
W	Tryptophan
X	Stop codon

µg	Microgram
µl	Microliter
3'-UTR	3' Untranslated Region
A.D.	Autosomal Dominant
A.R.	Autosomal Recessive
ADAMTS 13	A Disintegrin And Metalloproteinase With A Thrombospondin Type 1 Motif, member 13)—also known as von Willebrand factor-cleaving protease
Ala	Alanine
Amp	Amplicon
Ap1	Apple 1 Domain

Ap2	Apple 2 Domain
Ap3	Apple 3 Domain
Ap4	Apple 4 Domain
APC	Activated Protein C
aPTT	Activated Partial Thromboplastin Time
Arg	Arginine
Asp	Aspargine
bp	Base Pairs
BPB	Bromophenol Blue
BT	Bleeding Time
Cp	Crossing Point
CRM	Cross Reacting Material
CT	Closure Time
Cys	Csyteine
DDAVP	1-desamino-8-$_D$-arginine vasopressin
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotides
dTTP	Deoxythyminetriphosphate
dUTP	Deoxyuraciltriphosphate
EtBr	Ethidium Bromide Bromophenol Blue
EtOH	Ethyl Alcohol
Ex.	Exon
F8	Factor VIII gene
F11	Factor XI gene
F9	Factor 9 gene
FFP	Fresh Frozen Plasma
FIX	Factor IX Protein
FV	Factor V Protein
FVa	Active FV Protein
FVIIa	Activated Factor VII Protein
FVIII	Factor VIII Protein
FVIIIa	Active Factor VIII Protein

FXI	Factor XI Protein
FXI:C	Factor XI Concentration
FXI:Ag	Factor XI Antigen
FXIa	Active Factor XI Protein
FXII	Factor XII Protein
Glu	Glutamine
GPIbα	Glycoprotein Ib α
HB	Hemophilia B
HC	Hemophilia C
His	Histidine
HK	High Molecular Weight Kinninogen
Hm	Homozygous
HMW	High Molecular Weight
HMWM	High Molecular Weight Multimers
HRMA	High Resolution Melting Analysis
Ht	Heterozygous
ID	Identity
Ile	Isoleucine
kb	Kilobases
kD	Kilo Dalton
LC	Light Cycler
Leu	Leucine
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
mRNA	Messenger Ribonucleic Acid
ng	Nanogram
Nm	Nanometer
OMIM	Online Mendelian Inheritance in Man
PCR	Polymerase Chain Reaction
Phe	Phenylalanine
PK	Plasma Kinninogen

PMP	Platelet Derived Micro Particles
pol	Polymorphisism
Prom	Promoter
PZR	Polimeraz Zincir Reaksiyonu
R.E.R.	Rough Endoplasmic Reticulum
RE	Restriction Endonuclease
RIPA	Ristocetin Induced Platelet Aggregation
Rpm	Revolutions per Minute
S^{-1}	Per second
SDS	Sodium Dodecyl Sulphate
sec	Second
Ser	Serine
Ser	Serine
SNPs	Single Nucleotide polymorphisms
SP	Serine Protease
Taq	Thermus Aquaticus
TBE	Tris-Boric Acid-EDTA
TE Buffer	Tris-EDTA Buffer
Ter.	Terminating
TF	Tissue Factor
TGN	Trans Golgi Network
Tm	Melting Temperature
TM	Thrombomodulin
Tris-HCL	TRIS-Hydrochloride
U	Unit
UdL^{-1}	Unit Per Deciliter
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
Val	Valine
vWAgII	Von Willebrand Antigen II
VWD	Von Willebrand Disease
VWF	Von Willebrand Factor

VWFAg	Von Willebrand Factor Antigen
VWFRCo / VWF:RiCof	Von Willebrand Factor Ristocetin Activity
WPB	Weibel-Pallade Bodies
wt	Wild Type

1. INTRODUCTION

1.1. Von Willebrand Factor and Von Willebrand Disease

1.1.1. Upon vascular damage

Hemostasis can be realized as a balance between the coagulants and anticoagulants. In the result excess blood loss upon injury is prevented, as well as agglutination of blood in undamaged vessels. Hemostasis is stimulated when subendothelial proteins are exposed to flowing blood. The primary action is taken by platelets. They adhere to subendothelial von Willebrand factor protein and collagen. This binding tethers platelets to the site of injury and activates them. Activated platelets recruit other platelets in action and a platelet plug is formed. Meanwhile, signals sent by platelets cause vessel constriction and excessive blood loss is prevented [Horne, 2005, Ruggeri, 2003]. However, platelet plug is not stable enough to provide proper healing. Concomitant to primary hemostasis, a subendothelium protein Tissue Factor (TF) is exposed to blood and starts a series of proteolytic reactions in order to activate prothrombin in the thrombin form. In turn, thrombin converts soluble fibrinogen into insoluble fibrin and produces a fibrin clot on the wound. These reactions contain a number of zymogens which are inactive in the circulating blood; cofactors that couple with enzymes and inhibitors that restrict the coagulation in the site of injury or prevent excessive plug formation [Horne, 2005, Dahlback, 2000, Provan and Gribben, 2005]. This is called the *extrinsic pathway of coagulation*. On the other hand, coagulation can be triggered by the contact phase proteins (FXII, FXI, prekallikrein and high molecular weight kininogen) and this pathway is called *the intrinsic pathway of coagulation.* (Provan and Gribben, 2005) The reactions of the pathway are shown in Figure 1.1

Beside the coagulation cascade, a series of anticoagulation reactions take place in order to localize coagulation in the site of injury and to prevent excessive plug formation. The initial step is activation of thrombomodulin (TM). Thrombin bound to TM converts Protein C into Activated Protein C (APC). Consequently, APC attacks to FVIIIa and FVa and inactivates them. Protein S and FV function as cofactors of APC (Provan and

Gribben, 2005).

FXI is a serine protease in this cascade, which is activated by FXII, High Molecular Weight Kininogen (HK) and thrombin. In turn it activates Factor IX, by cleaving it in the presence of calcium ions. [Selignsohn, 2007] (Figure 1.1)

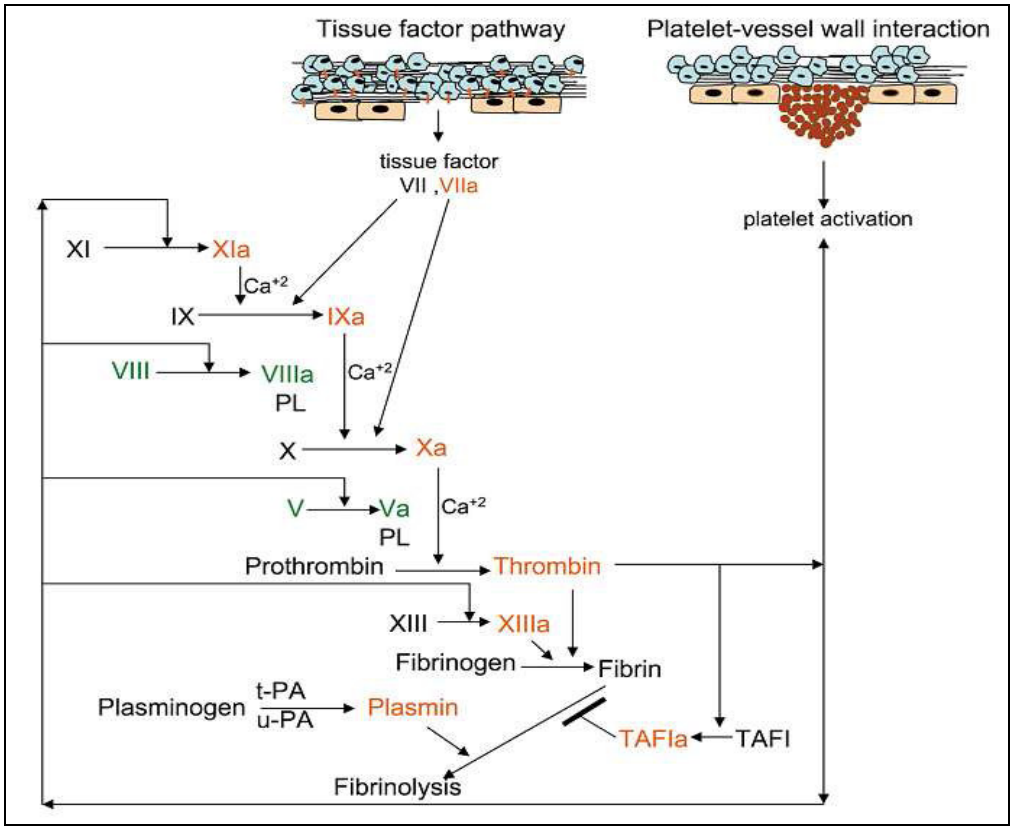

Figure 1.1. The coagulation cascade (Provan and Gribben, 2005)

1.1.2. The von Willebrand Gene and Protein

The gene encoding von Willebrand Factor (VWF) spans a 178kb region on chromosome 12, has 52 exons and transcribes an mRNA of 8,2 kb. The signal peptide and propeptide region of the protein is encoded by 17 exons about 80 bp in length, whereas, the mature peptide and the 3' UTR is product of 35 exons which are about 100 bp in length (Mancuso, 1989). The VWF is comprised of repeating domains that are arranged in the following order, D1-D2-D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK. These repeating units are thought to have arisen from gene duplications and some of them have specific functions as shown in the figure (Provan and Gribben, 2005).

The exons vary in length from 40 bp (exon 50) to 1347 bp (exon 28) and have an average of 146 bp, if the exceptionally long exon 28 is excluded. Fifty one introns have lengths from 97 bp (intron 29) to 19,9 kb (intron 6), with the average of 3,3 kb(Mancuso, 1989).

There are a number of repetitive sequences within the gene, encompassing fourteen ALU repeats and direct repeats that vary from 8 bp to 25 bp in length. Intron 40 contains a simple repeat of TCTA about 670 bp in length and a similar but less restricted sequence repeat of 100 bp is seen in intron 42. Additionally, the first 100 bp of intron 50 contains a 26-28 bp repeat (Mancuso, 1989).

The molecular analysis of the gene is complicated by the presence of a pseudogene on chromosome 22, extending from exon 23 to exon 34 of VWF gene. The gene and the pseudo gene share 97 per cent homology with each other, suggesting a recent duplication event. Gene conversions with the pseudogene, in some cases, account for the vWD (Mancuso, 1989, Provan and Gribben, 2005, Keeney and Cumming, 2001).

von Willebrand Factor (VWF) is an adhesive glycoprotein and it is synthesized in endothelial cells and megakaryocytes, as a 2813 amino acids long preproprotein, which is approximately 350 kilodaltons (kD). The preproprotein contains 22-amino acid signal peptide and 741-amino acid propeptide. The VWF propeptide is identical to von Willebrand antigen II, which is a plasma protein with unknown function independent of VWF (Mancuso, 1989).

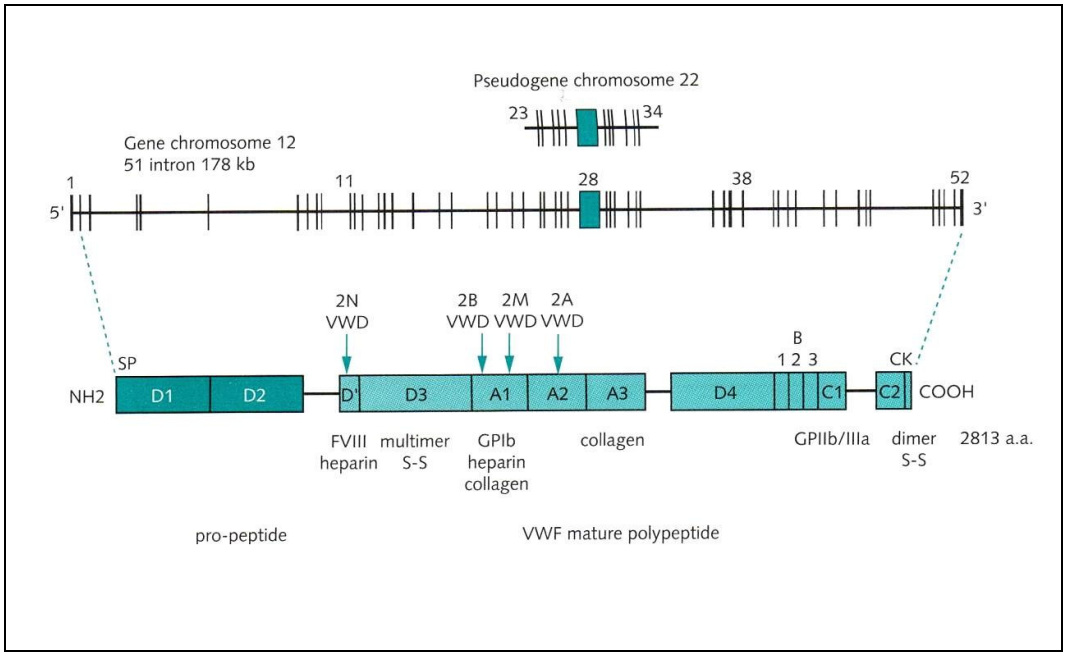

Figure 1.2. Domain structure of the VWF gene and pseudogene (Provan and Gribben, 2005)

Pre-pro-VWF undergoes a series of modulations to become a mature peptide (Figure 1.3). First of all the signal peptide is cleaved in the R.E.R. The pro-VWF chains dimerise at C-terminus, but this dimerisation is dependent on an initial glycosylation. Molecules dimerise upon the formation of disulphide bridges between cystein residues in the C-terminal domains. Afterwards, 12 N-linked and 10 O-linked oligosaccharide chains added to amino acid chain during glycosylation is processed in the Golgi. Oligosaccharide side chains make up 20 per cent of total mass and it is thought that they are crucial for VWF's structural and functional integrity. These side chains maintain the multimer integrity, protect the protein from proteolysis and modulate protein's interactions with platelets and collagen (Millar and Brown, 2006).

In the Trans Golgi network (TGN), dimers undergo N-terminal multimerisation, which starts with a non-covalent interaction between pro-VWF. In turn, disulphide bridges in the amino terminus of the mature protein stabilize the multimer at different sizes which ranges 0.5 to 20 Megadaltons. The multimers may appear as thin filaments up to 1300 nm long or as coiled molecules with a cross-section of 200–300 nm. 741 amino acid propolypeptide sequence is important for proper multimerisation of the molecules and targeting. However, it is cleaved during this process, though this cleavage is not required

for multimerisation (Keeney and Cumming, 2001, Millar and Brown, 2006, Ruggeri, 2007).

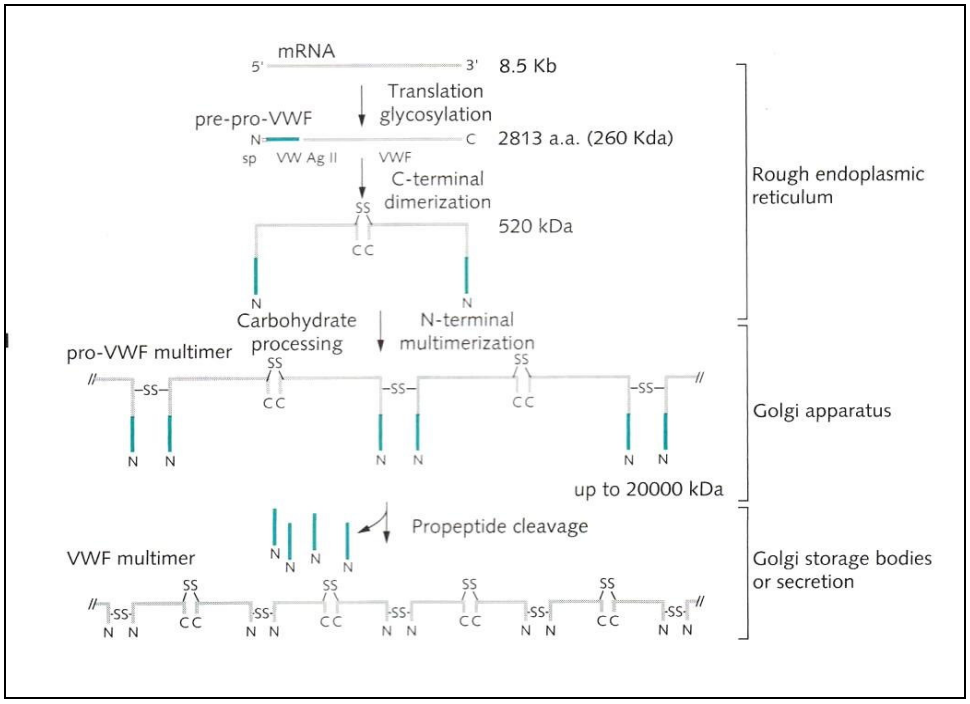

Figure 1.3. Type and location of Post-Transcriptional modifications of VWF (Provan and Gribben, 2005)

Majority of endothelial cell derived VWF is secreted into the circulation, which makes up 95 per cent of plasma VWF. The remainder is stored in the Weibel-Palade bodies of endothelial cells and secreted upon stimulation. Platelet VWF is stored in the alpha granules (Keeney and Cumming, 2001, Provan and Gribben, 2005, Ruggeri, 2007).

1.1.3. Function of von Willebrand Factor in Hemostasis

VWF functions in thrombus formation in two ways: directly by interacting with platelets and by adhering them to the site of vascular injury and in an indirect way as a carrier for coagulation factor VIII and by preventing its rapid clearance from plasma. However, its essential role is in the platelet adhesion (Ruggeri, 2007).

Integrity of blood vessels is continuously surveyed by platelets, although flow rate can be as high as 40,000 S^{-1} (in stenosed arteries). Due to this hemodynamic process

called axial migration, platelets flow near the endothelial walls of blood vessels. When vessel damage occurs, VWF directly binds to subendothelial structures and forms a bridge between flowing platelets and endothelium, through binding of A1 domain to Platelet's GPIbα receptor (Reininger *et al*).

This binding has two advantages. First, it immobilizes platelets and provides other receptors like glycoprotein VI or integrin to bind their ligands. The second advantage is observed in fast flowing blood. The flow rate is maximal in the center of a lumen and decreases towards the walls. This action divides the lumen into layers of flowing blood at different speeds. This velocity difference creates a shearing flow which is a sliding motion between layers, which consequently, imposes a torque on the platelets upon tethering by VWF. This torque makes platelets turn, and activates the signaling pathway of platelets and starts production of highly procoagulant Platelet Derived Micro Particles (PMP), which are thought to spread the coagulation (Reininger *et al,* 2006). Additionally, the shear stress makes immobilized VWF uncoiled and exposes domains to interact with their corresponding receptors (Reininger *et al,* 2006).

Secreted VWF undergoes proteolytic cleavage by ADAMTS 13, which cuts multimers at the peptide bond within the A2 domain through proteolytic cleavage. Thus, high molecular weight multimers (designated as ultra large VWF), which are more effective in coagulation, are not present in circulation. Shear stress promotes ADAMTS 13 activity. This fact has the crucial advantage of preventing coagulation without damage. On the other hand at the site of injury, VWF is secreted from Weibel-Palade bodies. This secreted multimer mixture contains required high molecular weight multimers (HMWM), which are directly recruited to the injury site where rapid platelet adhesion and aggregation is required (Ruggeri, 2007, Hobbs and Lopez, 2008).

1.1.4. The von Willebrand Disease

In 1926, a physician called von Willebrand discovered a bleeding condition in people of Aland Islands, between Sweden and Finland. It was named as "pseudohemophilia" because it was inherited in an autosomal dominant manner, distinct from classic hemophilia (OMIM 193400, Keeney and Cumming, 20018). However, the later discovery

that some people with this condition also had low FVIII activity caused uncertainty about the different roles of FVIII and VWF. After molecular cloning of VWF gene and VWF protein analysis, its role in primary hemostasis, interactions with platelets and FVIII was described in detail.

As mentioned above VWD is a congenital disorder and it is due to quantitative and qualitative defects in von Willebrand Factor, which is a heteromultimeric glycoprotein. It is the most common bleeding disorder with estimated prevalence between 3-4 per 100,000 and 1,3 per cent. The exact estimation is complicated due to the incomplete penetrance of the disease phenotype and extreme variability in clinical symptoms of mild VWD (Keeney and Cumming, 2001).

VWD patients generally suffer from mucosal and trauma related bleeding. Severely affected patients rarely experience hemartroses and soft tissue hematomas. People with these symptoms and a family history of bleeding are suspected to have VWD (Provan and Gribben, 2005). Beside common screening tests, several assays are applied to diagnose vWD and differentiate its subtypes (Table 1.1).

Table 1.1 Tests for the laboratory diagnosis of von Willebrand disease (adapted from Veyradier, A. *et al,* 1998)

Screening tests	**Discriminating tests**
Bleeding time (BT)	Ristocetin-induced platelet aggregation (RIPA)
Closure time (CT) PFA-100	Multimeric analysis
Platelet count	Platelet VWF
Activated partial Thromboplastin time	VWF binding to GPIb, collagen, F.VIII
Specific tests	DNA analysis
VWF antigen (VWFAg)	Antibodies to VWF
VWF Ristocetin cofactor activity (VWFRiCof)	vWAgII
F.VIII clotting activity	

Mutations at the VWF locus can affect VWF synthesis, its biosynthetic assembly, its stability and its interaction with specific proteins, each of which result in different disease phenotype. Consequently, there are several subtypes of the disease, which are grouped into

6 subgroups. These groups are classified as quantitative (type 1 and 3) and qualitative (type 2s) vWD (Provan and Gribben, 2005). Subtypes of the disease are summarized in Table 1.2.

In type 1, comprising 70 per cent of all cases, VWF level are significantly reduced. Accordingly, FVIII activity is reduced but multimer pattern and VWF activity is not altered. It is inherited in an autosomal dominant manner, with incomplete penetrance. Due to this fact, people with the same mutation, even members of the same family do not show similar bleeding tendency (Keeney and Cumming, 2001, Veyradier *et al,* 1998). It is difficult to diagnose type 1 vWD and discriminate patients from normal individuals with lower VWF level. Thus molecular genetic analysis is important in diagnosis and treatment. The VWF gene mutations that result in type 1 VWD are distributed throughout the gene (Veyradier *et al,* 1998 and Sadler *et al,*2005).

Type 3 is the most severe quantitative type, in which VWF level is dramatically low or it is totally absent. It is generally inherited in an autosomal recessive pattern, or it is due to compound heterozygotsity of type 1 mutations. Its prevalence is estimated to be 0,5 in 1 million and comprise 5 per cent of all cases. Molecular defects are large deletions and nonsense, missense and frame shift mutations that may occur throughout the gene (Veyradier *et al,* 1998).

Type 2 mutations are qualitative defects of VWF and comprise 20 per cent of vWD patients. There are four distinct subtypes. Type 2A is characterized by low affinity of VWF for GP1b, absence of intermediate and high molecular weight multimers. The patients are diagnosed by low VWF Ristocetin activity (VWF:RiCof) ratio over VWF antigen (Keeney and Cumming, 2001). Candidate mutations for type 2A vWD are accumulated around proteolytic site containing A2 domain and inherited in an autosomal dominant pattern. Additionally some other rare subtypes of vWD (IIC and IID) are also summed under this group although they may have some different modes of inheritance and etiology but show the similar phenotypes (Veyradier *et al,* 1998).

In type 2B high molecular weight multimers are not reduced but affinity of VWF for platelets is increased. Due to this majority of high molecular weight multimers are bound

to platelets and can be observed as low in the plasma. In the laboratory this phenotype is indentified by enhanced Ristocetin Induced Platelet Agglutination (RIPA) analysis, however, VWF:Ricof may be normal. On the other hand thrombocytopenia is also observed in these patients. Molecular defects are located in the A domain (Veyradier *et al,* 1998).

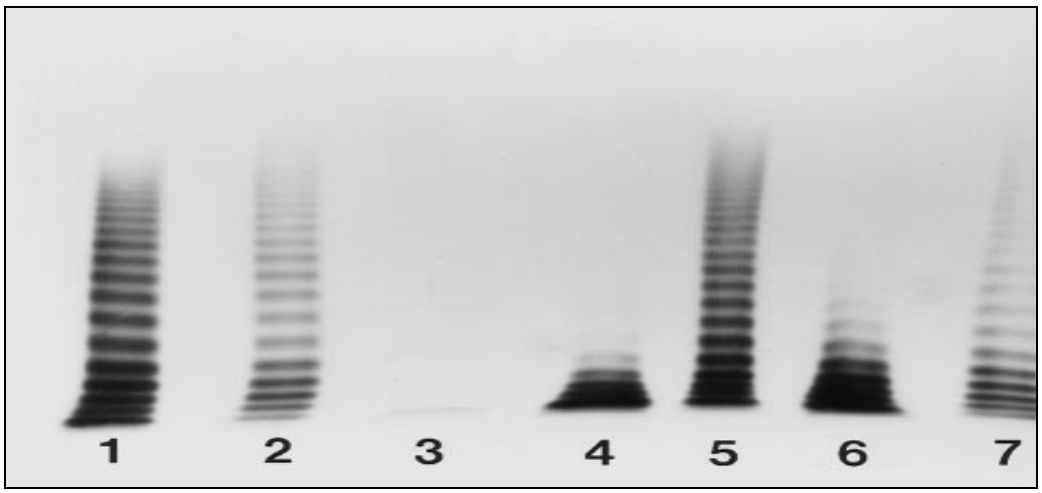

Figure 1.4. Multimeric Distribution of plasma VWF in different disease subtypes. Lane 1: Normal plasma. Lane 2: Type 1 vWD, Lane 3: Type 3 vWD, Lane 4: Type 2A vWD, Lane 5: Type 2M, Lane 6: Type 2B, Lane 7: Type 2N with compound heterozygous type 1 (Veyradier *et al,* 1998).

In type 2M patients VWF affinity for GP1b is reduced but not because of reduced HMW multimers. VWFAg/VWF:Ricof value is decreased. One rare type called Vicenza, is characterized by low level of plasma VWF level and abnormally high molecular weight multimers. Type 2M phenotype mutations are also located in the A1 loop domain. However mutations leading to Vicenza type are accumulated in exons 26 and 27 (Keeney and Cumming, 2001, Veyradier *et al,* 1998).

In type 2N patients VWF has drastically reduced affinity for FVIII. Consequently, plasma FVIII is not protected against proteolytic activity of activated protein C. Generally, these patients but they show long Activated Partial Thromboplasin Time (aPTT). Plasma VWF is normal but FVIII/VWF ratio is reduced. Thus, these patients are generally misdiagnosed for hemophilia A. VWF FVIII binding assay would help the diagnosis. The disease is inherited autosomal recessively (Veyradier *et al,* 1998).

Table 1.2. von Willebrand Disease subtypes (adapted from Keeney and cummings, 2001, Veyradier *et al,* 1998).

vWD Type	Frequency	Mode of Inheritance	Molecular Defect	Molecular basis
Type 1	1-30 :10000 most common >70 % of all cases	A.D. Incomplete penetrance	Partial Quantitative deficiency of VWF	Missense mutations all through the gene
Type 3	1-5:10^6	A.R.	Virtually complete deficiency of VWF	Large deletions, nonsense, missense, frame shift mutations cis-defects in mRNA expression
Type 2A	approximately 10-15 % of vWD cases	A.D.	Decreased platelet dependent function absence of HMW multimers	Missense mutations clustered within VWF A2 repeat. Defect in intracellular transport. Increased proteolysis in plasma after secretion
Type 2B	Uncommon <5% of cases	A.D.	Increased affinity for GPIb	Missense mutations clustered in VWF A1 repeat
Type 2M	Under estimated	A.D.	Decreased platelet dependent function HMW multimers are present	Missense mutations and small in frame deletions in VWF A1 repeat
Type 2N	uncommon	A.R.	Markedly decreased affinity for FVIII	Missense mutations within the N-terminus of mature VWF spanning exons 18-20

For patients with VWD it is difficult to predict bleeding, especially for mild VWD patients. FVIII level is the best predictor of soft tissue bleeding, whereas, platelet dependent VWF activity measurement is used for prediction of mucosal bleeding.

In treatment of VWD, the two defects that are targeted to be corrected, are decrease in VWF level, and associated FVIII deficiency. The main therapeutic approach in

treatment of VWD is the use of desmopressin (1-desamino-8-$_D$-arginine vasopressin, DDAVP). However, replacement products that contain VWF are used for treatment of patients with qualitative defects of VWF (Type 2) and severe deficiencies (Type 3), or to sustain VWF level in patients with milder disease (Manucci, 1998 and Konkle, 2007).

Desmopressin stimulates the VWF release from the storage bodies by an unknown mechanism and causes a transient increase in the VWF level, as well as the FVIII level, approximately 3-6 times higher than their basal levels. The effect of the drug lasts 8-10 hours and the administration can be repeated in 12-24 hours depending on the patient's response. But patients who have frequent desmopressin infusion become less responsive to the treatment. Additionally, there are reports of myocardial infarction and stroke in hemophiliacs. Thus, this drug that causes hypercoagulablity should be used with caution in elderly patients (Manucci, 1998, Bond and Bevin, 1998).

Replacement products containing VWF is the treatment alternative for the patients that do not give response to DDAVP. Fresh frozen plasma (FFP) used to be administered to VWD patients, contains low amounts of VWF and FVIII. Thus, large amounts are infused to achieve hemostasis limits. Cyroprecipitate contains 5-10 times more VWF and is used instead of FFP. However, virocidal methods are not applicable for cryoprecipitate and use of cryoprecipitate increases the risk of blood borne infection (Manucci, 1998).

Virus inactivated FVIII-VWF concentrates are current choice of treatment, because they contain large amounts of both FVIII and VWF. However, recent studies have shown that this approach elevates the FVIII level more than predicted, mainly due to the stabilization of endogenous FVIII with exogenous VWF, increasing the risk of thrombosis in VWD patients. Thus, treatment with FVIII-VWF concentrates is questioned and concentrates that contain only VWF are tested for efficiency in treatment of VWD (Manucci 1998 and Konke, 2007).

On the other hand, some rare patients with severe VWF deficiency (Type 3) develop anti-VWF alloantibodies, following multiple transfusions of VWF concentrates. These patients are treated with high doses of recombinant FVIII, which is totally devoid of VWF. Because FVIII devoid of its VWF carrier is rapidly cleared from plasma, continuous

administration is required in emergency conditions (Manucci and Federicii, 2001). Different approaches for treatment VWD types and subtypes are listed in the Table 1.3.

Table 1.3. Management of different types and subtypes of VWD (adapted from Manucci, 1998).

VWD Type/Subtype	Treatment of Choice	Adjunctive Therapy
Type 1	Desmopressin	Antifibrinolytics
Type 2A	Factor VIII-VWF Concentrates	Antifibrinolytics
Type 2B	Factor VIII-VWF Concentrates	Antifibrinolytics
Type 2M	Factor VIII-VWF Concentrates	Antifibrinolytics
Type 2N	Desmopressin	Antifibrinolytics
Type 3	Factor VIII-VWF Concentrates	Platelet concentrates, desmopressin
Type 3	Recombinant FVIII with alloantibodies	

1.2. Factor XI Protein and Its Deficiency (Hemophilia C)

1.2.1. Factor XI Deficiency (Hemophilia C)

Factor XI (FXI) deficiency was first described in a Jewish family in 1953. Two sisters bled after dental extraction and tonsillectomy and a maternal uncle bled after dental extraction, but not after infantile circumcision. Moreover, four other individuals among 13 family members of four generations also showed low FXI level. The case was different from hemophilia A and B, as it was observed in both sexes and no spontaneous bleeding, or muscle/joint bleeding was among the symptoms. Therefore, the deficiency was named as hemophilia C (Seligsohn, 2007).

The symptoms are trauma or surgery related bleeding, especially at the sites of elevated fibrinolytic activity, for example, oral and nasal cavities. On the other hand bleeding is less frequently seen at the sites of no local fibrinolytic activity, even after surgeries like circumcision, orthopedic surgery, etc. For postpartum hemorrhage, a recent

study with 62 women with severe FXI deficiency showed that 70 per cent of them had 93 uneventful deliveries without blood component therapy (Seligsohn, 2007).

Majority of severely affected individuals show excessive bleeding after invasive procedures, while, mild FXI deficiency patients are less prone to bleeding. However, there is not a direct correlation between FXI plasma level and bleeding tendency. Bleeding symptoms vary significantly between patients with the same FXI plasma level and even in the same patient (Bolton-Maggs, 2000).

After optimization of Activated Partial Thromboplastin Time (aPTT) test, severe or mild deficiencies could be distinguished. Severe FXI deficiency (FXI level below 15-20 UdL-1) result from homozygous and compound heterozygous mutations, the patients are more prone to bleeding. On the other hand, heterozygous mutations cause mild FXI deficiency (FXI level between 20-70 UdL-1). However, bleeding tendency of patients is not related to FXI level. Some patients with mild FXI deficiency show excessive bleeding, controversially; some patients with severe deficiency do not bleed even after surgery. Some patients with mild FXI deficiency generally do not bleed and for this reason they are not diagnosed. Due to these controversial facts, the inheritance of FXI deficiency is also disputed. In some cases diagnosis is based on patients' bleeding scores and people who have low FXI but no bleeding tendency (generally heterozygotes) do not get diagnosed. From this perspective, disease is defined as an autosomal recessive disorder. On the other hand, people that bear heterozygous mutations, also have low FXI levels, and may or may not show bleeding. From this perspective, the disease is defined as an autosomal dominant disorder (Bolton-Maggs, 2000).

Lack of correlation between FXI level and bleeding tendency can be attributed to some factors but none of them have been confirmed by adequate evidence. The first possibility is the variant FXI molecules. Some mutations do not interfere with the production or secretion of the protein. Thus, the protein level is high but FXI proteins are enzymatically inactive or bear some other defects. Though this is confirmed by some antigen tests (CRM+ and CRM -), it is not validated in all cases (Kravtsov *et al*, 2004).

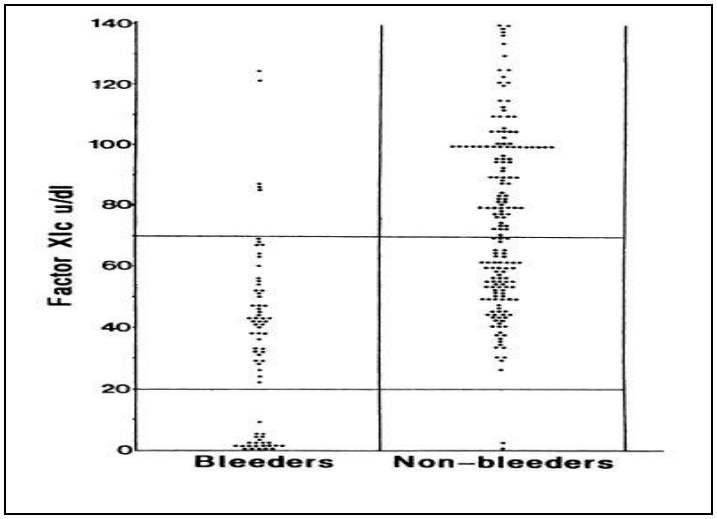

Figure 1.5. The relationship between bleeding tendency and FXI:C level. The data obtained from a British study of 128 FXI patients. Upper line represent the lower end of normal level and lower line represents the cut-off between mild and severe FXI deficiency. Of 128 patients, 45 (35 per cent) are bleeders (obtained from, Bolton-Maggs,2000).

Another possible explanation is the activity of platelet FXI. Though FXI in circulation is produced by liver, an alternatively spliced form of FXI (lacking exon 5) is produced and excreted by platelets. The activity is less than one percent of plasma FXI activity. However, these FXI proteins stay bound to platelet membrane and there are some reports indicating the function of platelet bound FXI in blood coagulation. But, some cases of platelet defects combined with FXI deficiency that do not show bleeding were reported. This model does not explain the case by its own (Bolton-Maggs, 2000 and Martinic *et al,* 1999).

There are reports about patients with FXI deficiency who also have VWD and FVIII deficiency. A recent study from Israel revealed that majority of mild FXI deficiency patients also have von Willebrand disease. However, this study was contradicted by another study in Britain, which showed an insignificant correlation. Though there are attempts of developing a model, which accounts for age, sex, blood group, VWF:Ag level beside FXI level as predictors of bleeding, a 100 per cent significant model has not been established (Bolton-Maggs, 2000, O'Brien *et al*, 2007).

Moreover, individuals with FXI deficiency show bleeding after dental extraction, tonsillectomy, adenoidectomy, nasal surgery and prostate surgery, all of which are areas of increased fibrinolytic activity. This may contribute to the bleeding tendency of patients (Seligsohn, 2007).

FXI deficiency is common among Ashkenazi Jews. With heterozygote frequency of eight per cent, it is the most common inherited disorder in this population. However, four mutations (type I-IV), one missense (phe 283 leu, Type III), one nonsense (Glu 117 Ter, Type II), one splice site (exon 14/intron N; type I) and a deletion (a 14bp deletion at the exon 14 / intron N splice site; type IV) are observed in these people. Type II and III are more common compared to types I and IV. Because of frequent consanguineous marriages, the mutations are carried over generations. The high frequency of heterozygosity also implies some kind of heterozygote advantage or positive selection in favor of heterozygotes. Although this case is not confirmed. Beside Ashkenazi Jews, type II mutation was also reported in Iraqi Jews, indicating that this mutation is more ancient than others (www.factorxi.com and Bolton-Maggs, 2000).

In other races the disease is not common and frequency is not determined but, an increasing number of mutations are being reported in other populations that are not Jews. The majority of mutations are single point mutations and accumulated in apple 4 and SP domain (statistics are listed in table 1.4. and 1.5). 127 causative mutations corresponding to 273 FXI deficient patients are listed in the database (www.factorxi.com). These mutations result in two distinct phenotypes. Type I phenotype is characterized with low FXI activity (FXI:C) and also low FXI antigen level (FXI:Ag). This implies that the protein is either in low amounts or not present in the plasma. The causative effect of the mutation can be structural. On the other hand, in Type II phenotype, FXI antigen level is normal but protein activity is low. This indicates that the mutant protein is present in the plasma in normal amounts, however, it has reduced or no activity. This fact suggests that the mutation has functional effect (www.factorxi.com).

Table 1.4. FXI mutation distribution among domains (The statistics are obtained from FXI database: www.factorxi.com).

Domain	Graphical Demonstration	Number of Mutations	Percentage
Apple 1		17	11%
Apple 2		17	11%
Apple 3		24	16%
Apple 4		24	16%
Introgenic Region		24	16%
Linker Region		3	2%
Signal Peptide		2	1%
SP		41	27%

Table 1.5. Distribution of mutations according to the mutation type (The statistics are obtained from FXI mutation database: www.factorxi.com).

Mutation Type	Graphical Demonstration	Number of Mutations	Percentage
Deletion		9	6%
Insertion		4	3%
Missense		81	53%
Nonsense		21	14%
Polymorphism		25	16%
Splice Site		11	7%
Unknown		1	1%

Individuals with FXI deficiency require specific treatment after surgery, accidents and dental extractions. Therapeutic approaches include the use of antifibrinolytic agents, FXI replacement therapies with fresh frozen plasma or FXI concentrates fibrin glue and desmopressin. The efficacy of each varies from patient to patient and each of them has drawbacks beside advantages (Bolton-Maggs,2000).

Antifibrinolytic agents are structurally similar to amino acid lysine and inhibit binding of plasminogen to fibrin and therefore, its conversion to plasmin. They can be administered intravenously, orally or topically at the areas of high fibrinolytic activity. Aminocaproic acid and tranexaminic acid, are most widely used antifibrinolytic agents. Tranexaminic acid is widely used after dental extraction due to its ease of administration as tablet or mouthwash (www.factorxi.com, Gomez and Bolton-Maggs,2008).

FXI replacement is achieved by use of FFP or FXI concentrate, though their contra-indications are not evaluated. Early cases of FXI deficiency were treated with FFP, however, after 1980s FXI concentrate was available. FXI concentrate become treatment of choice because it shortens the infusion time and avoids unnecessary load of other coagulation factors present in FFP. Additionally, FXI concentrates are virally inactivated (Gomez and Bolton-Maggs,2008, Salomon and Seligsohn, 2004).

In some cases of severe deficiency, inhibitor development after replacement therapy was observed. Some studies report the successful usage of recombinant FVIIa concentrate in inhibitor developing patients (www.factorxi.com and Salomon *et al,*2006).

1.2.2. Factor XI Protein

FXI is unique among other serine proteases for being a homodimer zymogene, formed by two identical subunits that are connected by a disulfide bond. Each subunit is an 80,000 kD protein, which is the product of *Factor 11 (F11)* gene located on chromosome 4, below prekallikrein gene. The protein is synthesized in the liver circulates in blood at a concentration of 3–7 µg/ml, as a complex with high molecular weight kinninogen and also alternatively spliced form is produced and present on the surface of platelets (Provan and Gribben, 2001, www.factorxi.com).

F11 gene is 23 kb long containing 15 exons. The gene is transcribed into 18 amino acid long signal peptide and 603 amino acids long mature protein, the first two exons are noncoding. Exons 3-10 comprise four apple domains that show sequence homology with each other and with other apple domains, also known as PAN modules, and exons 11-15 contain the catalytic site. The apple domains are 24-34 per cent identical in sequence. The protein shows sequence homology with prekallikrein (58per cent) especially in the

catalytic serine protease domain (81per cent homology). Both of them have potential N-Glycosylation sites and overall carbohydrate content of FXI is five per cent (Riley *et al*, 2006 and O'Connel *et al,* 2004).

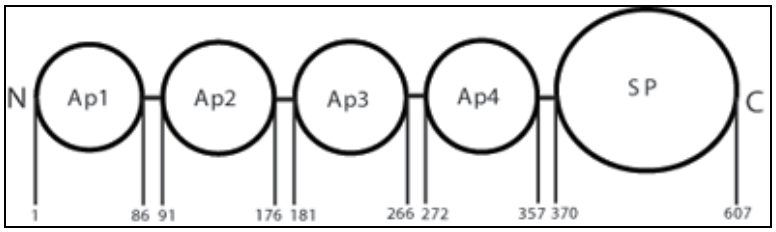

Figure 1.6. FXI Protein domain structure

After synthesis, FXI molecules form dimers by noncovalent interactions between apple 4 domains and a disulphide bond between Cys321 residues. Dimerisation is essential for secretion and may be essential for cleavage of its substrate; Factor IX. In circulation, FXI can be activated by Factor XII, Thrombin or undergoes auto activation, by cleavage of Arg369-Ile370 . As a result four subunits occurs, two heavy chains of apple domains (396 amino acids) and two light chains of serine proteases with His413, Asp462 and Ser557 the catalytic triad. FXI is activated when bound to activated platelets and activated FXI, activates FIX by cleaving Arg146 – Ala147 and Arg180 – Val181 bonds, sequentially, in the presence of calcium ions (Fujikawa, 2004 and O'Connel .*et al,*2004)

Apple 1 domain contains binding site for HK and PK and FXII binds to apple 4 domain. Moreover, Apple 2 and Apple 3 domains are responsible for binding Factor IX. After activation by Factor XII, these repeat domains comprise the heavy chains of the enzyme. Each apple domain contains functional sites; for instance, Apple 1 domain comprises a HK binding domain and a thrombin, another activator of FXI, binding domain (Riley *et al*, 2006, Gomez and Bolton-Maggs, 2008, and FXI site).

In the coagulation cascade, two mechanism yield thrombin generation, Tissue Factor (TF) mediated initiation phase, and FXI-containing amplification loop. The former produces a trace amount of thrombin, which is attacked by inhibitory proteins. Continued thrombin generation requires presence of FXI protein and activated platelets. Moreover clot lysis studies revealed that FXI indirectly inhibits fibrinolysis. On the other hand, FXIa is inhibited by a series of serine protease inhibitors (serpins), the most significant is the

Kunitz-type inhibitor protease nexin 2, found in the α-granules of platelets (Gomez and Bolton-Maggs, 2008, Nevaneetham *et al,* 2005)

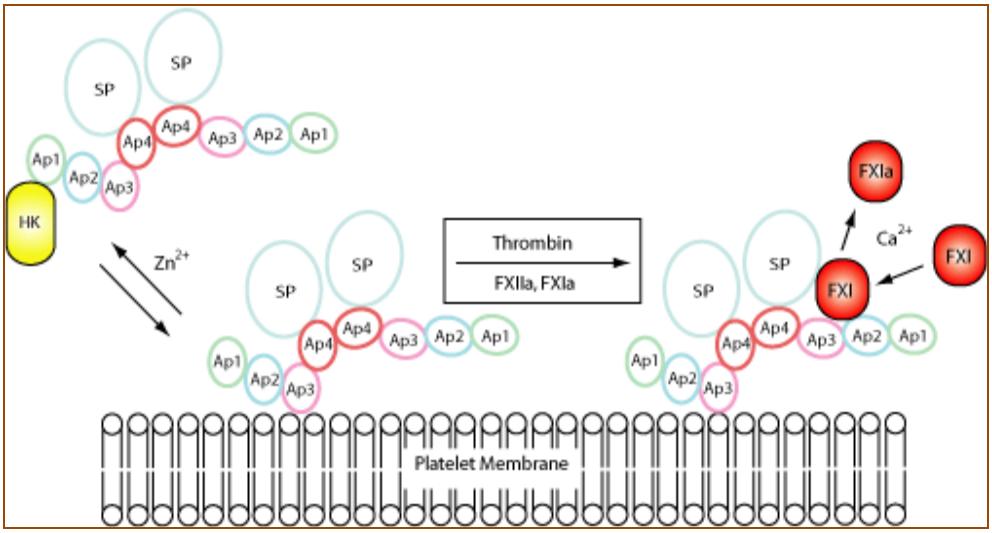

Figure 1.7. FXI Protein activation by Thrombin, FXIIa and FXIa in the presence of Zinc and Calcium ions.

2. PURPOSE

Coagulation is a balance between the actions of coagulation factors and anti-coagulants, and requires the fine adjustment of every factor. Presence of any factor in excess or less amount than normal shifts the balance between thrombosis or hemorrhage. Each case has serious clinical consequences. VWF and FXI are two important actors of this balance, and their deficiency result in several hemorrhagic conditions, though incomplete penetrance and variable expressivity complicates the laboratory diagnosis of both diseases. Co-existence of FXI deficiency with VWD and Hemophilia A has been reported, however, direct correlation have not been established.

Bleeding tendency of FXI deficiency patients is variable among different patients with the same level and FXI:C (activity, FXI:Ag) measurement cannot be used as the sole predictor of bleeding diathesis. Moreover, severely affected patients with certain mutation types have tendency to develop inhibitors following replacement therapy, but other severely affected patients with different mutations do not (Bolton-Maggs, 2000). Inhibitor development causes complications during surgery. Mutational analysis of the FXI gene could contribute to the explanation of the molecular pathology of the disease and could be a predictor of the bleeding diathesis of patients.

VWD is defined as the most common bleeding disorder and have several subtypes. Each subtype of VWD results from a mutation effecting different domains of the protein, hence, disrupting the expression, secretion or function of the molecule. The mutations are distributed throughout the gene. In order to decrease mortality and morbidity due to VWD, the establishment of the molecular pathology of the disease is essential.

In the framework of this thesis, we aimed to establish the DNA diagnosis for both FXI deficiency using direct DNA sequence analysis and VWD using a new approach, namely, real-time PCR followed by HRMA that combines the amplification and mutation screening steps. The application of HRMA is expected to greatly reduce the amount of work and time in the mutational analysis of a large gene like VWF gene

3. MATERIALS

3.1. Blood Samples

3.1.1. Blood Samples of Factor XI Deficiency and von Willebrand Disease Patients

Blood samples of FXI Deficiency and vWD patients and their family members were provided by the clinicians of following hematology centers: Department of Hematology, Cerrahpaşa Medical School and İstanbul Medical School, İstanbul University, İstanbul; Department of Hematology, Medical School, Hacettepe University, Ankara; Department of Hematology, Medical School, Ege University, Izmir. For the collection of VWD patient bloods, the approval of Ege University, ethical committee was taken.

3.2. Oligonucleotide Primers

3.2.1. Primers for PCR Amplification and DNA Sequencing

F11 and *VWF* gene specific primers were used in amplification of all exons and exon/intron boundaries. Sequences for *F11* and *VWF* gene were manually designed, avoiding overlapping with the pseudogene sequence. They were purchased from MEDEK inc. Turkey. The sequences of these primers used in this study are given in Table 3.1. and Table 3.2.

3.3. Enzymes

Taq DNA Polymerase	:	5 U/µl, Roche Diagnostics GmbH, Germany
Fok I Restriction Endonuclease	:	10 U/ µl, Amersham Biotech, U.S.A.

3.4. PCR Purification Kit

In order to obtain good quality of PCR product for direct DNA sequencing, GeneMark PCR Clean-Up Kit for PCR purification was purchased from GeneMark, Taiwan.

3.5. Gel Extraction Kit

For the cases of multiple fragment amplification, target fragment is isolated from two per cent agarose gel and purified, using QIAEX II Gel purification kit, Qiagen, U.S.A.

3.6. Buffers and Solutions

3.6.1. DNA Extraction

Lysis Buffer	:	155 mM NH_4Cl 10 mM $KHCO_3$ 0.1 mM Na_2EDTA (pH 7.4)
Nuclease Buffer	:	10 mM Tris-HCL (pH 8.0) 400 mM NaCl 2 mM Na_2EDTA (pH 8.2)
Sodiumdodecylsulphate (SDS)	:	10 per cent SDS (w/v) (pH 7.2)
Proteinase K	:	20 mg/ml in H_2O
Sodium Chloride (NaCl)	:	2,5 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)

3.6.2. Polymerase Chain Reaction (PCR)

10X PCR Reaction buffer with $MgCl_2$	:	100 mM Tris-HCl 500 mM KCl 15 mM $MgCl_2$ Roche Diagnostics GmbH, Germany
Magnesium Chloride ($MgCl_2$)	:	25 mM in dH2O Roche Diagnostics GmbH, Germany
Deoxyribonucleotides (dNTP)	:	100 mM of each dNTP Promega, USA
Dimethylsulphoxide (DMSO)	:	Stock solution Sigma, Germany

3.6.3. Agarose Gel Electrophoresis

10X TBE Buffer	:	0.89 M Tris-Base 0.89 M Boric acid 20 mM Na_2EDTA (pH 8.3)
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 0.5µg/ml Ethidium bromide
6X DNA Loading Dye (Fermentas, Lithuania)	:	10 mM Tris HCL (pH7.6) 0.03 % Bromophenol Blue (BPB) 0.03% xylene cyanol FF 60 % glycerol 60 mM EDTA
DNA Ladder	:	100 bp, Fermentas, Lithuania 500 bp, Fermentas, Lithuania

3.7. High Resolution Melting Analysis

HRM Analysis was conducted using of High Resolution Melting Master Kit, 500 reactions from, Roche Diagnostics GmbH, Germany. The kit contains:

2X Master Mix	:	FastStart *Taq* DNA Polymerase Reaction Buffer dNTP Mix (with dUTP instead of dTTP) High Resolution Melting Dye (LCGreen®)
$MgCl_2$	:	$MgCl_2$, 25 mM
H_2O, PCR Grade		

3.8. Equipment

Autoclav	:	Model MAC-601, Eyela, Japan
Balances	:	GM 512-OCE, Sartorius Inc., Germany
Centrifuges	:	Centrifuge 5415C, Eppendorf Germany Microfuge18 Centrifuge, Beckmann Coulter Inc. USA
Deep Freezers (-20 ºC)	:	Arçelik, Turkey
Documentation System	:	GelDoc Documentation System, BIO-RAD, USA
Electrophoretic Equipments	:	Mini Sub-Cell GT, BIO-RAD, USA

Heat Block&Magnetic Stirrer	:	Chiltern Hot-Plate Magnetic Stirrer HS31,
Real-Time PCR System	:	Light Cycler LC 480 96 well-plate platform, Roche Diagnostics GmbH, Germany
Magnetic Stirrer	:	Chiltern Hotplate Magnetic Stirrer HS31, UK
Ovens	:	Microwave Oven, DBK, Turkey
Power Supplies	:	EC 250-90 Thermo, USA
Refrigerator	:	4ºC Medicool, Sanyo, Japan Arçelik 4042T, Turkey
Spectrophotometer	:	ND 1000 Spectrophotometer, NanoDrop Inc. USA
Thermocyclers	:	Techne TC-512, UK
Water Bath	:	Köttermann, Laborteknik, Germany
Water Purification	:	WaTech Water Technologies, Turkey

3.9. Software

High Resolution Melting Analysis	:	GeneScan Program, Roche Diagnostics GmbH, Germany.
Cp Calculations	:	Absolute Quant/2nd Derivative Max

program, Roche Diagnostics GmbH, Germany.

Melting Peak Calculations : Tm Calculation Program, Roche Diagnostics GmbH, Germany.

3.10. DNA Extraction From Peripheral Blood Using MagNA Pure® Compact

Beside manual extraction which will be detailed below, MagNA Pure Compact Nucleic Acid Purification Machine, Roche Diagnostics, Germany, was used with MagNA Pure Nucleic Acid Isolation Kit, Large Volume, Roche Diagnostics, Germany to extract DNA from peripheral blood samples obtained from patients and their family members.

Table 3.1. Oligonucleotide primer pairs used in PCR and DNA sequencing of the *F11* gene analysis.

Primer Name	**Primer Sequence 5'-3'**
FXI-Prom-F	AGGAATCCTATCTGCTTAGGCCTCT
FXI-Prom-R	CCTTGAGAGAATTGCTTGCTTTCTC
FXI - 1F:	AGCAAGCAATTCTCTCAAGG
FXI-1R:	GCATTTCAAGGCGTATTTGC
FXI - 2F:	AAGTCTTAGTGTCAGTAAAC
FXI-2R:	TGTGGAGATTGCAGTGTG
FXI - 3F:	CCCAGTAAAATCCAACATAACGC
FXI-3R:	GTCTCCTCGATGTAGAAACATAAG
FXI - 4F:	TTCTTTTTGGCTTTCTGTGTGCTG
FXI-4R:	AGAACATATCTTACCGCTTATTTGGTG
FXI - 5F:	TCTGGAAGGTACTCATGTCTTCTG
FXI-5R:	TTCATCGACCACTCGAATGTCCTG
FXI - 6F:	ACATTTGTCTACTGAAGCACACCC
FXI-6R:	GGTCCGTTTCATCGTGAGCATAAG
FXI- 7F:	GATAGCTGGTGAATTGAGTCCCTG
FXI-7R:	CCTTACCTTTGAGATTCTTTGGGC
FXI – 8/9F :	GTCTCCTTAAAACATCTGAGAGTG
FXI-8/9R :	TATGGCTTACTTCCCTTCGTTG
FXI- 9/10F:	TGTGGTCTGCTGTCTAGTGTTC
FXI- 9/10R:	ACAACCTTAATGTGTATCCAGAGATG
FXI – 11F :	TGCTTCTGTTGCAGAGTGTACC

Table 3.1. Oligonucleotide primer pairs used in PCR and DNA sequencing of the *F11* gene analysis. (Continue)

FXI-11R :	GATGAACTAATAAAAACAGCCGTGG
FXI – 12F :	TCTTTCCCTCTGTTGTTTGCTC
FXI-12R :	TTCTCCGTACCTGTGTAATTCACTG
FXI – 13F :	AGAGTCTCTTCTGGAAAAGAGG
FXI-13R :	TTCTAGGATGGAGCACATATAACAAC
FXI – 14F :	CAAAATACTCTCCAGAAAGCCAAG
FXI-14R :	CAAAAAAACCCCAACGCATTAAGC
FXI-15R :	GACTGAATGCTCAAACAAACTACAAC

Table 3.2 Oligonucleotide primers for PCR amplification and HRM analysis of the *VWF* gene

Primer Name	**Primer Sequence 5'-3'**
VWFex1-HPLC-F1	AGGTACTTCTAATACCATTTCC
VWFex1-HPLC-R1	TCAGTCAGTCCTGCATCTTC
VWFex2-HPLC-F2	TACCTGGGCAGCAATGACC
VWFex2-HPLC-R2	AGACACACCTGCTGATTCC
VWFex3-HPLC-F3	CAGAGAGGTTTGAGCTGATG
VWFex3-HPLC-R3	TTTCCACTCAGACACTGTCC
VWFex4-HPLC-F4	GGGGCGTTTTCTGCTGAGA
VWFex4-HPLC-R4	CTAAAAATGACCCCCTTGTG
VWFex5-HPLC-5F	TAGAAGGTGGGAGAGACATC
VWFex5-HPLC-5R	AGTGAAGGTTTATGAGCAAGG
VWFex6-HPLC-6F	AGTTCTGGGACAGGCATAAC
VWFex6-HPLC-6R	TGAGACTGAGTCCTTCTGTC
VWFex7-HPLC-7F	CTCAGGGAGACACTAACGGA
VWFex7-HPLC-7R	CAGGACAGACCGTTCATC
VWFex8-HPLC-8F	ATGGCTGGTGAGATGATGCA
VWFex8-HPLC-8R	GTGCTGGCAAGGTCTCTGA
VWFex9-HPLC-9F	TCTATAGTTGTGGGATGAGTG
VWFex9-HPLC-9R	TCCAGGTTCTTTTCCACCTG
VWFex10-HPLC-10F	GAGCTCTAAATCCATTTGCATA
VWFex10-HPLC-10R	AACTTCTCGCTGCCTTGAGT
VWF-ex11-HPLC-11F	GCTTGCATATGCATTCCACC
VWF-ex11-HPLC-11R	GCAGCTATGCAGCACCTTG
VWF-ex12-HPLC-12F	TTGAGGCCTTTCTCTGATTAAG
VWF-ex12-HPLC-12R	TGCTAAGGGATGGGCTGTG
VWF-ex13-HPLC-13F	TGCTACCATCCTTTTGAGACA
VWF-ex13-HPLC-13R	ATTCTACCCAGAGCACAAGG

Table 3.2 Oligonucleotide primers for PCR amplification and HRM analysis of the *VWF* gene (Continue)

Primer Name	**Primer Sequence 5'-3'**
VWF-ex14-HPLC-14F	ACAACTATGCCGCTGCTTTC
VWF-ex14-HPLC-14R	GACCTCGAGATTCTGCGAG
VWF-ex15-HPLC-15F	ATTTCCAGGGGAGTGTTGAC
VWF-ex15-HPLC-15R	TTCGAAAAGCACACGTGGAC
VWF-ex16-HPLC-16F	AGTCCTAGAACCACAGTCCT
VWF-ex16-HPLC-16R	CAGCTCTGCTGTTTTAGAGG
VWF-ex17-HPLC-17F	CATTGGTAACGTTAGCAAGCT
VWF-ex17-HPLC-17R	TTCCACACGTGAGGAATCTG
VWF-ex18-HPLC-18F	GGTCCATTCTCTCCTTCACT
VWF-ex18-HPLC-18R	TGTTTCTTCCTCTCTCTGGC
VWF-ex19-HPLC-19F	GATCAGTCACTGTGGCCCT
VWF-ex19-HPLC-19R	AAAGGCAGAGAGTAACCAGG
VWF-ex20-HPLC-20F	AACTTGTCATCTCTGCCATGA
VWF-ex20-HPLC-20R	TAGAAAGAAACAGCACCCTCT
VWF-ex21-HPLC-21F	TAATGGTCTTCTCCTGGCAC
VWF-ex21-HPLC-21R	TCCTATTAAGTGGCAGAAGC
VWF-ex22-HPLC-22F	AGAGTGGAGGGAGGATCTG
VWF-ex22-HPLC-22R	GCAGAAAACACTCCAAAGGC
VWFex23-HPLC-F23	CTGAGCCGGAGAGCATGCT
VWFex23-HPLC-R23	CATTCCAGGAAGCAAGCTGCT
VWFex24-HPLC-F24	GTTGTGGGTGGTATGACCTC
VWFex24-HPLC-R24	GGCCAAGCCTTGGGACCG
VWFex25-HPLC-F25	CAGACTAAGAGCCAGAGTTC
VWFex25-HPLC-R25	GCATCTGAGAACATGAGCCG
VWFex26-HPLC-F26	CAGTGACTCATACCTGTAATC
VWFex26-HPLC-R26	ACTGCGTGTGGCTATTTACG
VWFex26-HPLC-F26N	ACATTATCTCCAGATGGCGC
VWFex26-HPLC-R26N	TCCATCCATCCCTATCCCAT
VWFex27-HPLC-F27	AGTTAAAAATGAGGCTTCCTCG
VWFex27-HPLC-R27	ATCACTTCAAACAACCCAGGA
VWFex28-HPLC-F1	GAAGTGTCCACAGGTTCTTC
VWFex28-HPLC-R1	GCAGTAGAAATCGTGCAACG
VWFex28-HPLC-F2	GGACATCTCGGAACCGCC
VWFex28-HPLC-R2	TACTTCACCTGGCTGGCAAT
VWFex28-HPLC-F3	CGTCAGAGCTGCGGCGCA
VWFex28-HPLC-R3	CAATGCCCACCGGGATCACA
VWFex28-HPLC-F4	CCTGAAGAAGAAGAAGGTCATT
VWFex28-HPLC-R4	ACAGTGACTTGTGCCATGTC
VWFex28-HPLC-F5	TCTGTGACCTTGCCCCTGAA
VWFex28-HPLC-R5	GCTGAAGGGGTACTCCACA
VWF-EX28-HPLC-R5New	TCCTTGCTCCTGTTGAAGTC
VWF-EX28-HPLC-F6New	GGACAAAATTGGTGAAGCCG
VWFex28-HPLC-F6	CAGTACTCCTACATGGTGACT

Table 3.2 Oligonucleotide primers for PCR amplification and HRM analysis of the *VWF* gene (Continue)

Primer Name	**Primer Sequence 5'-3'**
VWFex28-HPLC-R6	TCATCAGAGGCAGGATTTCC
VWFex28-HPLC-F7	CTGGTCTACATGGTCACCG
VWFex28-HPLC-R7	AGGTGCCAGCATACCAGGT
VWFex29-HPLC-F29	TAGGCCTGGTGGCCATTGT
VWFex29-HPLC-R29	CTAAACGGAACGAGAAAATGC
VWFex30-HPLC-F30	GAGGCTCTTTTTGTGGCTCT
VWFex30-HPLC-R30	TGCAGCTTCTGCATCCAGC
VWFex31-HPLC-F31	ACCGTTAAGACAGGGTGTCG
VWFex31-HPLC-R31	CATCCAAAAGTAACCCCAGC
VWFex32-HPLC-F32	TCAGGCCAGTCCATTTTGAG
VWFex32-HPLC-R32	TTTTGTTTCTTTGGCGGGTTTA
VWF-ex33-HPLC-F33	TCAGCCTCATGTCCCTATGT
VWF-ex33-HPLC-R33	GACCAAAGGAGGAAAAATTAAC
VWF-ex34-HPLC-F34	CTCCTTGCTGTGTAGGCCT
VWF-ex34-HPLC-R34	ACTTTTCTGCACAGCCAAGC
VWF-ex35-HPLC-F35	GTTTGGATAACGTCGCATCC
VWF-ex35-HPLC-R35	CTGACATTCTATTGCCTTACC
VWF-ex36-HPLC-F36	ATTCCTCATTGCTAGGACTA
VWF-ex36-HPLC-R36	TCAGAGTAGAGCAAGTAAGGT
VWF-ex37A-HPLC-F37A	CAGGGCCACTCAGTTTATC
VWF-ex37B-HPLC-R37A	TTGTGGCATTCAGCAAACAGT
VWF-ex37A-HPLC-F37B	ACTGTTTGCTGAATGCCACAA
VWF-ex37B-HPLC-R37B	GGTCTCCAGGATTTTCAGAG
VWF-ex38-HPLC-F38	TCAGCTGTGCCCATTTCACT
VWF-ex38-HPLC-R38	ATATCTCCCTTTTGACCCAAG
VWF-ex39-HPLC-F39	GAGCTGACCTCTGTGAATTC
VWF-ex39-HPLC-R39	AGAGGTGAAGATGGGTGGC
VWF-ex40-HPLC-F40	ACATTTCAAATCCCTCTGAGG
VWF-ex40-HPLC-R40	ACCTTTCAGCACCTTCAACG
VWF-ex41-HPLC-F41	GAGTAACCTTTCTGAACTCTG
VWF-ex41-HPLC-R41	ACCCAGATTCAGCTAGGTAG
VWF-ex42-HPLC-F42	CTATAGCATAGCTGAATACTTAC
VWF-ex42-HPLC-R42	GATAGAAGGATAAACTGACAGC
VWF-ex43-HPLC-F43	AACTCACCGCTTGTTTCAATG
VWF-ex43-HPLC-R43	AGATGCCCTCCTCTACTTTC
VWF-ex44-HPLC-F44	CAGGAGGCGTAGGTAAGAG
VWF-ex44-HPLC-R44	GAATGAGATGAAACCAAGGTC
VWF-ex45-HPLC-F45	TGAGAGAAGAGCACATTCCC
VWF-ex45-HPLC-R45	AGAGAGGCTTAAAGGTGGTG
VWF-ex46-HPLC-F46	CAAAGAGAGCAGCCTGCTC
VWF-ex46-HPLC-R46	ACTGCTTTACAATGACTTGCC

Table 3.2 Oligonucleotide primers for PCR amplification and HRM analysis of the *VWF* gene (Continue)

Primer Name	**Primer Sequence 5'-3'**
VWF-ex47-HPLC-F47	AGACAGGGTATGAGAGTGAG
VWF-ex47-HPLC-R47	TATTTAAAGACCGCAGTGAGG
VWF-ex48-HPLC-F48	AAATCAGCCTACTTACAATTGG
VWF-ex48-HPLC-R48	GAAGCAAGATGGTGATATGTG
VWF-ex49-HPLC-F49	AGTCAAGCTCATGGTTTGAAG
VWF-ex49-HPLC-R49	CAGAAGAATCTTGTTCTTAGAG
VWF-ex50-HPLC-F50	GTGACCTGAAAGCTGTCTAC
VWF-ex50-HPLC-R50	TTGCTAATGGGTTTCAAGGAG
VWF-ex51-HPLC-F51	CTGAAGAGTGTTCTCTAGAAC
VWF-ex51-HPLC-R51	TAGAATGTAACTAAGGATAGGTA
VWF-ex52A-HPLC-F52-A	TTCCCACCATTGTGAAGCTC
VWF-ex52A-HPLC-R52-A	TGGCATTGAGAACCTCATGG
VWF-ex52B-HPLC-F52-B	ACCATGAGGTTCTCAATGCC
VWF-ex52B-HPLC-R52-B	AGTATCTCACACTGACACTGA
VWF E18-F	GCAACTCTGAGTCTCTTGAA
VWF E18-R	CCTGCCTACAAGAAAACTGAA
VWF E19-F	GGCTTTAGATCAGTCACTGTG
VWF E119-R	GTGCACCCTCACTCCACCCGC
VWF E20-F	CTGTGTTCCTTCATTGCCTC
VWF E20-R	AGATCCACAGAACCCAACCT
VWF E21-F	AATCTTCTGGTCTGGTGAGA
VWF E21-R	CCTCATCCTCTTTAATGGCT
VWF E24-F	GTGGGTGGTATGACCTCC
VWF E24-R	GCCAAGCCTTGGGACCGT
hVWF-exon 28-nt 1-628F	GAAGTGTCCACAGGTTCTTC
hVWF-exon 28-nt 1-628R	ATGCCCACCGGGATCACAAT
hVWF-exon 28-nt 505-1022F	CTGAAGCCTCCCGCATCA
hVWF-exon 28-nt 505-1022R	AGTCACCATGTAGGAGTACT
hVWF-exon 28-nt 899-int28(14)F	GGACAAAATTGGTGAAGCCG
hVWF-exon 28-nt 899-int28(14)R	AGGTGCCAGCATACCAGGT

4. METHODS

4.1. Analysis of Genomic DNA

4.1.1. Manual DNA Extraction from White Blood Cells

In order to extract adequate amount of DNA ten ml peripheral blood was collected from patients and their relatives into K'EDTA containing tubes and kept at 4ºC in order to prevent coagulation before extraction. Nine ml of collected blood was used in the procedure and remaining one ml was stored -70ºC.

For the manual DNA extraction from white blood cells method, blood was initially collected into sterile 50 ml Falcon tubes, after addition of 30 ml lysis buffer (stored in 4ºC) the tubes were incubated at 4ºC for 20 minutes for 15 minutes for lysis of leukocyte membranes. Samples were then centrifuged at 5000 rpm for ten minutes at 4ºC to collect the nuclei. The supernatant was discarded and the pellet was washed with lysis buffer. Samples were centrifuged for an additional 10 minutes after addition of 10 ml lysis buffer in order to wash nuclear pellet.

Three ml nuclei lysis buffer, proteinase K(150 μg/ml) and SDS (0,14 per cent) were added to nuclear pellet, the samples were incubated at 37 ºC overnight for adequate disruption of nuclear envelope and degradation of proteins. Remaining proteins were salted out by addition of ten ml 2,5 M NaCl and centrifugation at 5000 rpm at room temperature for 20 minutes. After centrifugation the supernatants were taken into sterile falcon tubes and DNA was precipitated by addition of two volumes of absolute ethanol. DNA forming a white precipitate on the solution surface was fished out by the help of a sterile pipette tip and collected into sterile eppendorf tubes. After EtOH evaporated, DNA was dissolved in TE buffer and stored at 4ºC for further analysis. (Miller *et al*., 1988).

4.1.2. DNA Extraction from White Blood Cells with MagNA Pure Compact

DNA samples that were used in the HRM analysis were extracted from peripheral blood samples in the MagNA Pure compact, Roche Diagnostics, Germany. MagNA Pure Nucleic Acid Isolation Large Volume Kit, Roche Diagnostics, Germany was used as described in the user's manual.

In summary, one ml peripheral blood samples collected from patients and their relatives were added into sample tubes provided within the kit and loaded into the sample rack. After insertion of the reaction cartridges into cartridge rails, required protocol was selected from the protocol list. Then, patient IDs and barcode numbers for elution tubes were entered in the required fields of the program. Elution tubes were labeled and loaded into the elution tube line in the rack. Following a final control of the information entered, the run was started. In each run, DNAs from eight samples were extracted concomitantly.

4.1.3. Analysis by Agarose Gel Electrophoresis

100 ml of 0.5X TBE buffer was added adequate agarose (1 g for 1% gel and 2 g for 2 % gel) to prepare one per cent (w/v) agarose gel which is used to visualize whole genomic DNA and to prepare two per cent (w/v) agarose gel to visualize PCR products. After addition of agarose, the suspension was boiled for preparing a homogenous gel until no filaments of agarose was observed. As soon as the solution cooled down to about 50 ºC, five µl EtBr (10 mg/ml) was added in order to visualize DNA under ultraviolet (UV) light. Hot liquid was poured into the electrophoresis plate and appropriate comb was put into before the gel polymerizes, which is achieved in 15 minutes in room temperature. 1 µl DNA sample was mixed with 1 µl loading dye and 4 µl H_2O and loaded into the wells on the gel. The gel was submerged into the TBE buffer in the electrophoresis tank and run at 150 volts for 15 minutes.

4.1.4. Visualization of DNA Under Ultraviolet (UV) Light

DNA on the agarose gel was visualized in the GelDoc Documentation System, BioRad, USA and the gel images were stored in the "tiff" format.

4.1.5. Analysis by Spectrophotometer

The concentration of genomic DNA was measured by. NanoDrop spectrophotometer, NanoDrop Inc. USA calibrated with 2 µl of water after the cleaning of sample pedestal. 1.5 µl of water or elution buffer from MagNA Pure Nucleic Acid isolation Large Volume Kit were used as blank. 1,5 µl of DNA sample was loaded onto the sample pedestal. After measurement by the device the program calculates DNA concentration with the use of observed absorption. If the sample was concentrated beyond the measurement limit of spectrophotometer it was diluted with sterile distilled H_2O.

4.2. Investigation of Mutations

4.2.1. PCR Amplifications of *F11* gene

The promoter sequence and the coding regions of the *F11* gene were amplified in 15 amplicons. Optimization of the PCR amplification of the promoter, exon 1 and exon 2 regions were done within the framework of this thesis. For all amplicons 100 ng genomic DNA was used as template. The reaction mixture contained 1X PCR buffer with 1,5 mM $MgCl_2$ (final concentration), 0,2 mM each dNTP, 0,5 mM of each primer and 1,5 U Taq DNA Polymerase. In a final volume of reaction mixture was 50 µl.

The thermal cycle comprised an initial denaturation step of 5 min at 94^{o}C. It was followed by 30 cycles of amplification step, which consisted incubations at 30 seconds at 94 ^{o}C, 45 seconds at appropriate annealing temperature (table 4.1) and 60 seconds at 72 ^{o}C. Thermal cycle ends with a final elongation step of 5 minutes at 72 ^{o}C.

Thermal cycle for *F11* gene amplicons

Initial Denaturation	94°C	5 min.	
Amplification	94 °C	30 sec.	30 cycles
	A.T*.	45 sec.	
	72 °C	60 sec.	
Final elongation	72 °C	60 sec.	

*A.T.= Annealing temperature

Figure 4.1. Standard thermal cycle for amplification of *F11* gene amplicons

The PCR products were visualized on 2% agarose gels, and compared with a 100 bp DNA ladder (Fermentas, Lithuania) for length determination. The gels were checked under UV light and documented.

Table 4.1. Annealing temperatures of amplicons of *F11* gene.,

Amplicon	**Annealing Temperature**
Promoter	58 °C
Exon 1	60,3 °C
Exon 2	58,5 °C
Exon 3	56,6 °C
Exon 4	55 °C
Exon 5	55 °C
Exon 6	55,6 °C
Exon 7	55,6 °C
Exon 8/9	63,9 °C
Exon 9/10	61,9 °C
Exon 11	61,9 °C
Exon 12	62,6 °C
Exon 13	60,3 °C
Exon 14	58 °C
Exon 15	57 °C

4.2.2. PCR Amplification of *VWF gene* exon 28

1379 bp long exon was divided into three overlapping amplicons for PCR amplification. PCR amplifications were conducted with the PCR primers shown in Table 3.2. 75-100 ng genomic DNA was used as template for PCR reaction which contained 1X PCR buffer with 1,5 mM $MgCl_2$ (final concentration), 0,2 mM each dNTP, 0,5 mM of each primer and 1,5 U Taq DNA Polymerase in a final volume of 50 µl. The thermal cycle comprised an initial denaturation step of 5 minutes at 94°C. 30 cycles contained denaturation fort 30 seconds at 94 °C, annealing for 30 seconds at 58, 3 °C and synthesis for 30 seconds at 72 °C. Thermal cycle ended with a final elongation step for 5 minutes at 72 °C.

Thermal cycle for VWF Exon 28

Initial Denaturation	94°C	5 min.	
Amplification	94 °C	30 sec.	30 cycles
	58.3 °C	30 sec.	
	72 °C	30 sec.	
Final elongation	72 °C	60 sec.	

Figure 4.2. Standard thermal cycle for amplification of VWF exon 28

The PCR products were visualized on 2% agarose gel compared with a 100 bp DNA ladder (Fermentas, Lithuania) for length determination. The gels were checked under UV light and documented.

4.2.3. Long PCR Amplification of *VWF gene* exon 28

In order to amplify the whole exon a long PCR reaction was designed with the same reaction conditions and with primers hVWF exon 28-nt 1-628 F and hVWF exon 28-nt 899-int 28 R. The altered thermal cycle comprised the steps indicated in Figure 4.3.

Thermal cycle for VWF Exon 28 long PCR

Initial Denaturation	94°C	5 min.	
Amplification	94 °C	30 sec.	
	60 °C	30 sec.	30 cycles
	72 °C	30 sec.	
	94 °C	45 sec.	
	60 °C	45 sec.	30 cycles
	72 °C	60 sec.	
Final elongation	72 °C	60 sec.	

Figure 4.3. Thermal cycle for VWF gene Exon 28 long PCR amplification

4.2.4. Purification of PCR Products for DNA Sequencing

In order to prepare PCR products for DNA sequencing they were purified using GeneMark PCR Clean Up Kit (GeneMark, Taiwan), as described in the user's manual Briefly, equal volume of Binding Buffer (BB) was added to the PCR products and shortly vortexed. The solution was transferred into spin column, which was inserted into collection tube, centrifuged at top speed and the filtrate was discarded. The membrane was washed two times with 700 ml Washing Buffer and centrifuged. After discarding the filtrate the column was centrifuged for 5 minutes in order to get rid of excess ethanol in the wash buffer. The spin column was transferred into a sterile microcentrifuge tube. 50 ml Elution Buffer was applied on top of the membrane. The column was incubated at room temperature for a minute and centrifuged for a minute. The filtrate was transferred into a labeled sterile microcentrifuge tube.

Purified PCR products were checked on 2 per cent agarose gel and visualized under UV light.

4.2.5. Purification of PCR Products from Agarose Gels

Target DNA fragments are extracted from two per cent agrose gel using QIAEX II gel extraction kit as described in user's manual. Briefly, all of the PCR product (approximately 45 µl) was loaded on two percent agarose gel and run until fragments are separated. Target fragment is excised from the gel using a scalpel and weighed. The gel slice is put in a microcentrifuge tube and three volumes of Buffer QX1 added to the tube and resuspended by vortexing. 30 µl QIAEX II was added to the tube and incubated at 50 ^{o}C for 10 minutes. Sample was centrifuged for 30 seconds and supernatant was removed. Pellet was washed with 500 µl Buffer PE. The pellet was air dried and resuspended in 10 mM Tris-Cl. The sample was centrifuged for 30 seconds and supernatant was pippeted into a clean tube.

4.2.6. DNA Sequencing

Adequate amount of purified PCR products were sent to Refgen Biotechnology inc. Ankara for automated sequencing. The results were obtained online as ABI document and analyzed in the Finch TV version 1.4.0, (Geospiza Inc. USA) chromatograph analyzing program.

4.2.7. HRM Analysis of *VWF* Gene

In order to analyze *VWF* gene by HRM analysis, the amplification conditions for each amplicon was optimized so that each specific amplification reaction entered the logarithmic increase step before the 35th cycle.

4.2.7.1. Reaction Mixture. For HRM analysis, HRM Master Mix, (Roche Diagnostics, Germany), kit was used and the reaction mixture was prepared as described in the user's manual. The reaction mixture contained DNA, 1X Master Mix, which contained specific Taq Polymerase enzyme, buffer and dye, primer pair (listed in Table 3.2), MgCl2, DMSO (when required) and H2O in 20 µl reaction volume. The concentration of each item was adjusted for each amplicon and listed in the Table 4.2.

In order to have an internal control two reactions were conducted for each sample in replicate. Moreover, to introduce an artificial heterogeneity, spike reactions were also conducted by adding DNA sequence of which was known, to each sample of DNA as additional control, so that, wild type or mutant homozygous samples could be differentiated.

Reaction mixture was loaded into different wells on 96 well plate and inserted into LC480 Real Time Platform.

4.2.7.2. Reaction Thermal Profile. The thermal cycle contained three programs, for, pre-incubation, amplification, high resolution melting followed by cooling. Programs other than amplification were the same for all amplicons. Program details are indicated in Table 4.2. The fluorescence history for all three programs is shown in Figure 4.4.

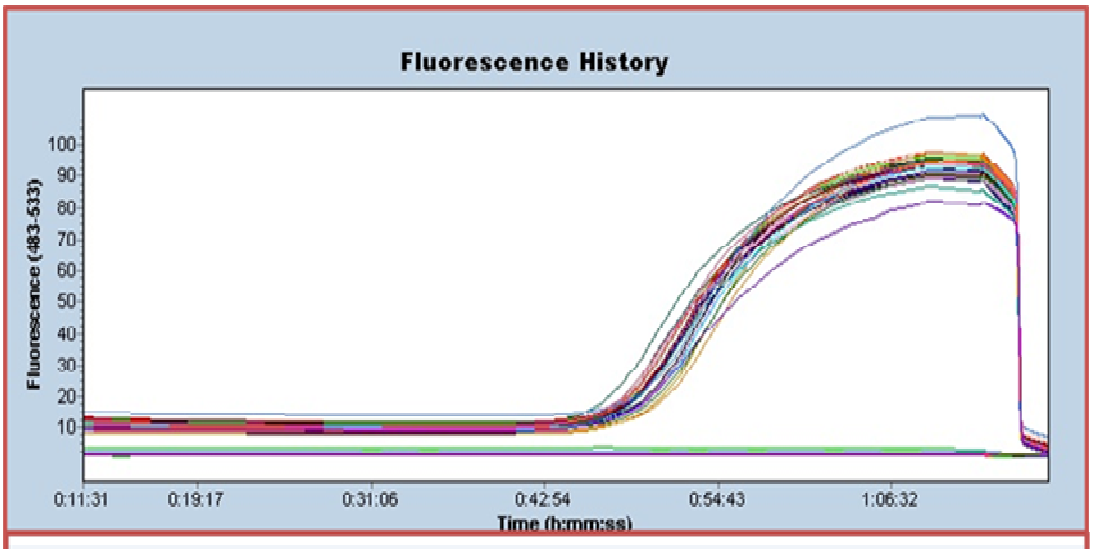

Figure 4.4. Florescence history obtained in a HRM reaction.

Table 4.2. Thermal profile of HRMA reaction (hold times differs in indicated range between amplicons)

Program	Target (^{o}C)	Acquisition mode	Hold (mm:ss)	Ramp rate (^{o}C/s)	Acquisition (per ^{o}C)
Pre-incubation	95	None	10:00-15:00	4.4	none
Amplification (45 cycles)	95	None	00:10-00:30	4.4	none
	Primer dependent	None	00:10-00:30	2.2	none
	72	Single	00:10-00:30[1]	4.4	none
High Resolution melting	95	None	01:00	4.4	none
	40	None	01:00	2.2	none
	65	None	00:01	1	none
	95	Continuous	--	--	25

4.2.7.3. Evaluation of PCR amplification. After the run was completed the reactions that showed amplification were evaluated for specificity and whether they entered logarithmic increase phase before 35th cycle. In order to evaluate specific melting curve for each reaction well was drawn by Tm determination program. Specific amplification (single amplicon in each reaction) was indicated by single melting peak and Tm value for each reaction (Figure 4.2). Double Tm values within 2.5 ^{o}C, also indicated single amplicon (personal communication with product representative). For the second part absolute quantification/ 2nd derivative program was used. When the program was run for selected wells (i.e. reactions) Cp values in the result screen indicated the cycle at which the reaction entered the log phase.

[1] Depent on the lenght of amplicon and determined by dividing amplicon lenght by 25.

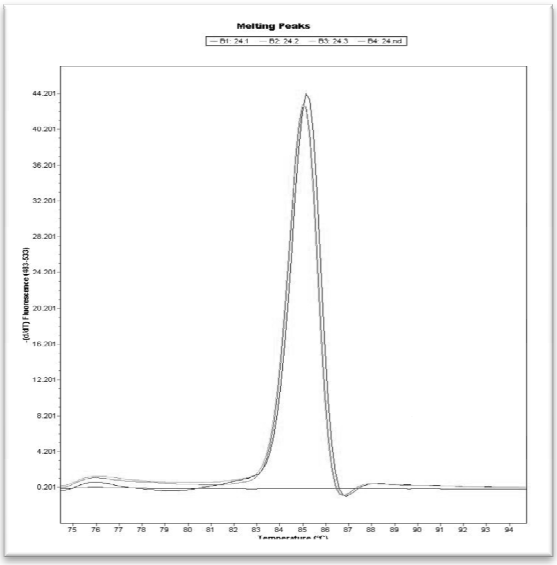

Figure 4.5. Melting peak for three reactions and a negative control. Single peak indicates specific amplifications.

Additionally, PCR products were also run on 2% agarose gel and visualized under U.V. light to check the product length. And selected amplicons are purified and sent for sequencing to see if the target genomic region is amplified.

4.2.8. Restriction Enzyme Analysis

In order to confirm the mutation in *F11* exon 13, PCR products were cut by Fok 1 enzyme. When cut by Fok I enzyme PCR product of exon 13 produces two fragments, 150 bp and 50 bp in length. The enzyme abolishes the cut site of normal sequence.

After PCR amplification, 10 µl of PCR product was used in the reaction, 20 µl in total. Reaction mixture also contained 2 µl buffer and 1 µl enzyme. The mixture is incubated at 37 ^{o}C overnight.

Fok I Restriction Reaction		
PCR product	**10** µl	O/N at 37^{O}C
Buffer	2 µl	
Enzyme	1 µl	
H_2O	7 µl	

Figure 4.6. Fok I restriction enzyme digestion reaction mixture.

5.RESULTS

5.1. Mutational Analysis of *Factor 11* Gene

Two families were included in the investigation for mutations in the *F11* gene. Promoter sequences and the coding region of *F11* gene were amplified in 15 amplicons. PCR products of all amplicons are shown in Figure 5.1. The product sizes and the ranges of the amplified regions are given in the Table 5.1. The schematic presentations of the amplicons are shown in Figure 5.2.

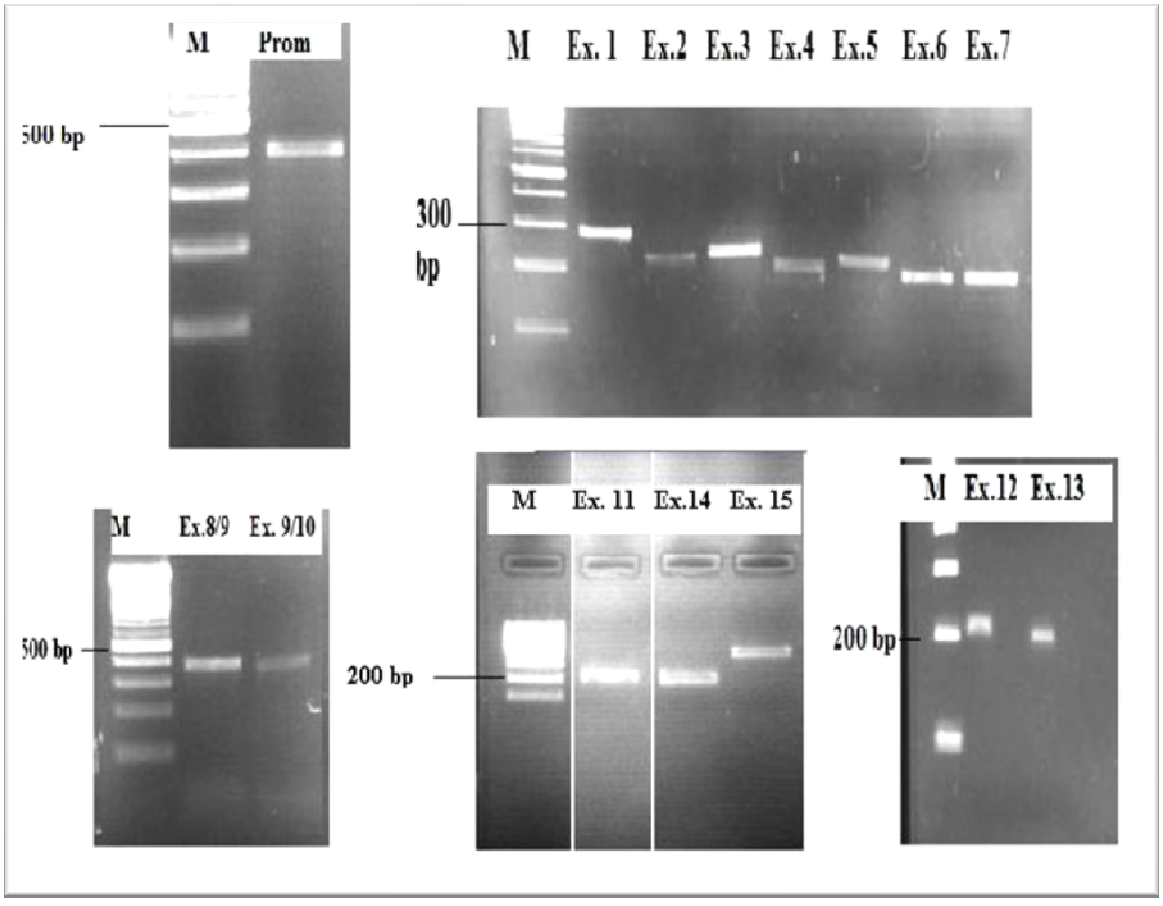

Figure 5.1.PCR products of 15 amplicons of *F11* gene visualized on 2 % agarose gel. (M: DNA length marker, 100 bp)

Table 5.1. PCR amplicons of *f11* gene

Amplicon	Length (bp)	Amplified region	Amplicon	Length (bp)	Amplified region
Exon1	**292**	**c.1-c172**	Exon2	**231**	**c.172-228**
Exon 3	**237**	**c.228-391**	Exon 4	**211**	**c.391-498**
Exon 5	**235**	**c.498-658**	Exon 6	**211**	**c.660-768**
Exon 7	**211**	**c.768-928**	Exon 8/9	**359**	**c.936-1201**
Exon 9/10	**359**	**c.1189-1293**	Exon 11	**214**	**c.1293-1477**
Exon 12	**214**	**c. 1477-1653**	Exon 13	**199**	**c.1653-1749**
Exon14	**206**	**c.1757-1889**	Exon 15	**398**	**c.1889-2217**
Promoter	**329**	**-370 - -42**			

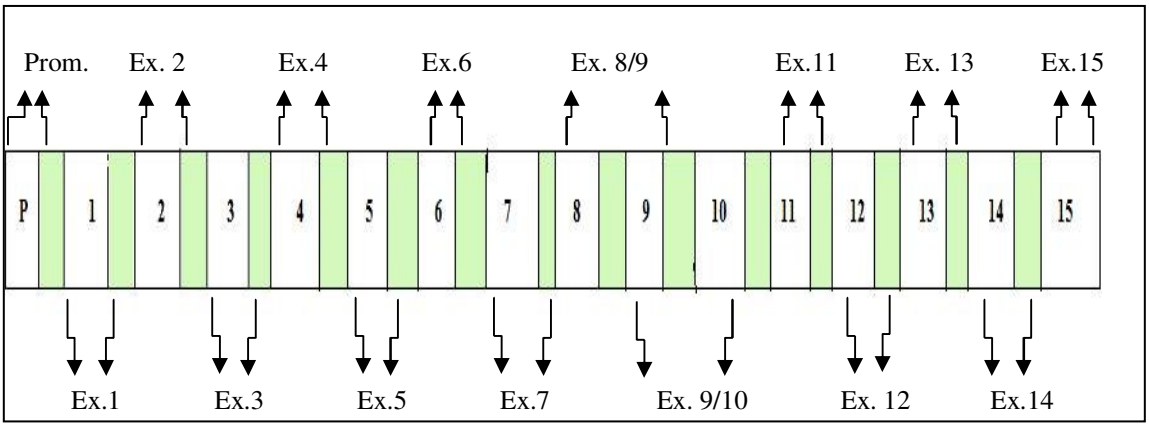

Figure 5.2. Amplification schema for *F11* gene.

5.1.1. Family 1

The first patient (1HC1) was an 18 years old male. In his pre-operation survey he was diagnosed to have FXI deficiency (FXI:Ag 12%) (Hemophilia C). He was of "O" blood group and his FVIII:C and VWF levels were below the normal range. In earlier investigations mutational analysis of *F8* gene by PCR and complete DNA sequencing did not reveal any changes in patient 1HC1. Sequencing of his VWF exon 28 dit not reveal any

change and his DNA sample was also included in the mutational screening of the VWF gene by HRM analysis. Hematological data of the family is given in Table 5.2.

Table 5.2. Hematological data of the members of family 1

Coagulation Factor	FXI	FVIII	VWF	VWF: ric	VWF: Ag	FVII	FV	Blood group
Normal Range / Family members	50-120	50-120	50-160	50-150	50-150	50-120	70-120	
1 HC 1 (Index)	12,2	47,9	69	79	64	82,5		O
1 HC 2 (Mother)	56	34	56			75	66	
1 HC 3 (Father)	40	30 89	57	98	106	78	96	
1 HC 4 (Sister)	66	39 68	59	69	57	154	57	

Molecular genetic analysis of the *F11* gene was carried out on patient 1HC1. The amplification of the promoter sequence and the coding regions was followed by DNA sequence analysis. A nonsense mutation (c. 1556 G>A) was identified in exon 13 in heterozygous condition. The mutation was in the serine protease domain and caused the truncation of the protein at Triptophan 501 residue (if signal peptide is not counted) (Figure 5.3). Truncated FXI is probably not produced and disease is caused by haploinsufficiency. The same mutation was identified in the father (1HC3) in heterozygous condition, but mother (1HC2) and the sister (1HC4) were homozygous normal. The mutation was cited in the FXI mutation database (Iijima, 2000, www.factorxi.com/www.factorxi.org).

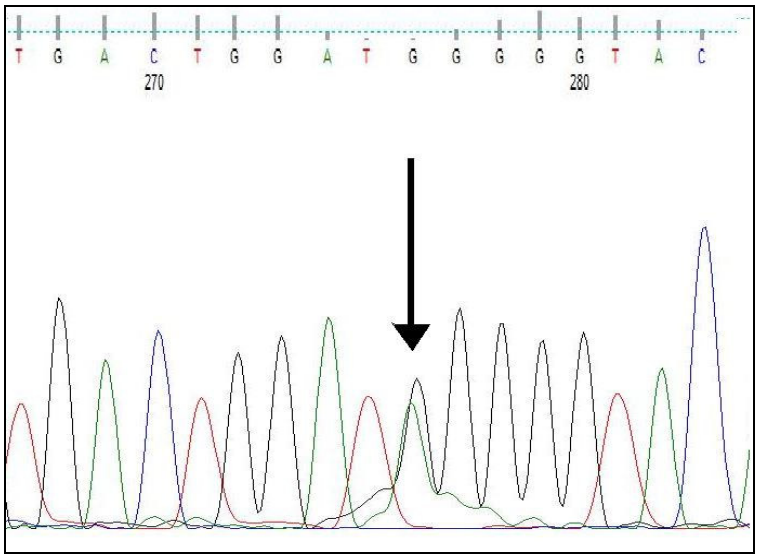

Figure 5.3. DNA sequence analysis of *F11* exon 13 in patient 1HC1. The arrow indicates the c.1556 G>A transition in heterozygous condition.

The mutation in exon 13 was also confirmed by Restriction Enzyme digestion. The mutation abolished the Fok I site, which normally produces two fragments of 150 bp and 50 bp in length. The uncut normal sequence fragment (200 bp) and the longer fragment of mutant sequence cut by Fok I enzyme are seen in the heterozygous patient and in his father (Figure 5.4.). Short 50 bp fragment is not visible.

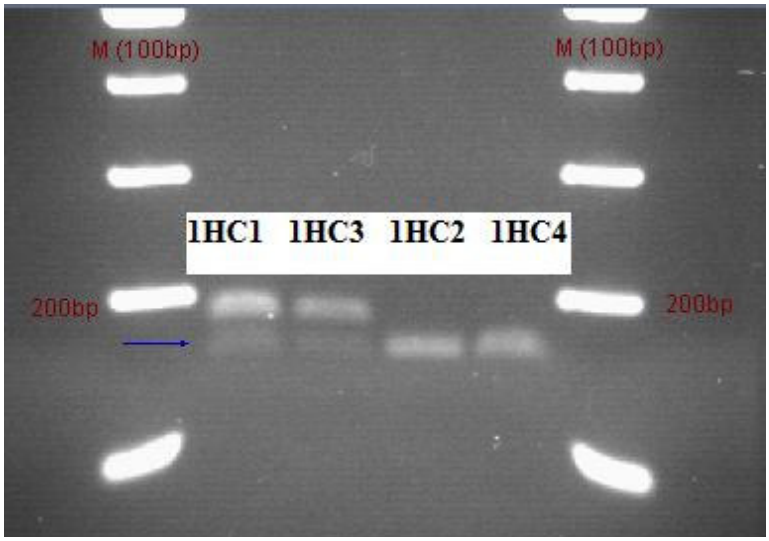

Figure 5.4. RE analysis of exon 13 PCR products in Family 1. Arrow indicates the lower band, which is produced in the result of restriction reaction.

Additionally, in exon 8, one polymorphism (c.901 T>C, Phe 203 Leu (301)) was detected in heterozygous condition. The polymorphism was reported in the FXI polymorphism database. The *F11* genotype of patient 1HC1 and the family pedigree are given in Table 5.3 and in Figure 5.5, respectively.

Table 5.3. *F11* phenotype of patient 1 HC 1

1 HC 1 FXI Sequencing Results					
Exon1	Target Sequence	**c.1-c172**	Exon2	Target Sequence	**c.172-228**
	Sequence Read	all		Sequence Read	all
	Comment	**No change**		Comment	**No change**
Exon 3	Target Sequence	**c.228-391**	Exon 4	Target Sequence	**c.391-498**
	Sequence Read	all		Sequence Read	all
	Comment	**No change**		Comment	**No change**
Exon 5	Target Sequence	**c.498-658**	Exon 6	Target Sequence	**c.660-768**
	Sequence Read	all		Sequence Read	c.714-768
	Comment	**No change**		Comment	**No change**
Exon 7	Target Sequence	**c.768-928**	Exon 8/9	Target Sequence	**c.936-1201**
	Sequence Read	all		Sequence Read	c.989-1194
	Comment	**No change**		Comment	**c.901 T>C Phe 203 Leu (301)** Ht. polymorphism
Exon 9/10	Target Sequence	**c.1189-1293**	Exon 11	Target Sequence	**c.1293-1477**
	Sequence Read	c.1202-1293		Sequence Read	All
	Comment	**No change**		Comment	**No change**

Table 5.3. *F11* phenotype of patient 1 HC 1 (Continued)

Exon 12	Target Sequence	**c. 1477-1653**	Exon 13	Target Sequence	**c.1653-1749**
	Sequence Read	All		Sequence Read	All
	Comment	**No change**		Comment	**c.1556 G>A mt.W501X (519)** heterozygous recurrent mutation
Exon14	Target Sequence	**c.1757-1889**	Exon 15	Target Sequence	**c.1889-2217**
	Sequence Read	c.1800-1889		Sequence Read	All
	Comment	**No change**		Comment	**No change**
Promoter	Target Sequence	**-370 - -42**			
	Sequence Read	-340- -42			
	Comment	**No change**			

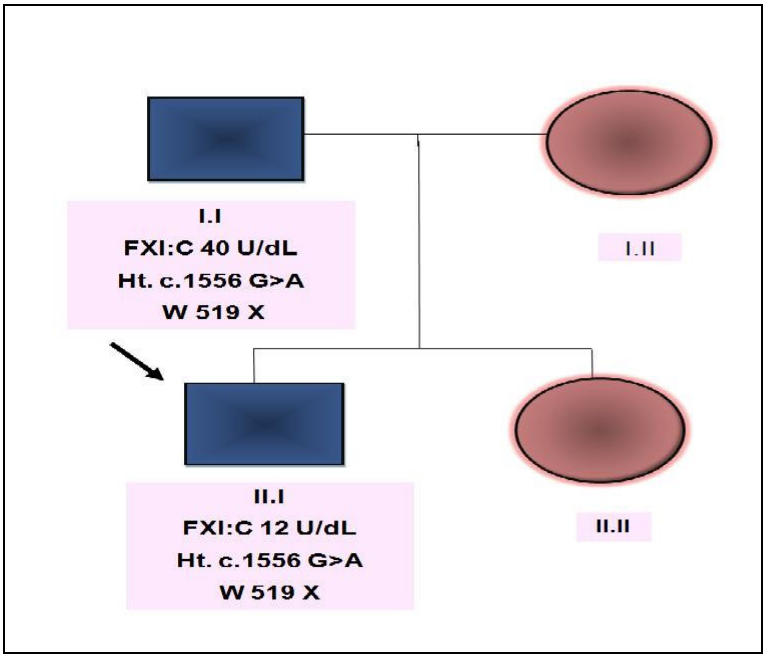

Figure 5.5. Pedigree for Family 1. Arrow indicates the index patient (1HC1)

In the result of molecular analysis of *F11* gene in family 1, FXI deficiency in patient 1HC1 was confirmed and the autosomal dominant inheritance of the disease was demonstrated. Moreover, father was also diagnosed to have FXI deficiency, though he had no bleeding history. As far as the hematological data imply there are some other coagulation factor deficiencies present in the phenotype of the family members, who have low FVIII and VWF levels. Additionally, mother and the sister have low FV levels. This data demonstrates that the family is a good example to study interactions among procoagulants and relationship with the phenotype.

5.1.2. Family 2

The second patient (2HC5) was a pregnant female who had been diagnosed to have Hemophilia C. She was referred to our laboratory for prenatal diagnosis. Hematological data of the index patient and her father is given in the Table 5.4.

Table 5.4. Hematological data of family 2.

Screen	Normal Value	Index patient	Father
FVIII	53-170(%)	134(%)	
FIX	60-170(%)	118(%)	
FXI	**70-150(%)**	**21(%)**	**24(%)**
aPTT	**25-40 sec**	**44,2 sec**	
Blood group		O Rh+	ARh+

When the promoter sequence and coding regions of *F11* gene of the index patient were analyzed by direct DNA sequence analysis, a A>C transversion (c. 151 A>C) was identified (Figure 5.6.). The family members including patient's parents, two brothers, the spouse and the fetus were analyzed for this mutation. The same mutation was also identified in the paternal parent and fetus in heterozygous condition (Figure 5.6.). the patient did not have any other changes in the *F11* gene. Her genotype is shown in Table 5.5. The pedigree of the family is given in Figure 5.7. When nucleotide sequence of human *F11* gene was compared with its homologs in seven other mammals it is seen that the c.151

adenine residue is conserved in all of these species Figure 5.8. The mutation was not present in FXI mutation database (www.factorxi.com), i.e. it is a novel mutation.

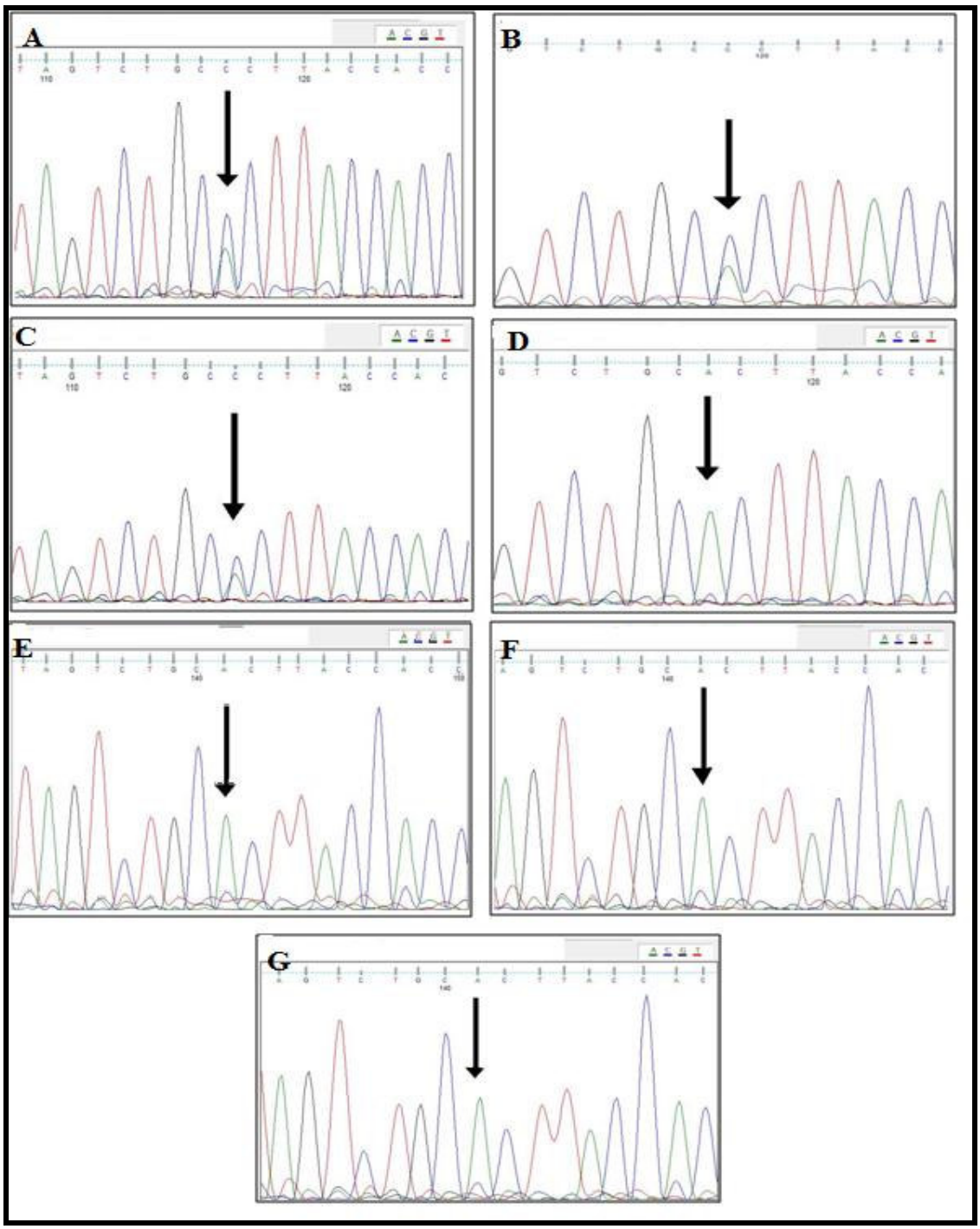

Figure 5.6. *F11* Exon 3 DNA sequence analysis of the members of family 2. Arrows indicate the conversion. Panels A-G belong to index, fetus, father, mother, the spouse and two brothers, respectively.

Table5.5. *F11*genotype of patient 2 HC 5

2 HC 5 FXI Sequencing Results					
Exon1	Target Sequence	**c.1-c172**	Exon2	Target Sequence	**c.172-228**
	Sequence Read	All		Sequence Read	All
	Comment	**No Change**		Comment	**No Change**
Exon 3	Target Sequence	**c.228-391**	Exon 4	Target Sequence	**c.391-498**
	Sequence Read	All		Sequence Read	All
	Comment	**c151 A>C T33P** Ht. novel mutation		Comment	**No Change**
Exon 5	Target Sequence	**c.498-658**	Exon 6	Target Sequence	**c.660-768**
	Sequence Read	All		Sequence Read	All
	Comment	**No Change**		Comment	**No Change**
Exon 7	Target Sequence	**c.768-928**	Exon 8/9	Target Sequence	**c.936-1201**
	Sequence Read	All		Sequence Read	All
	Comment	**No Change**		Comment	**No Change**
Exon 9/10	Target Sequence	**c.1189-1293**	Exon 11	Target Sequence	**c.1293-1477**
	Sequence Read	All		Sequence Read	1308-1477
	Comment	**No Change**		Comment	**No Change**
Exon 12	Target Sequence	**c. 1477-1653**	Exon 13	Target Sequence	**c.1653-1749**
	Sequence Read	All		Sequence Read	All
	Comment	**No Change**		Comment	**No Change**
Exon14	Target Sequence	**c.1757-1889**	Exon 15	Target Sequence	**c.1889-2217**
	Sequence Read	1787-1889		Sequence Read	All
	Comment	**No change**		Comment	**No Change**
Promoter	Target Sequence	**-370 - -42**			
	Sequence Read	-340- -42			
	Comment	**No Change**			

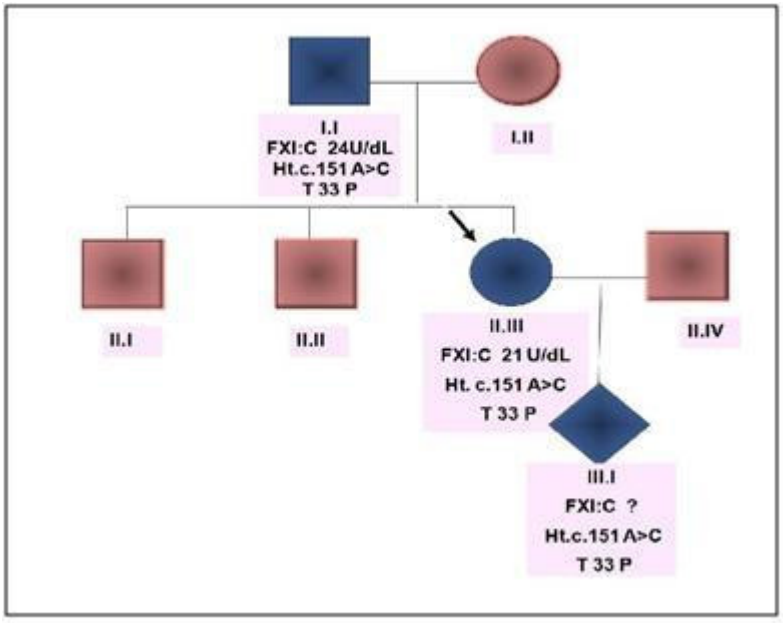

Figure 5.7. Pedigree for Family 2. Arrow indicates the index patient.

The c.151 A>C transversion resulted in the substitution of T with P at position 33 corresponding to the Apple 1 domain of the FXI protein. Apple domains are present in many other non-related human proteins and also FXI homologs are present in other species. Amino acid comparisons in 7 other species showed that T 33 is evolutionally conserved, indicating the functional significance of the amino acid in protein structure (Figure .8).

Bos_taurus	TGCCAAATCATCTGCACCCACCACCCAAGGTGCTT
Canis_familiaris	TGCCAGCTTGTCTGCACCCACCACCCACGCTGTTT
Homo_sapiens	TGCCAGGTAGTCTGCACTTACCACCCAAGATGTTT
Macaca_mulatta	TGCCAGGTGGTCTGCACTCACCACCC
Monodelphis_domestica	TGCCAAATAGTGTGTACCTATCACCCAAGATGTTT
Mus_musculus	TGCCGCTTGGTCTGCACTCACCACCCACGGTGCTT
Pan_troglodytes	TGCCAGGTAGTCTGCACTTACCACCCAAGATGTTT
Rattus_norvegicus	TGCCAGCTGGTCTGCACACCACCCACGTTGCTT

ACC/ACT/ACA encodes amino acid threonine

Figure 5.8. Nucleotide sequence comparison of human *F11* gene with its homologues in seven mammalian species. C151 Adenine residue is shown in blue.(obtained from www.ensembl.org)

HRMA was conducted to search for the presence of the mutation in the normal population. Exon 3 of the patient 2HC5, the fetus, one family member who do not carry the mutation and 9 unrelated people were amplified and high resolution melting analysis was conducted in LC 480 real-time platform. GeneScan software analysis revealed the patient and the fetus to have distinct melting curves from the healthy family member (Figure 5.9) The HRMA analysis grouped 9 normal individuals with the healthy family member, indicating that they do not bear the mutation (figure 5.10). The whole analysis was conducted in a time period as short as 90 minutes. Results of the HRMA showed that the c.11 A>C is not a polymorphism and is the causative mutation for FXI) deficiency in the family.

Mutational analysis of *F11* gene by direct DNA sequencing in family 2 and HRMA of the mutation in normal population resulted in the identification of a novel mutation, and also revealed that the apparently healthy father, who never bled and did not undergo any surgery had actually FXI deficiency. Although the fetus also diagnosed to have FXI deficiency, the pregnancy was not terminated due to the mild phenotypic effect of the mutation.

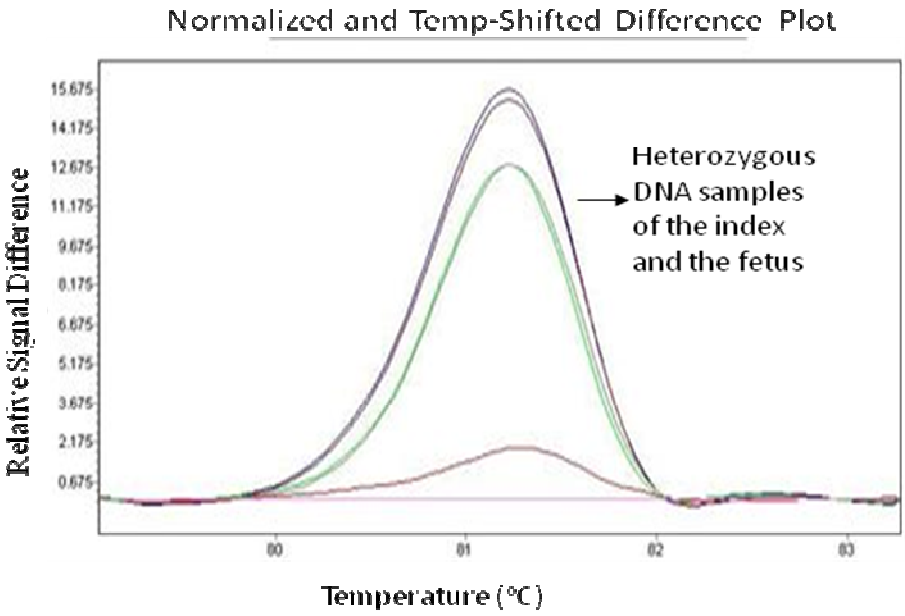

Figure 5.9. Normalized and temperature shifted difference plot generated after HRMA analysis of exon 3 of the *F11* gene of index patient (blue), fetus (green) and an unaffected family member (red). Index patient and the fetus have the heterozygous genotype and are grouped together.

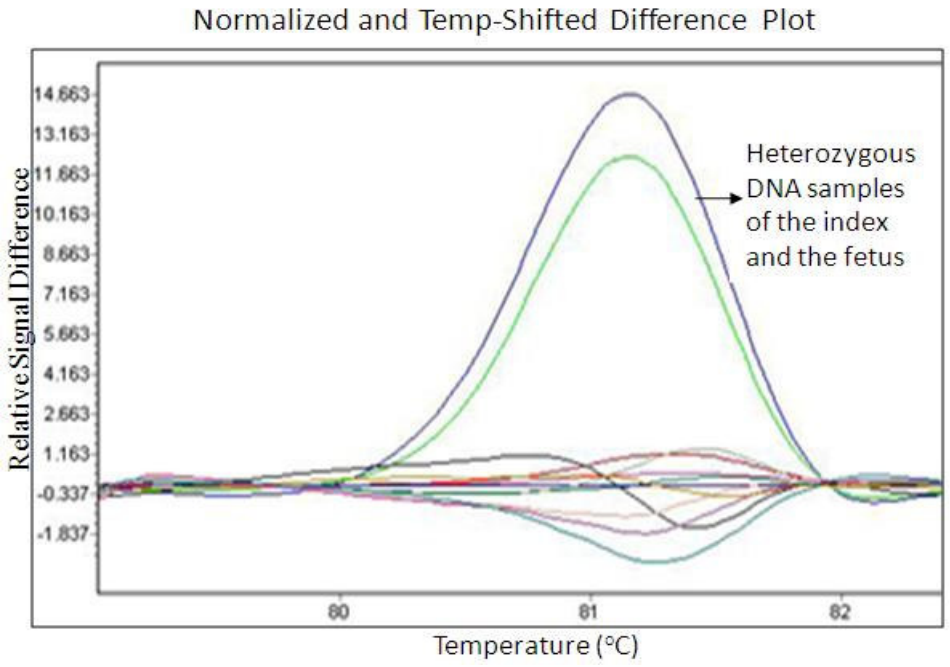

Figure 5.10. Normalized and temperature shifted difference plot generated in HRMA of exon 3 of the *F11* gene in normal individuals. HRM analysis grouped 9 apparently healthy individuals with the healthy family member.

5.2. Mutational Analysis of *VWF* Gene Exon 28

Exon 28 is the longest and highly polymorphic exon of the *VWF* gene (app.1500 bp), and bears many mutations listed in the databases. The amplicons of exon 28 and PCR products on agarose gel are given on Table 5.6. and Figure 5.11, respectively.

Table 5.6. Amplicons for VWF exon 28.

Amplicon	Region	Primer Pair	Length
VWF-28/1	c3675-57 to c.4256	hVWF-exon 28-nt 1-628F hVWF-exon 28-nt 1-628R	640
VWF-28/2	c. 4126 to c.4642	hVWF-exon 28-nt 505-1022F hVWF-exon 28-nt 505-1022R	516
VWF-28/3	c.4518 to c. 5053+14	hVWF-exon 28-nt 899-int28(14)F hVWF-exon 28-nt 899-int28(14)R	550

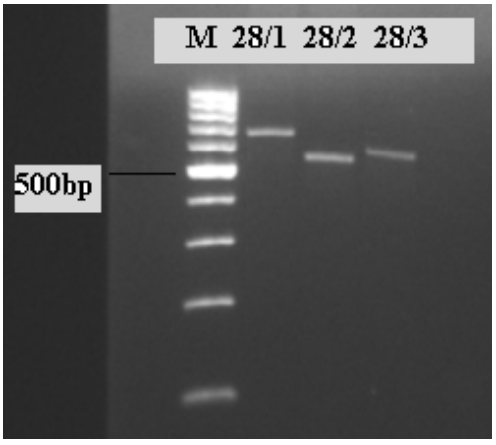

Figure 5.11. PCR products for three amplicons of exon 28 observed on 2 per cent agarose gel. (. M: 100 bp DNA length marker, 28/1: VWF exon 28 amplicon 1, 28/2: VWF exon 28 amplicon 2, 28/3: VWF exon 28 amplicon 3.)

Since Exon 28 has the highest mutation rate in the VWF gene, two patients (1HC1 and 8HBT194) suspected to have VWD were initially analyzed for the presence of mutations in Exon 28. Patient 1HC1 has FXI deficiency and also has low levels of FVIII and VWF. He has a recurrent nonsense mutation in *F11* gene, which was identified within the framework of this thesis. Patient 68HB194 has hemophilia B due to R403P mutation in exon 8 of *F9* gene, which was identified earlier in our laboratory.

In patient 1HC1 amplicon 2 could not be amplified because of a polymorphism in the primer binding site of the forward primer. Exon 28 was then amplified by long PCR in this patient. As a result the targeted amplicon and some other much smaller fragments were amplified. The target band of approximately 1500bp was extracted from the gel and purified (Figure 5.12). Direct DNA sequencing was limited with approximately 800 bp, thus, the PCR product was sequenced with both forward and reverse primers, and the complete sequence of the exon was read. Both patients did not have a mutation in exon 28 of their VWF genes.

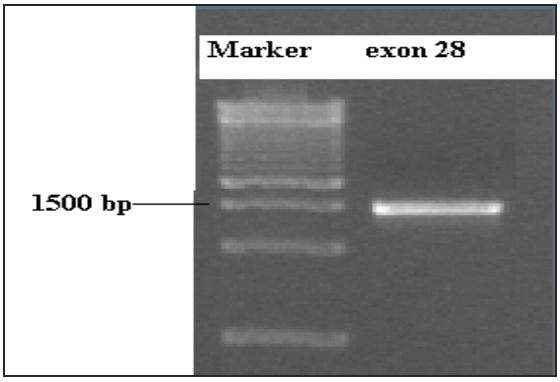

Figure 5.12. Image of long PCR product for *VWF* gene exon 28, observed in 2 per cent agarose gel. Marker: 500 bp DNA length marker

The sequence alteration in exon 28 observed in patient 1HC1 is shown in Figure 5.13. The polymorphic sites of VWF exon 28, and genotypes of the patients 1HC1 and 68HBT194 are listed in Table 5.7. Heterozygosity was observed at five sites, only one (c.3927 C>A) transition observed in patient 1HC1 was not listed in VWF database (http://www.vwf.group.shef.ac.uk). This change resides in codon 1309 coding for I, but do not casuse an amino acid substitution. Heterozygosity of four plymorphisms in two

patients indicated the presence of both alleles in the Turkish population.

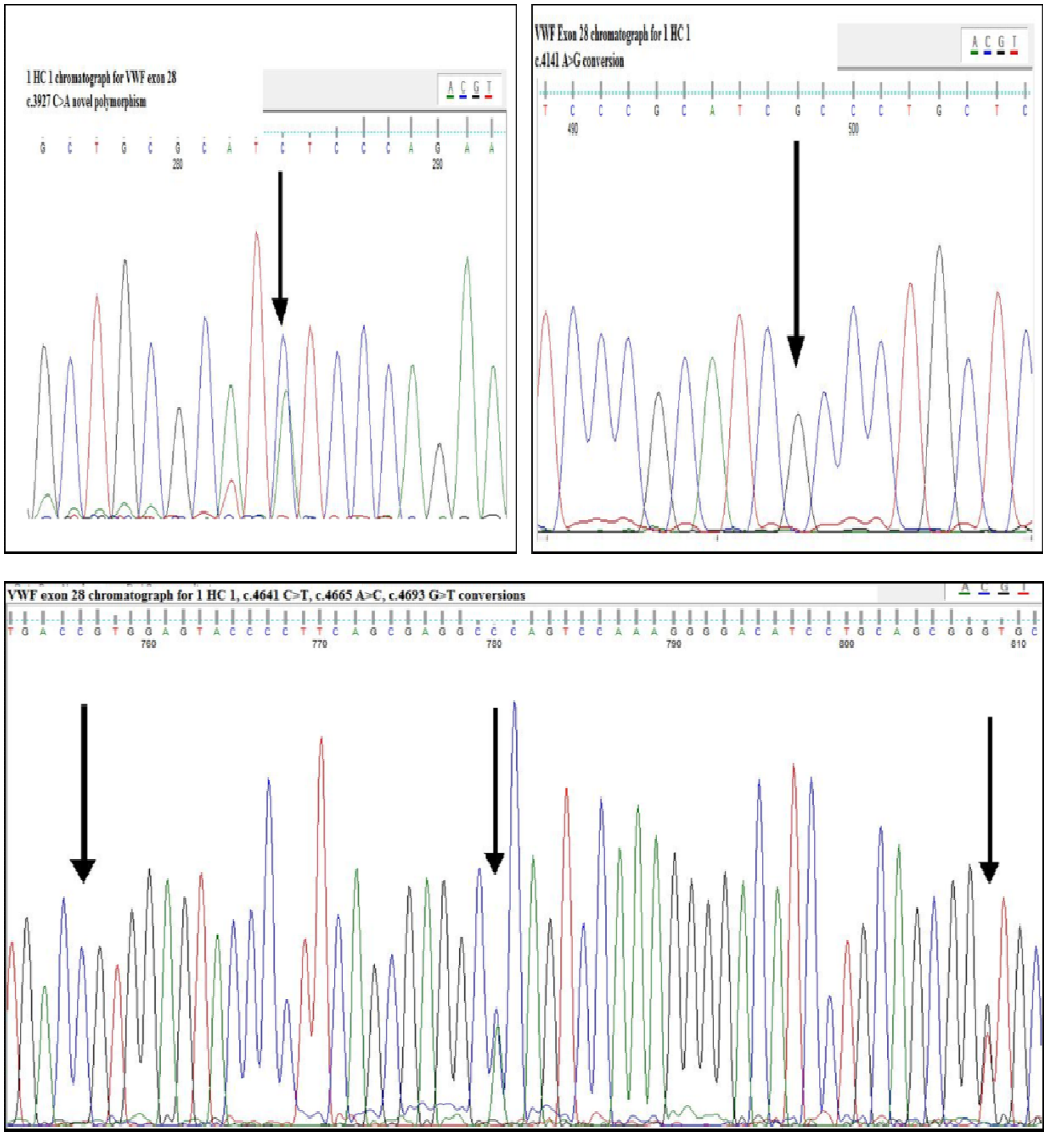

Figure 5.13. DNA sequence analysis of VWF exon 28 in patient 1HC1, arrows indicate the polymorphic sites.

Table 5.7. The genotypes of patients 1HC1 and 68HBT194 for exon 28 of the VWF gene. C.3927 C/A novel polymorphism (yellow). Polymorphic sites with both alleles detected (bold). (na. not applicable)

cDNA nucleotide number	Alleles	Amino Acid Number	Patient I 1 HC 1	Patient II 68 HBT 194
3795	G/A	P 1265	G/G	G/G
3801	G/A	L 1267	G/G	G/G
3927	**C/A**	**I 1309**	**C/A**	**C/C**
3951	T/C	A 1317	C/C	C/C
4011	G/A	P 1337	G/G	G/G
4074	C/T	S 1358	C/C	C/C
4129	G/T	A/V 1377	G/G	G/G
4138	A/G	I/V 1380	A/A	A/A
4141	**G-/A+**	**A/T 1381**	**G/G**	**G/A**
4196	G/A	R/H 1399	G/G	G/G
4304	A/G	N/S 1435	A/A	A/A
4414	G/C	D/H 1472	G/G	G/G
4500	G/A	A 1500	G/G	G/G
4503	C/T	F1501	C/C	C/C
4517	C/T	S/L 1506	C/C	C/C
4641	**C+/T-**	**T 1547**	**C/C**	**C/T**
4665	**A-/C+**	**A 1555**	**C/A**	**C/A**
4693	**G/T**	**V/L 1565**	**G/T**	**G/T**
4707	C/T	R1569	C/C	C/C
4713	G/C	E/H 1571	G/G	G/G
4716	T/C	G 1572	C/C	C/C
4716	C/T	G 1572	C/C	C/C
4719	C/T	G 1573	C/C	C/C
4728	C/A	T 1576	C/C	C/C
4751	A+/G-	Y/C 1584	A/A	A/A
4801	C/A	P/T 1601	C/C	C/C
4801	C/A	P/T 1601	C/C	C/C
4855	C/T	L1619	C/C	C/C
4875	G/T	V 1625	G/G	G/G
	G/T	I/T 1628	T/T	T/T
4926	T/C	I1642	T/T	T/T
4927	G/A	G/S 1643	G/G	G/G
4944	T/C	P1648	T/T	T/T
5046	A/T	P 1682	na	na

5.3. High Resolution Melting Analysis (HRMA) of the VWF gene

Due to the length of the von Willebrand Factor gene and high prevalence of VWD, a rapid mutation detection system was required for complete gene analysis in large number of patients. High Resolution Melting Analysis is suitable for this purpose using 96 well plates on LC 480 platform. It allows the analysis of large number of patients and also possibly all amplicons of the VWF gene of the same patient in the same run in a closed tube system, in a time scale of approximately 90 minutes, since, different amplicons with same amplification thermal profile can be amplified in the same run, resulting in screening of different amplicons for one individual at the same time.

In order to optimize High Resolution Melting Analysis of the VWF gene, 52 exons were amplified amplification in 60 amplicons in lengths between 200-350 bp in. Three exons, exon 28, exon 37 and exon 52 were divided into more than one amplicon due to amplicon length limitation of the method. On the other hand, in order to differentiate exon 26 from pseudo gene it was amplified first in 841 bp fragment and after that this fragment was used as template for a shorter (260 bp) amplicon which was more feasible for HRMA. Additionally, primers for the exons between 23 and 34 were designed over the sequences that show difference between the *VWF* gene and its pseudogene (on chromosome 22). Melting temperatures for each primer were designed around 60 ^{o}C. As PCR thermal programs optimized in conventional thermal cyclers using standard Taq Polymerases cannot be adopted to Light Cycler platform amplicons were directly optimized in LC 480, using Roche HRM Master Mix reaction mixture. Initially, each amplicon was tested in the same template program with the template reaction mixture containing (30ng DNA, 2 mM MgCl2, 0,2 µM Primers without DMSO). Later on with changes in amplification and reaction conditions, 38 of 60 amplicon were amplified properly for HRM analysis.

After PCR and HRM reactions were completed, the amplification was evaluated for two criteria: Cp value and melting peaks. In order to give proper HRM analysis, it was necessary that amplicons should enter log phase before the 35th cycle of a 45 cycles PCR amplification step, which is determined by using 2nd derivative Max program as Cp values. Then, whether amplicons showed single melting peak was determined using the Tm

calculation program. This indicated that PCR product contained single DNA fragment (Roche HRM Kit user manual).

Following the tests for proper PCR amplification, PCR products in the multiwell plate was purified, loaded on two per cent agarose gel, to determine the length of the product. Purified PCR product, corresponding to 24 successfully amplified amplicons were sequenced to ensure the amplification of the target DNA region.

5.3.1. HRMA for VWF Exon 28

Due to HRMA limitations, exon 28 was divided into seven slightly overlapping amplicons (Table 5.8). Five amplicon, 28-1,28-2,28-3,28-4 and 28-7 were successfully amplified. Exon 28 sequences for two patients that had been determined by direct sequencing were used for the validation of HRMA of exon 28. Exon 28 genotypes of two patients are summarized in Table 5.9. HRMA results of 5 exon 28 amplicons were consistent with the direct sequencing results. Result of amplicon 3 is given as an example (Figure 5.14). Homozygous AA genotype was not observed in our samples.

Table 5.8. HRMA amplicons of Exon 28. Optimizations were completed for the underlined amplicons.

Amplicons of Exon 28	**Length (bp)**
Amplicon 1	203
Amplicon 2	270
Amplicon 3	240
Amplicon 4	225
Amplicon 5	290
Amplicon 6	225
Amplicon 7	261

Table 5.9. The genotypes of patients 1HC1 and 68HBT194 revealed by HRMA

Amplicon	Polymorphism	Patient 1	Patient 2
Amp 1	No change	wt	wt
Amp 2	c.3927 C>A	Ht pol (C/A)	Wt (C/C)
Amp 3	c.4141 A>G	Hm pol (G/G)	Ht pol (G/A)
Amp 4	No change	wt	wt
Amp 7		wt	wt

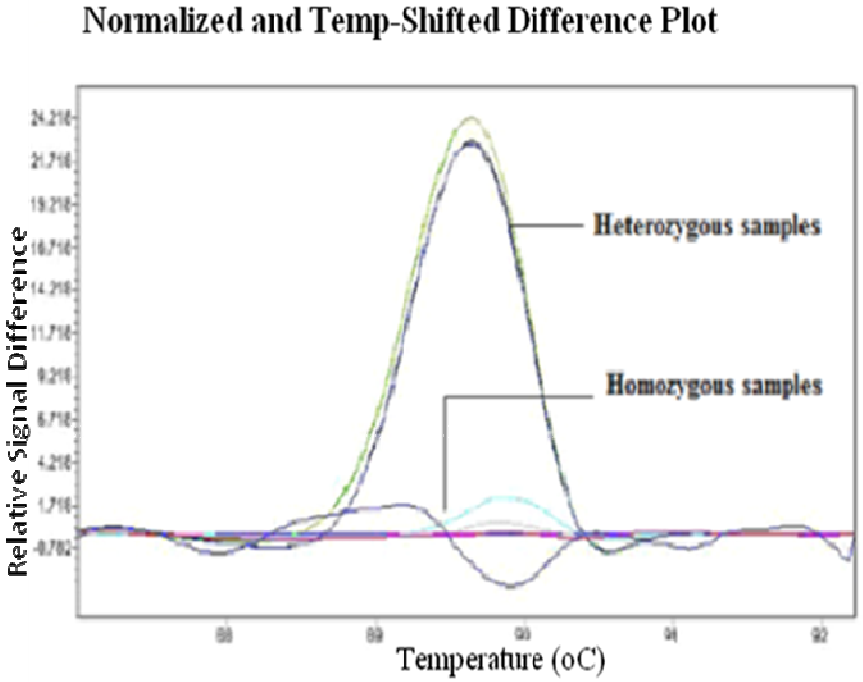

Figure 5.14. Normalized and temperature shifted difference plot for amplicon 3 of VWF gene exon 28. Individuals with known heterozygous genotype were distinct from homozygous individuals. Patient 1HC1 was grouped with homozygous samples and patient 68HBT194 was grouped with heterozygous samples.

5.3.2. HRMA for the Rest of VWF Gene

Optimizations were carried out by changing the annealing temperature, DNA, $MgCl_2$, primer and DMSO concentrations. In the result, 38 amplicons had acceptable amplification by HRMA, including amplicons 5 and 6 of exon 28.

Following optimization of individual amplicons, it was possible to amplify some amplicons in same thermal and reaction condition. As a result, 37 amplicons can be amplified in 7 groups as summarized in Table 5.10. Amplicon 11 was successfully amplified in two thermal and reaction condition and was therefore included in more than one group. Amplicon 21 was amplified with two different primer sets, so that, thermal and reaction conditions for each primer set were different from each other. In result, amplicon 21 was also included in more than one group.

Table 5.10. HRMA amplification groups of VWF gene amplicons with corresponding thermal and reaction conditions.

Groups	**Amplicons**	**Thermal condition**	**Reaction conditions**
Group 1	Exon 1 Exon 4 Exon 11 Exon 21 Exon 22	95 °C 10 min 95 °C 10 sec 62-56 °C 15 sec 72 °C 15sn	30 ng DNA 0,3 uM Primer 3 mM MgCl2 0 % DMSO
Group 2	Exon 2 Exon 5 Exon 6 Exon 7	95 °C 10 min 95 °C 10 sec 62-54 °C 10 sec 72 °C 15 sec	30 ng DNA 0,2 uM Primer 2 mM MgCl2 0 % DMSO
Group 3	Exon 8 Exon 11 Exon 13 Exon 17	95 °C 15 min 95 °C 10 sec 60-54 °C 15sec 72 °C 15 sec	30 ng DNA 0,3 uM Primer 2,5 mM MgCl2 7,5 % DMSO
Group 4	Exon 28-1 Exon 28-2 Exon 28-3 Exon 28-4 Exon 28-7	95 °C 10 min 95 °C 10 sec 64-60 °C 10 sec 72 °C 15 sec	30 ng DNA 0,2 uM Primer 2,5 mM MgCl2 0 % DMSO
Group 5	Exon 28-5 Exon 28-6	95 °C 15 min 95 °C 10 sec 62-54 °C 15 sec 72 °C 15 sec	30 ng DNA 0,5 uM Primer 5 mM MgCl2 0 % DMSO
Group 6	Exon 19 Exon 21 (Old primers)	95 °C 10 min 95 °C 10 sec 62-54 °C 10 sec 72 °C 15 sec	30 ng DNA 0,2 uM Primer 2 mM MgCl2 0 % DMSO

Table 5.10. HRMA amplification groups of VWF gene amplicons with corresponding thermal and reaction conditions (Continued).

Group 7	Exon 24 Exon 30 Exon 31 Exon 35 Exon 36 Exon 37-B Exon 38 Exon 40 Exon 41 Exon 43 Exon 44 Exon 45 Exon 48 Exon 50 Exon 51 Exon 52-A Exon 52-B	95 °C 15 min 95 °C 10 sec 62-54 °C 15 sec 72 °C 15 sec	30 ng DNA 0,3 uM Primer 3 mM MgCl2 5 % DMSO

5.3.3. VWD Patient Screening

After amplification of 38 amplicons were optimized, 10 vWD patients (three of which is Type 3 and the rest is Type 1) and a healthy control DNA were screened using HRMA for 14 amplicons (exons 1, 2, 4, 5, 6, 7, 8, 11, 19, 21, 28-1, 28-2, 50, 52-A). When the amplifications were evaluated, exon 6 and 11 were omitted from analysis because of low Cp value and the presence of two bands on the gel.

In the result of HRM analysis of 10 patients for 12 amplicons, all patients' PCR products had the same melting pattern and grouped into one for 9 amplicons. On the other hand, for exons 50 and 19, patients were grouped into three and for exon 8, there were two groups. The results are summarized in Table 5.11.

Table 5.11. HRM analysis results for 10 patients and a healthy control. Patients having the same melting curve were included in the same group (G1G3) and represented by the same color. Samples that are analyzed also by direct DNA sequencing were labeled as "seq". (C: Control sample)

Patient No. / Exon	**4 VWD 6**	**5 VWD 7**	**6 VWD 10**	**7 VWD 11**	**8 VWD 14**	**9 VWD 17**	**10 VWD 19**	**11 VWD 22**	**12 VWD 25**	**13 VWD 26**	**C**
1	G1 Seq	G1 Seq	G1	G1	G1	G1	G1	G1	G1	G1	G1 seq
2	G1	G1	G1	G1	G1	G1	G1	G1	G1	G1	G1
4	G1 seq	G1 Seq	G1	G1	G1	G1	G1	G1	G1	G1	G1 seq
5	G1	G1	G1	G1	G1	G1	G1	G1	G1	G1	G1
7	G1 seq	G1 Seq	G1	G1	G1	G1	G1	G1	G1	G1	G1
8	G1	G1	G1	G1	G2 seq	G1	G1	G1	G1	G2 seq	G1 seq
19	G1 seq	G2	G3 Seq.	G1 seq	G3	G2	G3	G2	G3	G2 seq	G2 seq
21	G1	G1	G1	G1	G1	G1	G1	G1	G1	G1	G1
28-1	G1	G1	G1	G1	G1	G1	G1	G1	G1	G1	G1
28-2	G1	G1	G1	G1	G1	G1	G1	G1	G1	G1	G1
50	G2 seq	G1 Seq	G1	G1	G1	G1	G1	G1	G3	G1	G1 seq
52-A	G1 seq	G1	G1	G1	G1	G1	G1	G1	G1	G1	G1 seq

In order to confirm HRM results, two patients' and healthy control's PCR products were sequenced for exons 1, 4, 7 and 52-A and found to have the reference sequences. In the same way samples will also be sequenced for amplicons 2, 5, 8, 21, 28-1, 28-2. For exon 19 patients were grouped into three. After sequencing of selected PCR products marked as "seq" on the Table 5.12. a nucleotide change in intron 19 (c2546+25 C>T) was observed. Patients, 4VWD6 and 7VWD11 (Group 1) were wild type, 5VWD7, 9VWD17,

11VWD22, 13VWD26 and healthy control (Group 2) were heterozygous for the change and 6VWD10, 8VWD14, 10VWD19 and 12VWD25 (Group 3) were homozygous mutant. Sequencing results confirmed the grouping of the HRM analysis. The change was not listed in VWF polymorphisms database, suggesting that it may be a novel polymorphism. The HRMA of exon 19 is shown in Figure 5.15. The chromatograms of three genotypes are shown in Figure 5.16.

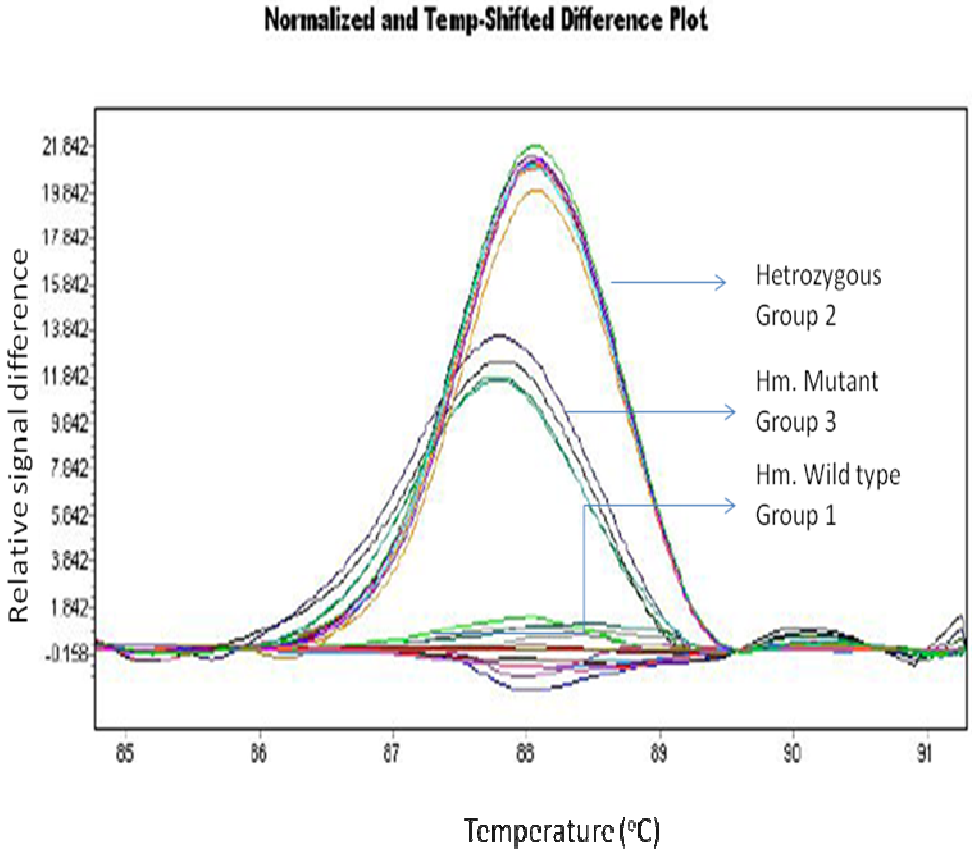

Figure 5.15. Normalized and temperature-shifted difference plot of patient scanning for exon 19. Patients in group 1 are represented as Hm. Wild Type, patients in group 2 are represented as heterozygous and patients in group 3 are represented as Hm. Mutant.

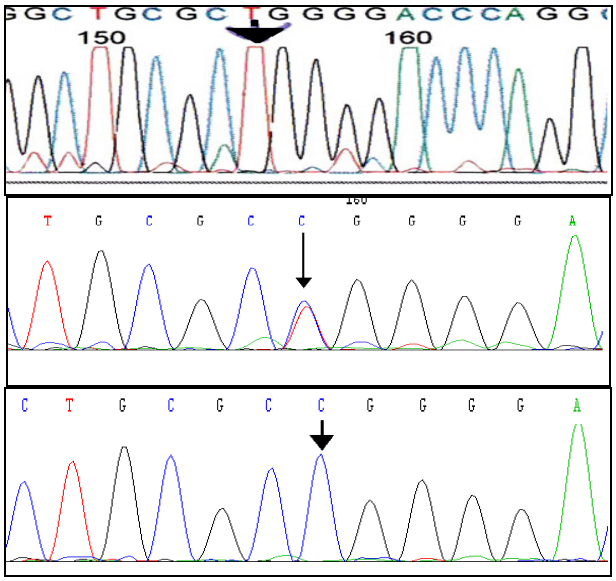

Figure 5.16. Chromatograph of Exon 19 for three different patiens with different genotypes. Panel A: Homozygous mutant (T/T), Panel B: Homozygous wild-type (C/C) and Panel C: Heterozygous (C/T) for c2546+25 C>T transition.

HRMA of exon 8 is observed in Figure 5.17 that grouped patients 8VWD14 and 13VWD26 separately. In the result of DNA sequencing analysis, the PCR product could only be read after 50th base of the amplicons. The DNA sequence analysis of both patients and the healthy control revealed a heterozygous change (c. 932 C>A) (Figure 5.18). The change was a silent change that was not reported before in neither polymorphism nor mutation database in Sheffield University VWF database (www.vwf.group.shef.ac.uk). The sequencing of the samples should be repeated using reverse primers to see if there is any change in the first 0 bases of the amplicon and whether this change is responsible for grouping of two patients separately from the others including the control. Additionally, sequencing of other group 1 patients are also necessary to see whether they all have the c.932 C>A in heterozygous condition along with the control. This would confirm the polymorphic nature of the change.

Signal difference between curves of different groups of exon 8 amplicons is not significant when compared to HRMA of other amplicons that produced different grouping of patients; implying that HRMA result for exon 8 may be false. Additionally, spike reactions when control DNA was mixed with all patient DNA samples did not reveal any difference suggesting that patients in Group 1 are actually heterozygous.

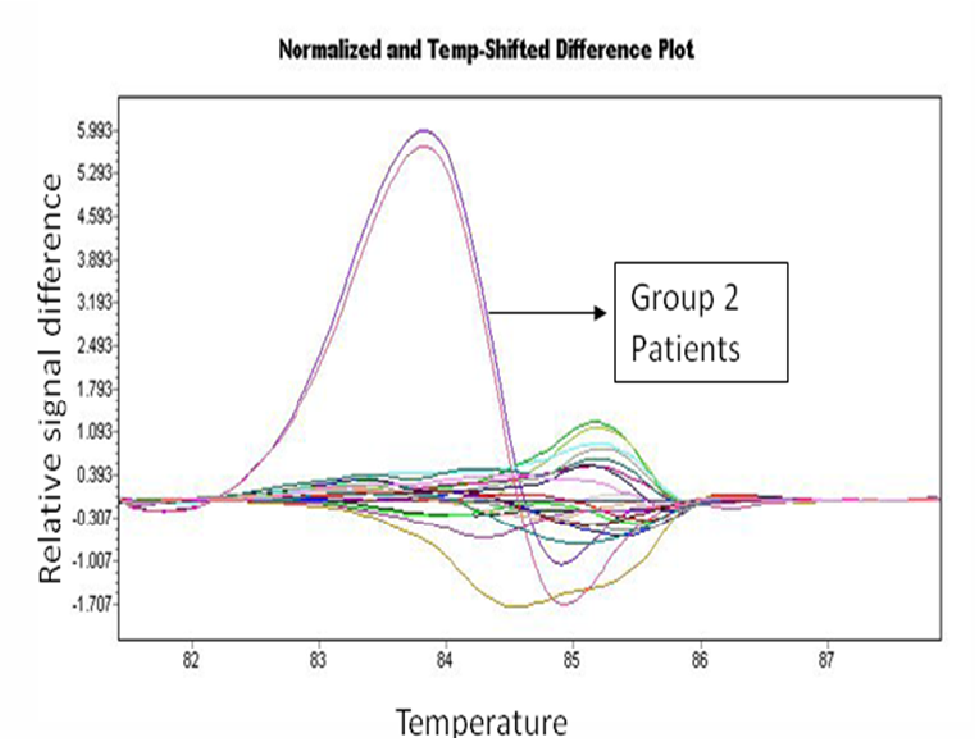

Figure 5.17. Normalized and temperature-shifted difference plot for exon 8. The peaks correspond to patients 8VWD14 and 13VWD26 were grouped together.

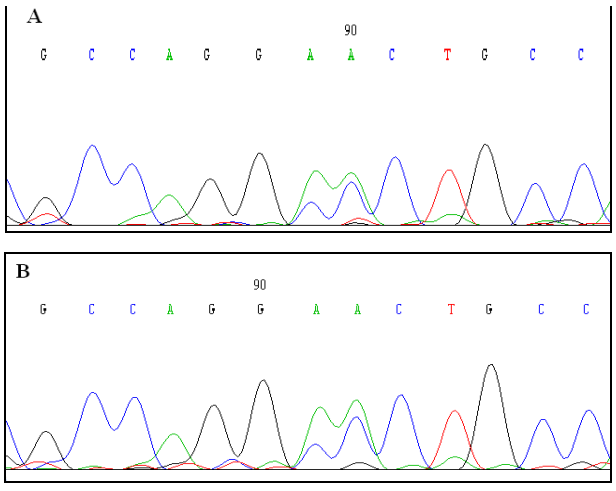

Figure 5.18. Exon 8 chromatographs for patient 13 vwd 26 (Panel A) and Control (Panel B). Arrow indicates the observed change c. 932 C>A in heterozygous state.

The grouping pattern of HRMA of exon 50 is shown in Figure 5.19. Samples for 4VWD 6 (Group 2), 5VWD7 (Group 1) and Control (Group 1) were sequenced, but the sample with the most significant difference grouped in group 3 was not sequenced due to

the loss of PCR product during purification. On the other hand, though samples for Control and 4VWD 6 were grouped into different groups, the chromotographs of the amplicons were readable only after 50th base of the exon and no change was observed. Whether the change that gave rise to the shift in melting curve resided in the first 0 bases remains to been by sequencing with the reverse primer. Additionally, sequencing of 12VWD25 may reveal a change in the DNA sequence.

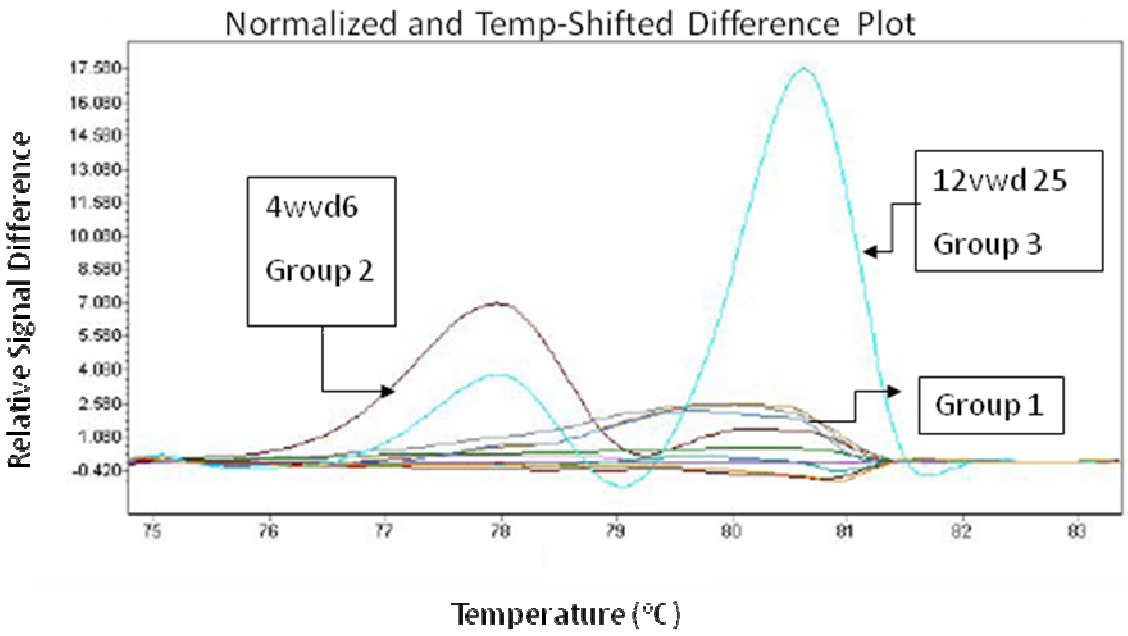

Figure 5.19. Normalized and temperature-shifted difference plot of patient scanning for exon 50. Patients 4 vwd 6 and 12 vwd 25 show different melting curve.

A summary of HRMA of the VWF gene is given in Table 5.12.

Table 5.12. Summary of the HRMA of the VWF gene. Optimized amplicons are colored and the amplicons that have same amplification conditions are shown in the same color. groups.

Exon no	Optimized?	Patient Screen	HRM difference
1	**Yes**	Done	No
2	**Yes**	Done	No
3			
4	**Yes**	Done	No
5	**Yes**	Done	No
6	**Yes**	Done	No
7	**Yes**	Done	No
8	Yes	Done	8 vwd 14 13 vwd 26 not discriminated from chromotographs
9			
10			
11	Yes	done	Not reliable
12			
13	Yes		
14			
15			
16			
17	Yes		
18			
19	Yes	Done	3 groups for c.2546+24 C-T conversion
20			

Table 5.11. HRMA of VWF gene (Continued)

21	**Yes**	Done	
22	**Yes**		
23	Wrong reverse primer		
24	**Yes**		
25	Wrong reverse primer		
26	Will be amplified in two PCRs		
27	Primers are not present		
28-1	**Yes**	Done	No
28-2	**Yes**	Done	No
28-3	**Yes**		
28-4	**Yes**		
28-5	**Yes**		
28-6	**Yes**		
28-7	**Yes**		
29			
30	**Yes**		
31	**Yes**		
32			
33	**Yes**		
34			
35	**Yes**		
36	**Yes**		
37-A	**Yes**		
37-B	**Yes**		
38	**Yes**		

Table 5.11. HRMA of VWF gene (Continued)

39			
40	Yes		
41	**Yes**		
42			
43	**Yes**		
44	**Yes**		
45			
46	Wrong reverse primer		
47			
48	**Yes**		
49	**Yes**		
50	**Yes**	Done	4 vwd 6 12 vwd 25 different
51			
52-A	**Yes**	Done	
52-B	**Yes**		

6. DISCUSSION

FXI and VWF proteins are two important actors of hemostasis. The first is a plasma zymogen that takes a crucial step in intrinsic pathway of coagulation cascade. And the latter is a highly adhesive protein that takes the immediate action upon vascular damage and creates a platelet plug on the wound. Consequently, deficiencies of both factors weaken the hemostasis, resulting in a series of complications characterized generally by excessive bleeding after trauma.

Hemophilia C phenotype is complicated due to incomplete penetrance and variable expressivity. Additionally, plasma FXI level is not a good indicator of bleeding diathesis of patients. This fact may be due to the actions of several factors on hemostasis, not merely in the cardiovascular system but also the others like immune system. Reports of coexistence of FXI deficiency with Hemophilia A or VWD is suggestive of some yet unidentified interactive pathways. Information on detailed interactions of different factors would be highly valuable for the diagnosis and treatment of coagulation defects.

Molecular analysis of *F11* gene generally begins with the search for mutations that lead to type I-IV, especially in the Ashkenazi Jewish population. However, in other populations, causative mutations for FXI deficiency are distributed throughout the gene. Thus, it is important to analyze the whole gene.

Additionally, the inheritance of the FXI deficiency is also debated. It was defined as an autosomal recessive disorder, however there has been accumulating numbers of reports indicating the autosomal dominant inheritance of the disorder. This may be due to the fact that heterozygous mutations generally result in mild FXI deficiency. As Mild FXI deficiency patients may or may not bleed, they may have not been diagnosed for FXI deficiency. However, heterozygous mutations result in a decrease in the FXI level.

In contrast to FXI deficiency, VWD is the most common bleeding disorder. However, a large number of individuals with mild VWD remain undiagnosed due to variability of the disease phenotype. Moreover, plasma VWF level is not stable and shows a considerable temporal variation in an individual or personal variation among different individuals. Thus, plasma VWF level is not a significant indicator of the disease.

VWF is a large adhesive plasma protein, which is synthesized in endothelial cells and magakaryocytes as a large Multimeric complex. In has two distinct functions; recruits platelets to damage site and besides carries and stabilizes FVIII in plasma. When these functions and the multimeric structure of the protein are taken into consideration it is not surprising that protein interacts with several factors and bears several functional sites along the protein. This fact has a crucial consequence; a defect in any of these functional sites results in a different disease phenotype, either quantitative or qualitative. Recently, VWD working commission summarized all these phenotypes in three main categories according to mild deficiencies (Type I), severe deficiencies (Type III) and qualitative defects (Type II with many subtypes) of VWF protein. Some of the subtypes result from the mutations in particular regions on the molecule, but mutations result in type I VWD are distributed throughout the VWF gene, which spans 17,8 kb region on chromosome 12. Thus, mutational analysis of the whole gene is necessary in especially in type I VWD, though it is a cumbersome task.

6.1. FXI Deficiency Patients

The first patient (1HC1), an 18 years old male, was diagnosed to have FXI deficiency (Hemophilia C), he also had FVIII and VWF level slightly under limits. His *F8* gene and exons 18^{th}-21^{st} and 24^{th} exons of the VWF gene had been screened earlier in our laboratory, but no mutation had been observed. The molecular genetic analysis of the *F11* gene was also established.

Upon PCR amplifications of amplifications of the promoter sequences and all coding region of the *F11* gene, a nonsense change; W 501 X, which result from c. 1556 G>A substitution was observed in heterozygous condition, creating premature stop codon in the catalytic site. The mutation confirmed by the F*ok*I restriction enzyme digestion. As a result first two amino acids of catalytic triad; His413, and Asp462 was present in the defected monomers but the third component; Ser557 is missing. Thus, monomers expressed from the defected allele were not functional. On the other hand, defected allele may cause the disease either by poor secretion/stability of the truncated protein or by decreased mRNA levels (Solda, 2005). This mutation was reported before, and confirmed by RE digestion in our study. A functional study would determine the molecular pathology of the disease in patient 1HC1.

Mutation is also screened in other family members, father, mother and a sister, by restriction digestion. The restriction pattern for patient's father was same as the Patient's. On the other hand patient's mother and sister showed the wild type banding pattern. After the sequencing of father's *f11* gene exon 13, same mutation was also observed in heterozygous state. Father's FXI plasma level is 40 per cent, slightly below than the normal limit and he does not show any bleeding diathesis. This may be an example of variable expressivity of the disease gene.

The second patient was a pregnant woman, who (2 HC 5) had been diagnosed for FXI deficiency and referred to our laboratory for prenatal diagnosis. She has a low FXI activity (21 %) and elongated aPTT. In the result of *F11* gene mutational analysis, c.151 A>C substitution was observed in the third exon in heterozygous condition which causes T 33 P amino acid conversion in Apple 1 domain, and it was not listed in FXI mutation database. After screening of other family members, father, mother, two brothers, her spouse and the fetus, mutation was also observed in father's and the fetus' genotype also in heterozygous state. Father was not symptomatic; however laboratory measurement of FXI level has shown that he also had low FXI level (24 %). The fetus was also had the mutation in heterozygous state. Moreover, the father is diagnosed for thrombophilia and during her

pregnancy, thrombosis occurred in the placenta of fetus. It may be argued that the father's thrombophilia tendency may compensate for FXI deficiency.

The mutation identified in this family is conserved in other mammalian serine proteases. A population screen was also conducted and the conversion was not observed in screened in 100 apparently healthy individuals (200 alleles in total). These finding suggest that the mutation is novel and the causative defect in the FXI protein.

A functional study in hepatocyte cell lines is underway to reveal its molecular pathology.

6.2. VWF Exon 28 Analysis

Consistent with the size of the protein, VWF gene is a large gene which spans 17,8 kb region and comprises 52 exons. Beside its length the gene is also highly polymorphic. Exon 28 is the longest exon of the gene (1347 bp) and bears a large number of mutations listed in the database (169 entries) (www.vwf.group.shef.ac.uk). Because of this, after the exons responsible for Type 2N VWD (Exons 18, 19, 20, 21 and 24) molecular analysis of exon 28 was decided to be optimized. Due to its length the exon was divided into three overlapping amplicons and the amplification conditions were successfully optimized. All three amplicon can be amplified in same thermal cycle and with same reaction conditions.

For patient 1HC1 and another patient 68HBT194, VWF level was below the normal. Their VWF gene exons 18, 19, 20, 21 and 24 had been screened before and no mutation was observed. Their VWF exon 28 was also amplified and sequenced. No mutation was observed. However one novel and four recurrent polymorphisms novel polymorphisms were genotyped. Table 5.7. summarizes two patient's genotype for known polymorphisms.

Number of polymorphic differences observed in two patients highlights the highly polymorphic nature of the gene.

6.3. The High Resolution Melting Analysis of the VWF Gene

VWD is the most common congenital bleeding disorder, especially type I VWD. Though other types of VWD generally result from the mutations in particular regions of the gene, mutations responsible for Type I VWD are distributed throughout the gene. Thus, whole gene mutational analysis is required to identify the molecular pathology. However, the length of the gene complicates the analysis with traditional methods, like direct DNA sequencing or other gel based methods.

Fluorescent melting analysis of PCR products in conjunction with real time PCR was first introduced at late nineties. Many melting techniques used expensive fluorescent labeled oligonucleotide probes that only screen the region covered by the probe. In addition the entire PCR product can also be screened by the use of DNA saturating dyes like SYBR Green I and LCGreen® and several different real time platforms (Hermann, 2006).

The HRMA is based on the fact that different PCR products generally have different melting temperatures depending on the GC content, length and the sequence of the amplicon. In first stage experiments SYBR Green was used as saturating dye, however, it failed to identify small changes like single nucleotide polymorphisms (SNPs). However, after the development of LCGreen® dye, which is saturately, intercalates double stranded DNA and give better resolution, the specificity of the analysis was increased. Recent reports of SNP genotyping with LCGreen® claim that most homozygous sequence variations produces detectable *Tm* shifts compared to wild type. On the other hand heterozygous samples produce distinct melting curves. Homozygous samples generally have sharp, symmetric melting curves, whereas, heterozygous samples produces more

gradual complex melting curves due to the presence of different homo and heteroduplex species A recent cross platform comparison of HRM technologies done by Hermann *et al.* reported that LCGreen® is the suitable dye for this analysis (Hermann, 2006). For this purpose we used Roche High Resolution Melting Master Mix Kit, which includes LCGreen® in Lighcycler 2.0 platform for the HRMA of Turkish VWD patients.

HRMA of the VWF gene began with the analysis of exon 28, since it was highly polymorphic and already sequenced in two patients. The result of HRMA of exon 28 were consistent with the findings of direct DNA sequencing. The HRMA analysis of the rest of the exons of the VWF gene was then attempted. For the rest of the gene we did not have reference sequences, nor were the amplicons optimized for PCR. Since, Polymerase of the kit is not the identical with standard *Taq* Polymerase, thus it not always to apply optimal amplification conditions of thermal cycler to real-time platform. Optimization of exons for HRMA was directly conducted on light cycler with Roche HRM Master Mix.

For some amplicons we did not obtain any amplification, or they entered logarithmic phase at very late cycles (e.g. 38), even at very high primer and magnesium concentrations and very low annealing temperatures. And for some amplicons we observed multiple melting peaks and also more than one band on the agarose gel. These primers require further optimization.

Out of 60 amplicons 37 were amplified properly and 24 of these 37 amplicons were sequenced and confirmed the amplification of the correct genomic region. However, chromatographs for some of these amplicons were not read easily and it should be determined whether the problem is in sequencing or in amplification of the amplicons.

Up to now, 69 patients' blood samples arrived to our department for VWF gene mutational screening (190 blood samples for patients and family member). We started screening initially with 11 patients (3 of them are diagnosed for type 3 and the rest are diagnosed for Type 1 VWD) additionally one apparently healthy individual. In preliminary studies of HRMA, analysis of each amplicon was conducted in replicate as an internal

control. Additionally, patient DNAa were added some control DNA to introduce an artificial heterozygosity to possibly different homozygous samples (homozygous wild type or mutant). Consequently, for each patient three reactions was run to screen each amplicon. Thus up to now, two amplicons were scanned for 12 samples in the same run, in 90 minutes.

12 different amplicons were analyzed in 12 samples. When the GeneScan program grouped all PCR products in same group, i.e. did not show any thermal difference, three PCR products for different individuals were sequenced to confirm the results. When GeneScan program reported different groups, individual samples from each group were sequenced in order to identify the sequence variation.

Up to this point, GeneScan of three different amplicons reported a sequence variation among 11 individuals. One of them was in exon 19 and was confirmed also by sequencing. However, for exon 8, sequencing did not reveal any sequence alteration. When the difference plot was examined it was observed that, relative signal difference was 5,9 for exon 8 scanning and it was not significant when compared with 21,8 signal difference on the plot for exon 19 scanning. On the other hand, for exon 50, relative signal difference between sequenced PCR products were also not significant, except the PCR product of patient 12VWD25 (Group 3), that was slightly significant (17,5) in this case the sequencing result was expected to confirm a sequence change, however, sequencing was not possible for this patient.

In summary, HRMA method has been validated by the data obtained from the analysis of VWF exon 28 and patient screening for 10 other amplicons including exon 19, results of which were consistent with sequencing data. These preliminary analyses showed that HRMA is a rapid screening method and for optimized amplicons it is promising for the screening of VWF gene mutations. Though, HRMA and DNA sequence analysis of exon 8 remains to be repeated and exon 50 of patient 12VWD25 (Group 3) should be sequenced with reverse primers.

Moreover, HRMA results for amplicons 2, 5, 21, 28-1 and 28-2 should be confirmed by DNA sequencing of patient samples and control. 10 analyzed patients need to be screened for already validated amplicons and highly established amplicons (1, 2, 4, 5, 7, 19, 21, 28-1 and 28-2 should be analyzed in all 60 patients. Meanwhile optimization studies for rest of the amplicons need to be completed with revised primers and target sequence amplification should be checked using direct DNA sequencing.

Establishment of a rapid screening method for mutational analysis of VWF gene would be a significant achievement in the molecular diagnosis of bleeding disorders and the evaluation of interactions among the procoagulants. Upon completion of VWF mutational analysis, it will be possible to examine coexistence of FXI, FIX and FVIII deficiency with VWD, since; many patients included in this study have low levels for more than one factor.

7. REFERENCES

Baronciani, L., G. Cozzi, M. T. Canciani, F. Peyvandi, A. Srvastava, A. B. Federici, and P. M. Mannucci, "Molecular Defects in Type 3 von Willebrand Disease. Updated Results from 40 Multiethnic Patients", *Blood Cells, Molecules and Diseases,* Vol.30, pp.264-270, 2003.

Berber, E., "Investigations to Elucidate the Molecular Mechanisms Controlling Transcription of the Factor VIII and von Willebrand Genes", PhD Thesis, Boğaziçi University,2002.

Bolton-Maggs, P. H. B., "Factor XI Deficiency and Its Management", *Hemophilia,* Vol.6, pp.100-109, 2000.

Bond L. and D. Bevin, "Myocardial Infarction in a Patient with Hemophilia A Treated with DDAVP" *New England Journal of Medicine*, Vol.318, page 121, 1988.

Chateigner-Boutin, A. L. and Small, I., "A Rapid High-Throughput Method for the Detection and Quantification of RNA Editing Based on High-Resolution Melting of Amplicons", *Nucleic Acid Research*, Vol.35, No.17, 2007.

Chou, L. S., E. Lyon and C. T. Wittwer, "A Comparison of High Resolution Melting Analysis with Denaturing High Performance Liquid Chromotography for Mutation Scanning", *American Journal of Clinical Pathology,* Vol.124,pp.330-338, 2005.

Croft, S., N. Beauchamp, D. Hampshire, A. Goodeve, D. Lillicrap, M. Hashemi, and U. Budde, The University of Shefield, ISTH SSC VWF Database, http://www.vwf.group.shef.ac.uk/index.html

Dahlback, B., "Blood Coagulation and Its Regulation by Anticoagulant Pathways: Genetic Pathogenesis of Bleeding and Thrombotic Diseases", *Journal of Internal Medicine,* Vol.257. pp. 209-223, 2005

Dahlback, B., "Blood Coagulation", *The Lancet,* Vol.355, pp.1627-32, 2000

Fujikawa, K., "Historical Perspective of Factor XI", *Thrombosis Research,* Vol.115, pp.441-450, 2005.

Goodeve, A., J. Eikenboom, G. Castaman, F. Rodeghiero, A. B. Fedrici, J. Battle, D. Meyer, C. Mazurier, J. Goudemand, R. Schneppenheim, U. Budde, J. Ingerslev, D. Habart, Z. Vorlova, L. Holmberg, S. Lethagen, J. Pasi, F. Hill, M. H. Soteh, L. Baronciani, C. Hallden, A. Guilliat, W. Lister and I. Peake, "Phenotype and Genotype of a Cohort of Families Historically Diagnosed with Type 1 Von Willebrand Disease in European Study, Molecular and Clinical Marlers for the Diagnosis and Management of Type 1 Von Willebrand Disease (MCMDM-1VWD)", *Blood,* Vol. 109, No. 1, pp. 111-121.

Gomez, K. and P. Bolton-Maggs, "Factor XI Deficiency", *Hemophilia,* Journal Compliation, pp.1-7, 2008.

Gudrun, H. R. and C. T. Wittwer, "Sensitivity and Specificty of Single-Nucleotide Polymorphism Scanning by High Resolution Melting Analysis", *Clinical Chemistry,* Vol.50:10, pp.1748-1754, 2004.

Herrmann, M. G., J. D. Durtchi, L. K. Bromley, C. T. Wittwer, and K. V. Voelkerding, "Amplicon DNA Melting Analysis for Mutation Scanning and Genotyping: Cross-Platform Comparison of Instruments and Dyes" *Clinical Chemistry,* Vol. 52, No.3,pp.494-503, 2006.

Hill, M., F. McLeod, H. Franks, B. Gordon and G. Dolan, "Genetic Analysis in FXI Deficiency: Six Novel Mutations and the Use of a Polymerase Chain Reaction-Based

Test to Define a Whole Gene Deletion", *British Journal of Hematology*, Vol.129, pp.825-829, 2005.

Hobbs, W. E. II and J. A. Lopez, "Shear Elegance: Regulation of Thrombus Growth by Shear Stress", *Blood,* Vol. 111., p.972, 2008.

Hoffmann, M., J. Hurlebaus, and C. Weilke, "High-Resolution Melting Curve Analysis on the LightCycler 480 PCR System", *Biochemica,* No.1, 2007

Hoffmann, M., J. Hurlebaus and C. Weilke, "Novel Methodsfor High-Performance Melting Curve Analysis Using the LightCycler 480 System", *Biochemica,* No.1, 2007

Horne, M. D., "Overview Of Hemostasis And Thrombosis; Current Status Of Antithrombotic Therapies" *Journal of Thrombosis Research,* Vol.117, pp. 15-17, 2005.

Iijima, K., A. Udagawa, H. Kawasaki, F. Murakami, T. Shimomura and S. Ikawa, "A Factor XI Deficiency Associated with a Nonsense Mutation (Trp501Stop) in the Catalytic Domain", *British journal of Haematology,* Vol. 111, pp. 556-558, 2000.

Keeney, S. and M. Cumming, "The Molecular Biology of von Willebrand Disease", *Clinical and Laboratory Haematology.* Vol. 23, pp.209-230, 2001

Konkle B. A. "Von Willebrand Disease: Treatment With or Without Factor VIII?" *Journal of Thrombosis and Haemostasis*, Vol.5, pp. 1113–4, 2007.

Kravtsov, Dmitri, V. W. Wenman, J. C. M. Meijers, M. F. Sun, M. A. Blinder, T. P. Dang, H. Wang and D. Galiani, "Dominant Factor XI Deficiency Caused by Mutations in the Factor XI Catalytic Domain" *Blood,* Vol.104, No.1, pp.128-134, 2004

Krypuy, M., G. M. Newnham, D. M. Thomas, M. Conron and A. Dobrovic, "High Resolution Melting Analysis for the Rapid and Sensitive Detection of Mutations in

Clinical Samples: KRAS Codon 12 and 13 Mutations in Non-Small Cell Lung Cancer", *BioMed Central Cancer,* Vol. 6:295, 2006

Kunicki, T. J., A. B. Fedirici, D. R. Salomon, J. A. Koziol, S. R. Head, T. S. Mondala, J. D. Chismar, L. Baronciani, M. T. Canciani and I. R. Peake, "An Association of Candidate Gene Haplotypes and Bleeding Severity in Von Willebrand Disease (VWD) Type 1 Pedigrees", *Blood,* Vol. 104, No. 8, pp. 2358-2367, 2004.

Mancuso, D. J., E. A. Tuley, L. A. Westfield, N. K. Worral, B. B. Shelton-Inloes, J. M. Sorace, Y. G. Alevy and J. E. Sadler, "Structure of The Gene for Human von Willebrand Factor"*, The Journal of Biological Chemistry,* Vol.264, No.33, pp.19514-19527, 1989

Manucci, P. M. and A. B. Federici, "Management of Inherited Von Willebrand Disease", *Best Practice and Research Clinical Hematology*, Vol. 14, No. 2, pp. 455-462, 2001

Manucci, P. M. "Treatment of Von Willebrand Disease", *International Journal of Clinical Laboratory Research,* Vol. 28, pp. 211-214, 1998.

Martinic, D., V. Kravtsov, and D. Galliani, "Factor XI Messenger RNA in Human Platelets", *Blood,* Vol. 94, No.10, pp.3397-3404, 1999.

Millar, C. M. and S. A. Brown, "Oligosaccharide Structures of von Willebrand Factor and Their Potential Role in von Willebrand Disease", *Blood Reviews,* Vol. 20, pp. 83-92, 2006.

Navaneetham D, L. Jin and P. Pandey "Structural and Mutational Analyses of the Molecular Interactions Between the Catalytic Domain Of Factor XIa and the Kunitz Protease Inhibitor Domain of Protease Nexin 2" *Journal of Biological Chemistry,* Vol. 280, pp. 36165–75, 2005.

O'Brien, L. A., P. D. James, M. Othman, E. Berber, C. Cameron, C. R. P. Notley, C. A. Hegadorn, J. J. Sutherland, C. Hough, G. E. Rivard, D. O'Shaunessey and D. Lillicrap, "Founder von Willebrand Factor Haplotype Associated with Type 1 von Willebrand Disease", *Blood,* Vol.102, No.2, pp.549-558, 2003.

O'Brien, S. H., A. K. Ritchey and M. V. Ragni, "Combined Clotting Factor Deficiencies: Experiences at a Single Hemophilia Treatment Center", *Hemophilia,* Vol.13, pp. 26-29, 2007.

O'Connel, N. M., R. E. Saunders, C. A. Lee, D. J. Perry and S. J. Perkins, "Structural Interpretation of 42 Mutations Causing Factor XI Deficiency Using Homology Modeling", *Journal of Thrombosis and Haemostasis*, Vol.3, pp.127-138, 2005.

OMIM, Online Inheritance in Men, Report for Von Willebrand Disease, 2008, http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=193400.

Peyvandi, F., R. J. Kaufman, U. Seligsohn, O. Salomon, P. H. B. Bolton-Maggs, M. Spreafico, M. Menegatti, R. Palla, S. Siboni, and M. Manucci, "Rare Bleeding Disorders", *Hemophilia,* Vol.12, pp.137-142, 2006.

Provan, D. and J. Gribben, (editors) "Molecular Hematology", 2nd edition, pp.330-335, 345-352, Blackwell Publishing, Malden, U.S.A., 2005.

Reininger, A. J., H. F. G. Heijnen, H. Schumann, H. M. Specht, W. Schramm and Z. M. Ruggeri, "Mechanism of Platelet Adhesion to von Willebrand Factor and Microparticle Formation under High Shear Stress", *Blood,* Vol. 107, No.9, pp.3537-3546, 2006.

Riley, P. W., H. Cheng, D. Samuel, H. Roder and P. N. Walsh, "Dimer Dissociation and Unfolding Mechanism of Coagulation Factor XI Apple 4 Domain: Spectroscopic and

Mutational Analysis" *Journal of Molecular Biology,* Vol.367, Issue 2, pp. 558-573, 2007.

Ruggeri, Z. M. "Von Willebrand Factor, Platelets and Endothelial Cell Interactions" *Journal of Thrombosis and Haemostasis,* Vol.1, pp.1335-42, 2003.

Ruggeri, Z. M., "Von Willebrand Factor: Looking Back and Looking Forward", *Thrombosis and Haemostasis,* Vol. 98, pp.55-62, 2007.

Sadler, J. E. and F. Rodeghiero, on the Behalf of the SSC Subcommitee on Von Willebrand Factor, "Provisional Criteria for the Diagnosis of VWD Type 1", *Journal of Thrombosis annd Haemostasis,* Vol. 3, pp. 775-777, 2005.

Salomon, O. and U. Seligsohn, "New Observations on Factor XI Deficiency", *Hemophilia,* Vol. 10, Supp. 4, pp. 184-187, 2004.

Saunders R. E., N. M. O'Connell, C. A. Lee, D. J. Perry and S. J. Perkins, "Factor XI Deficiency Database: An Interactive Web Database of Mutations, Phenotypes, and Structural Analysis Tools", *Human Mutation*, Vol. 26:1922005, 2005.

Seligsohn, U., "Factor XI in Heamostasis and Thrombosis: Past, Present and Future", *Thrombosis and Haemostasis,* Vol.98, pp. 84-89, 2007

Shida, Y., K. Nishio, M. Sugimoto, T. Mizuno, M. Hamada, S. Kato, M. Matsumoto, K. Okuchi, Y. Fujimura and A. Yoshioka, "Functional Imaging of Shear-Dependent Activity of ADAMTS13 in Regulating Mural Thrombus Growth Under Whole Blood Flow Conditions", *Blood,* Vol. 111, No. 3, pp.1295-1298, 2008.

Solda, G., R. Asselta, R. Ghiotto, M. L. Tenchini, G. Castaman and S. Duga, "A type II mutation (Gluu117Stop), Induction of Allele-Specific mRNA Degredation and Factor XI Deficiency" *Haematologica,* Vol. 90, pp.1716-1718, 2005.

Solomon, O., A. Zivelin, T. Livnat and U. Selignsohn, "Inhibitors to Factor XI in Patients with Severe Factor XI Deficiency", *Seminars in Hematology,* Vol.43, Supp. 1, pp.10-12, 2006.

Tarumi, T., D. V. Kravtsov, M. Zhao, S. M. Williams and D. Galliani, "Cloning and Characterization of the Human Factor XI Gene Promoter: Transcription Factor Hepatocyte Nuclear Factor 4α (HNF-4α) is Required for Hepatocyte Specific Expression of Factor XI", *Journal of Biological Chemistry,* Vol.277, No. 21, pp. 18510-18516, 2002.

Veyradier, A., E. Fressinaud and D. Meyer, "Laboratory diagnosis of von Willebrand Disease", *International Journal of Clinical Laboratory Research,* Vol. 28, pp.201-210, 1998.

Zhou, L., L. Wang, R. Palais, R. Pryor and C. T. Wittwer, "High Resolution DNA Melting Analysis for Simultaneous Mutation Scanning and Genotyping in Solution", *Clinical Chemistry,* Vol. 51, No. 10, pp. 1770-1777, 2005.