



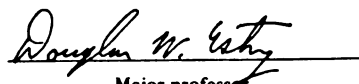
This is to certify that the
thesis entitled
IDENTIFICATION OF THE MOLECULAR DEFECT
OF GLYCOPROTEIN Ib ALPHA IN A PATIENT
WITH BERNARD-SOULIER SYNDROME

presented by

Aida A. Abujoub

has been accepted towards fulfillment
of the requirements for

Masters degree in Clinical Laboratory
Sciences


Major professor

Date May 2, 1997

PLACE IN RETURN BOX to remove this checkout from your record.
TO AVOID FINES return on or before date due.

DATE DUE	DATE DUE	DATE DUE
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____

MSU Is An Affirmative Action/Equal Opportunity Institution

c:\circ\datedue.pw3-p.1

**IDENTIFICATION OF THE MOLECULAR DEFECT OF GLYCOPROTEIN Ib α
IN A PATIENT WITH BERNARD-SOULIER SYNDROME**

BY

Aida A. Abujoub

A THESIS

**Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of**

MASTER OF SCIENCE

Medical Technology Program

1997

ID:

gly

be

the

an

m

by

an

co

en

ac

di

he

ABSTRACT

IDENTIFICATION OF THE MOLECULAR DEFECT OF GLYCOPROTEIN Ib α IN A PATIENT WITH BERNARD-SOULIER SYNDROME

BY

Aida A. Abujoub

Bernard Soulier Syndrome (BSS) is a rare congenital bleeding disorder. The glycoprotein (GP) Ib-IX-V complex, the receptor for von Willebrand factor, was found to be absent or dysfunctional in patients with BSS. At the molecular level a defect in any of the genes that encode GPIb α , GPIb β , GPIX, and possibly GPV could prevent assembly and cell surface expression of the entire complex.

In this research a family with BSS is being investigated to determine the molecular basis of GPIb α absence. The proband's platelets lack GPIb α as documented by two dimensional gel electrophoresis. Polymerase chain reaction (PCR) did not reveal any gross rearrangement (insertion or deletion) within GPIb α gene. Single stranded conformation polymorphism analysis indicated that a mutation could be present at the 3' end of GPIb α gene. Sequencing analysis identified a point mutation that changed amino acid proline 481 to a serine a change which could play a role in the development of the disease. PCR, restriction digest and sequencing analyses showed that the mother is heterozygote for a 39 bp repeat, whereas the proband is homozygote for this repeat.

To my mother and father
Adla and Abdel-Hamid Samara,
who instilled in me that knowledge
is the greatest achievement.

To my wonderful husband
Amin
for all his support, love and help.

To my precious children,
Rawan and Shadi.

This is for you all

ACKNOWLEDGMENTS

I would like to thank my advisor Dr. Douglas Estry for his time, guidance and financial support. Also I would to thank Dr. Joan Mattson for providing us with the blood samples and clinical data. A special thanks to Dr. John Gerlach and Dr. Paul Coussens for their guidance, suggestions, comments and for giving me the opportunity to use their labs to conduct my experiments. My deepest appreciation goes to my husband Dr. Amin Abujoub for his help, support and encouragement.

List of

List of

Introd

Mate

TABLE OF CONTENTS

List of Tables	vii
List of Figures	viii
Introduction	1
Overview of hemostasis	1
Bernard-Soulier Syndrome	5
Normal Biochemical and Molecular Characteristics of GPIb-IX-V	6
GPIb-IX complex and vWf.	11
Structural and Functional Defects of platelets in BSS	14
Platelet structure abnormality in BSS	14
Platelet function abnormality	14
Platelet membrane glycoprotein abnormalities	15
Molecular Defects Associated with BSS	16
Polymorphism in GPIb-IX	23
Family History	30
Objectives	30
Materials and Methods	33
Sample collection and DNA isolation	33
DNA amplification	35

Resu

Di

F

L

Agarose gel electrophoresis	37
PCR products purification	37
Heteroduplexing analysis	38
Single Stranded Conformation Polymorphism (SSCP)	39
Cloning	40
Preparation of large scale DNA	46
Sequencing	49
Sequencing analysis	50
Results	51
Polymerase Chain Reaction	51
Hetreoduplexing	53
Single Stranded Conformation Polymorphism (SSCP)	54
Cloning	59
Sequencing	62
Discussion	74
Localization of mutation(s) by heteroduplexing and Single Stranded Conformation Polymorphism	76
Sequencing and Sequence Analysis	79
Future Research	82
List of references	84

Table

Table

Table

Table

Table

LIST OF TABLES

Table 1. Mutations found in BSS.	24
Table 2. Gene frequency of molecular weight polymorphism in Japanese and American population.	27
Table 3. Tandem repeat polymorphism of individuals with ATG 145.	28
Table 4. Frequency distribution of GPIb- α alleles	29
Table 5. Sequences of primers used for amplification and sequencing.	35

Fig

Fig

Fig

Fig

Fig

Fi

Fi

Fi

Fi

Fi

Fi

Fi

F

F

LIST OF FIGURES

Figure 1. Assembly of GPIb-IX complex.	8
Figure 2. Polymorphism of GPIb-IX complex.	25
Figure 3. Pedigree of the studied family.	32
Figure 4. Cloning vector pUC19.	41
Figure 5. Purification of the closed circular plasmid DNA by ultracentrifugation using CsCl-ethidium bromide gradient centrifugation method.	48
Figure 6. PCR amplification of the first and the second fragments.	52
Figure 7. PCR amplification of the third fragment.	55
Figure 8. Heteroduplexing analysis gel.	56
Figure 9. Restriction digest of the first and the second PCR fragments.	57
Figure 10. Restriction digest of the third PCR fragment.	58
Figure 11. SSCP of the first and second fragments.	60
Figure 12. SSCP analysis of the third fragment.	61
Figure 13. Comparison of the third fragment nucleic acid sequences.	64
Figure 14. Comparison of the third fragment peptide sequences.	71

0

va

fi

c

c

W

INTRODUCTION

Overview of hemostasis:

Hemostasis is the process by which the body prevents the loss of blood from its vascular system. This process involves platelets, blood vessels, coagulation factors, fibrinolytic proteins and inhibitors that maintain a state of fluidity in the normal circulation as well as help form a physical barrier that limits the loss of blood from sites of vascular injury (Ratnof et al., 1984). The balance of hemostasis must be maintained to prevent bleeding or pathologic thrombosis.

Physical and biochemical properties of blood vessels help to maintain normal circulation. The physical properties include vasoconstriction, vasodilation and changes in vessel permeability. They separate plasma factors from the highly reactive elements present in the deeper layers of the vessel wall. These elements include adhesive proteins such as:

- a) collagen and von Willebrand factor (vWf) which promote platelet adhesion to the injured vessel wall (Hession et al., 1990).
- b) tissue factor, a membrane protein located in fibroblasts and macrophages that triggers blood coagulation (Hession et al., 1990).

Biochemical properties of the blood vessel include inhibition of blood coagulation by synthesizing and releasing heparan sulfate, which accelerates the activity of antithrombin

III.

and

disc

cyt

of

sec

gr

su

th

er

m

v

in

T

F

c

v

t

III, a plasma anticoagulant and the vascular endothelium synthesis of prostacyclin (PGI₂) and nitric oxide which cause vasodilation (Weiss et al., 1979)

Platelets play a fundamental role in this hemostatic process. Platelets are 2-4 μm, disc-shaped cells formed in the bone marrow by fragmentation of the megakaryocyte cytoplasm. Normal whole blood contains 150,000-400,000 platelets /μL and the life span of the average platelet is 9.5-10.5 days.

The platelet has a variety of functions. These include adhesion, aggregation, secretion and concentration of plasma clotting factors on both the membrane and within granules. Platelets adhere to an injured vessel and then spread on the exposed subendothelial surface. Adhesion at high shear rates depends on the presence of vWf and the platelets surface receptor complex GPIb-IX-V. Platelets do not adhere to normal endothelial cells but, in the case of injury, the adhesive proteins in the subendothelial matrix of blood vessels facilitate platelet adhesion to subendothelial collagen. Von Willebrand factor is made up of multiple functional domains, including domains involved in binding to GPIb and to the subendothelium of blood vessels (Clemetson et al., 1982). Thus vWf present in the subendothelium may directly mediate platelet adhesion and the plasma and/or platelet form of vWf may bridge platelets to other subendothelial components such as collagen and microfibrils. It is postulated that the association of vWf with matrix constituents may alter its properties and permit its direct binding to GPIb (Handin et al., 1989).

Secretion is the release of a wide variety of substances that can stimulate or inhibit platelets, contribute to cell adhesive events and modulate growth of cells of the vessel wall. Most of these substances are either secreted from preformed storage granules (alpha

and c

bodi

plate

brou

(TX

al..

the

thro

and

diff

198

vW

suc

pla

pla

gr

in

fo

o

fa

fi

c

and dense granules) or synthesized de novo from membrane phospholipids. The dense bodies release ADP (Greenburg et al., 1988), a weak agonist, which recruits other platelets to the forming aggregate. ADP induces secretion only when platelets are brought into close contact during aggregation (Charo et al., 1977). Thromboxane A₂ (TXA₂) is generated as a consequence of hydrolysis of membrane phospholipids (Kroll et al., 1989). It is a highly potent aggregation stimulating substance. TXA₂ is produced by the cyclooxygenase pathway from arachidonate. When added to platelets in vitro, thromboxane causes shape change, aggregation, secretion, phosphoinositide hydrolysis and an increase in the cytosolic calcium (Gerrad et al., 1981). Once formed, TXA₂ diffuses across the platelet plasma membrane and activates other platelets (Brass et al., 1987). The alpha (α) granules of platelets contain adhesive proteins such as fibrinogen, vWf, fibronectin, vitronectin, thrombospondin. They also contain growth modulators such as platelet factor 4 and platelet-derived growth factor (PDGF) (Snyder 1989), which play an important role in smooth muscle cell proliferation that may occur consequent to platelet interaction with vessel walls. Many coagulation proteins are present in α granules and are released by platelets. The alpha granules contain factor V which is involved in the generation of thrombin (Wencel-Drake et al., 1986).

Platelet aggregation is the process by which platelets interact with one another to form a hemostatic plug. Both fibrinogen and GPIIb-IIIa are required for aggregation to occur (Peerschke 1985, Phillips et al. 1988). GPIIb-IIIa complex belongs to the integrin family of adhesion receptors and functions as the platelet receptor for fibrinogen, fibronectin and vWf (Phillips et al., 1988). GPIIb-IIIa is the most abundant glycoprotein on platelet surface (50,000 - 80,000 copies / platelet). Patients with Glanzman's

Thre:

fail to

activa

weak

fibrin

1987)

recept

memb

fibrin

sugge

comp

al., 19

hemo

adhe

V co

defec

thron

(Nur

cell

form

Thrombasthenia have defective or abnormal amounts of GPIIb-IIIa, thus their platelets fail to bind fibrinogen and fail to aggregate (Nurden et al., 1974).

Soluble fibrinogen does not interact with resting platelets, but binds to platelets activated by various agonists (strong agonists such as thrombin and thromboxane A_2 , weak agonists such as ADP). Thrombin and thromboxane A_2 induce exposure of fibrinogen receptors by activation of phospholipase C and protein kinase C (Shattil et al., 1987). The intracellular molecular mechanism of the exposure of platelet fibrinogen receptor is not well understood but platelet activation might induce a change in the membrane micro environment around GPIIb-IIIa, allowing access of ligands, such as fibrinogen, to their binding site (Coller 1986). Another more recent theory theory suggests that platelet activation causes a conformational change within the GPIIb-IIIa complex thereby inducing formation or exposure of a ligand-binding pocket (Golden et al., 1990).

The importance of platelet membrane receptors is illustrated by the occurrence of hemorrhage in different diseases such as Bernard-Soulier Syndrome (BSS) where the adhesion process is impaired due to the fact that the patient's platelets lack the GPIb-IX-V complex (Nurden et al., 1983), in von Willebrand disease in which vWf is decreased or defective resulting in decreased platelet adhesion (Weiss et al., 1978), or Glanzman's thrombasthenia where GPIIb-IIIa is absent or defective therefore preventing aggregation (Nurden et al., 1974).

In addition the platelet plasma membrane plays an important hemostatic role in cell-cell interaction and in concentrating coagulation factors thus assisting in the formation of a fibrin clot. Although, the various components of the enzyme-substrate

comp

of rea

(Nur

serve

In ad

partic

Bern

infar

plate

next

esta

Syn

rece

botl

dys

et a

nor

co

He

complexes that constitute the coagulation pathway can interact in the fluid phase, the rate of reaction increases by at least 1000 fold in association with a phospholipid surface (Nurden et al., 1975). The platelet plasma membrane acts as a phospholipid surface and serves to enrich the local concentrations of reactants such as factor X, V and prothrombin. In addition the platelet α -granule contains some of the factor V, which when secreted participates in this process (Hoffman et al., 1991).

Bernard-Soulier Syndrome

Bernard-Soulier Syndrome (BSS) was first described in 1948 with the report of an infant who had severe mucocutaneous bleeding, a prolonged bleeding time with a normal platelet count and abnormally large platelets (Bernard J. and Soulier J., 1948). Over the next ten years additional patients were reported. In 1964 a review of 14 patients established the diagnostic criteria for the disease (Alagille et al., 1964). Bernard-Soulier Syndrome is a congenital bleeding disorder that is usually inherited in an autosomal recessive manner. Consanguinity is often noticed, and the disease is equally common in both sexes (Bernard 1983).

Bernard-Soulier syndrome is characterized by giant platelets with an absent or dysfunctional platelet membrane receptor GPIb-IX-V complex (Nurden et al., 1981; Ware et al., 1993), prolonged bleeding time, thrombocytopenia in most patients, large platelets, normal coagulation time and normal clot retraction. Platelet counts in these patients commonly range from 50,000/ μ L to near normal (normal value 150,000 - 400,000/ μ L). However platelet counts as low as 20,000/ μ l have been reported (Alagille et al., 1964).

The illness is both rare and ubiquitous, it has been observed in Europe (Italy,

Britain, France, Poland, Austria, Norway and Greece), in Asia (India, Japan, Kuwait and Turkey), in America (United States and Mexico), and in Africa (South Africa and Reunion) (Bernard 1983). The signs of the disease are usually noticed during the first months of life, but a few exceptions in which onset of symptoms occurs later have been reported (Bernard 1983). Hemorrhage, the only clinical sign of the disease, is varied. Epistaxes, ecchymoses and purpura are frequent. Menorrhagia and digestive hemorrhages are often observed. Interestingly hematuria and hemorrhages due to circumcision and retinal hemorrhages are rare (Bernard J. 1983).

Normal Biochemical and Molecular Characteristics of GPIb-IX-V:

The GPIb-IX-V complex is a major sialoglycoprotein on the platelet surface and contributes to the net negative charge of the platelet surface. GPIb is a high molecular weight GP composed of two subunits. Cleavage of intermolecular disulfide bonds results in the separation of a small 22 kDa, β -subunit from a large, 148 kDa, α -subunit (Modderman et al., 1992). The outer N-terminal 45 kDa region of GPIb α forms a compact globular domain containing binding sites for both vWf and thrombin (Wicki et al., 1985). Approximately 25,000 copies of the GPIb-IX complex and half as many of GPV are present on the platelet surface. Each of the three polypeptides α , β and IX span the platelet plasma membrane once, with the carboxyl terminus in the cytoplasm and the amino terminus toward the exterior (Lopez et al. 1987, Lopez et al. 1988, Hickey et al. 1989). The three polypeptides and GPV belong to the leucine rich family of glycoproteins and have a structural domain defined by one or more tandem repeats of a conserved leucine-rich sequence and conserved sequences flanking the repeats. GPIb α

has 7 leucine rich repeats (LRR). GPIIb β and GPIX each have one LRR, whereas GPV has 15 LRR (Lopez 1994; Sae-Tung et.al,1996).

The GPIIb-IX complex consists of two globular domains with an intervening rod-like region (Figure 1, adapted from Lopez 1994). The smaller globular domain represents the amino-terminal ligand-binding part of GPIIb α , and the larger globular domain consists of the GPIIb α transmembrane and cytoplasmic domains associated with GPIIb β and GPIX. The rod-like region that connects the two globular domains consists primarily of the macroglycopeptide part of GPIIb α .

The structure of GPIIb α can be divided into four distinct domains:

- 1) A globular domain at the amino terminus that contains the seven leucine rich repeats (LRR), consists of approximately 300 amino acids and has two N-glycosylation sites and few, if any, O-linked carbohydrate chains. This domain accounts for most, if not all, of the vWf binding to the complex and also contains a binding site for thrombin. This region also contains all of the intrachain disulphide bonds in GPIIb α , having seven of its nine cysteines. The cysteines form three disulphide bonds, leaving one free thiol. A disulphide bond links Cys-4 at the amino-terminal and Cys-17 just prior to the first LRR (Fox et al., 1988). Two more disulphide bonds follow the seven LRR and are formed by a pairing between Cys-209 with Cys-248 and Cys-211 with Cys-264. Because of the proximity of Cys 209 and Cys-211, the structure that results from this disulfide pairing consists of two loops, these loops are critical for the ability of the complex to bind vWf.

- 2) The macroglycopeptide is an 84KD peptide that separates the amino-terminal globular domain from the plasma membrane. The sequence of this region is dominated by threonine, serine and proline. Most of the threonine and serine residues are

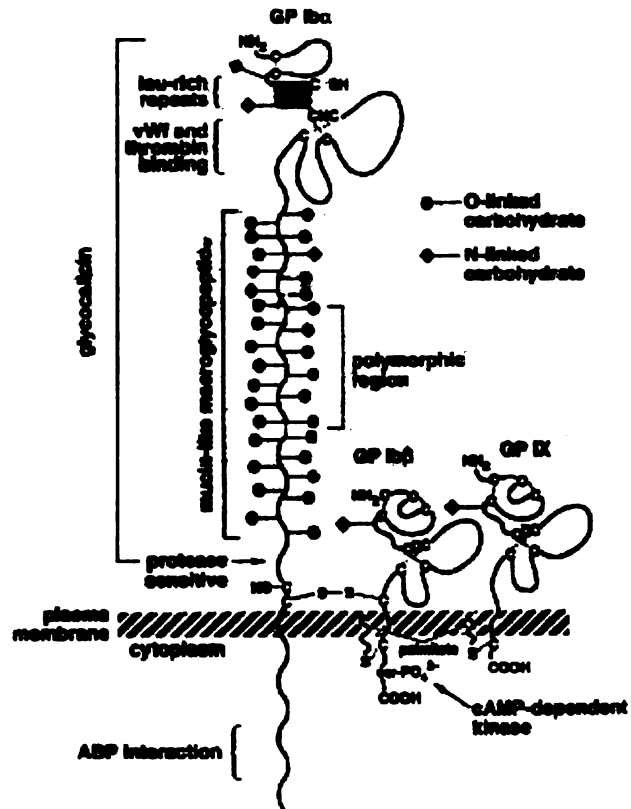


Figure 1. Assembly of GPIb-IX complex. This schematic diagram shows the different structure motifs of GPIb- α and its covalent association with GPIb- β . (Adapted from Lopez 1994).

glycosylated. Glycosylation accounts for approximately half of the molecular weight of the GPIb α polypeptide determined by SDS-polyacrylamide gel electrophoresis. Most of the carbohydrate chains in this region are modified by sialic acid which makes GPIb α the major contributor to the sialic acid concentration on the platelet surface (Judson et al. 1982, Tsuji et al. 1983, Korrel et al. 1984). This concentration was estimated to be eleven times that of the erythrocyte (Maddoff et al., 1964), giving the platelet surface a very high negative charge which generates electrostatic repulsion that keeps platelets apart as they circulate in the blood stream.

The extensive glycosylation and high proline content of this region prevent the formation of extended secondary structure (Shogren et al., 1989). The structure of the GPIb α resembles a palm tree, with the ligand binding domain representing the leafy top and the macroglycopeptide representing its rigid trunk. The elongated nature of this receptor is possibly one of the structural features that underlie its unique response to shear stress. Following the macroglycopeptide and preceding the hydrophobic transmembrane domain is a sequence of approximately 30 amino acids that has very few sites for O-glycosylation. This region contains the protease sensitive site. Cleavage at this site releases a water-soluble extra cytoplasmic domain of GPIb α known as glycocalicin.

3) The hydrophobic transmembrane segment consists of 29 amino acids flanked with charged amino acids which may serve to anchor the protein in the membrane (Lopez et al., 1987). 4) The cytoplasmic domain is approximately 100 amino acids. This domain connects the GPIb to the cortical cytoskeleton of the platelet. The interaction of GPIb-IX complex with the cytoskeleton plays a role in maintaining the distribution of the complex on the platelet surface and may be necessary for the proper spatial relations of the

complex with other proteins on the plain of the plasma membrane (Fox et al., 1989).

The structure of GPIb β and GPIX shows approximately 60% sequence similarity (identical or conserved residues) in their extra cytoplasmic domains but diverge completely in their cytoplasmic domains. The major differences between the overall structure of these smaller subunits and that of GPIb α are the presence of the macroglycopeptide domain in GPIb α , the number of LRR, and a much longer cytoplasmic domain in GPIb α . The cytoplasmic domain of GPIb β is approximately 34 amino acids and that of GPIX ranges between 6-8 amino acids. The disulphide pairings in GPIb β and GPIX, shown in figure 1 above, are drawn from homology with GPIb α and with other members of the leucine-rich family (Neame et al., 1989; Lopez 1994).

Each of the leucine rich glycoproteins in the complex GPIb-IX-V is encoded by its own gene. The α chain has been cloned, completely sequenced (Lopez et al., 1987; Wenger et al., 1988), and localized to chromosome 17 (Wenger et al., 1989). Also, the cDNA for GPIX has been cloned, sequenced (Hickey et al., 1989; Miller et al., 1992), and localized to chromosome 3 (Hickey et al., 1993). The gene encoding GPV has been cloned (Lanza et al., 1993), but its chromosomal location has not been determined (Lopez, 1994). GPIb β has been localized to chromosome 22 and it was cloned and sequenced (Lopez et al., 1988 b). GPIb α and GPV each have one intron, where as GPIX has two introns, all of which are in the 5' untranslated regions of the gene immediately upstream of the ATG initiation codon, which leaves the coding sequence uninterrupted (Lopez, 1994).

GPIb-IX complex and vWf

Platelet adhesion to vWf in the subendothelium is the major function of the GPIb-IX complex, evidence for this function is as follows:

- 1) In the congenital absence of GPIb, as in BSS, platelets fail to bind to vWf when stimulated with Ristocetin. Ristocetin is a peptide antibiotic that is used to diagnose BSS. Some data indicates that it binds to the receptor whereas other data shows that it binds to the ligand. (Coller et al. 1977, Coller et al. 1978, Jenkins et al. 1979)
- 2) Selective proteolytic digestion of GPIb decrease ristocetin-induced vWf binding.
- 3) Monoclonal antibodies against GPIb selectively inhibit ristocetin-induced vWf dependent platelet agglutination (Oscar et al., 1984).
- 4) Inhibition of platelet binding to immobilized vWf in the presence of monoclonal antibodies against the GPIb-IX complex (Weiss et al., 1986).

Although vWf circulates freely in plasma, under normal conditions it does not associate with GPIb-IX. The binding appears to require a change in either the ligand (vWf) or the receptor (GPIb-IX), induced either by shear or by immobilization of vWf on the subendothelial matrix. For platelet aggregation to occur, shear stresses must reach levels high enough to allow the GPIb-IX/vWf interaction to occur in the fluid phase, with a subsequent events resulting in the formation of a platelet aggregate. Shear stresses high enough to induce the fluid-phase interaction between GPIb-IX complex and vWf are not found in normal physiologic situations (Maoke et al., 1988). It appears that either one or both vWf and GPIb-IX molecules are acting as mechanoreceptors capable of sensing shear (vWf) and adapting a conformation (GPIb-IX) favorable for the interaction to occur

(Peterson et al., 1987).

Many studies have been done to identify the site on GPIb-IX complex to which vWf binds. Synthetic peptides that block certain areas of GPIb α were used for this purpose (Vicenti et al., 1990). These studies along with others that involved recombinant GPIb α polypeptide (Cruz et al., 1992) suggest that the vWf binding domain comprises more than simple linear sequences. The studies together with naturally occurring mutations provided evidence that the two loops formed by two disulphide bonds in the carboxyl terminus of this region are essential for vWf binding to GPIb-IX complex. The first loop appears to regulate the binding of vWf to the second loop, possibly through steric effects. Another possibility is that discontinuous stretches of sequences from both loops combine to form the binding site for vWf (Lopez, 1994).

The GPIb-IX complex plays a role in stabilizing the plasma membrane and thus helps maintain the discoid shape of platelets (Fox et al., 1988). This occurs by interaction between the cytoplasmic portion of GPIb-IX and the submembraneous cytoskeleton. Platelets from individuals with BSS lack this membrane-cytoskeleton interaction which may be the cause of large and irregularly-shaped cells (Okita et al., 1985). Membrane fluidity studies have shown that BSS platelets are much more deformable than normal platelets (McGill et al., 1984) and form surface filopods three times longer than normal ones when aspirated into micropipettes (White et al., 1984). This could be due to the absence of interaction between GPIb-IX and actin binding protein (ABP). This loss of membrane stability may predispose platelets to early destruction when exposed to the rigours of traversing the circulation which could be the reason for low platelet counts in these patients (Hartwig et al., 1991). Anchorage of the GPIb-IX complex to the skeletal

framework of the cell may also affect the ability of the complex to mediate platelet adhesion. This could happen in two ways, first attachment of the complex to the submembraneous region may result in an orderly spatial distribution of the receptor on the platelet surface. This arrangement could be important for the optimal interaction of the complex with its ligand vWf, which binds to platelet best when it is in the very large multimer form (Federici et al., 1989). Second, the attachment of complex to cytoskeleton may regulate the affinity with which vWf binds to the complex, independent of any distribution effect (Coller et al, 1982).

Glycoprotein V is another platelet membrane glycoprotein that is rich in leucine, has large amounts of carbohydrate and provides antigenic determinants on the platelet surface for antiplatelet antibodies (Gerald et al., 1993). GPV is an 82 KDa glycoprotein which was first identified because it is a substrate for thrombin on intact platelets. Thrombin cleavage causes liberation of a large (MW 69,000) water soluble fragment of GPV (Modderman et al.,1992). Therefore, GPV is a logical candidate to be a thrombin receptor. However, experiments have shown that even platelets that lack GPV (on a congenital, proteolytic or immunologic basis) respond to thrombin in a nearly normal fashion. This implies that GPV may influence the effect of thrombin on platelets, but is not likely to be an actual thrombin receptor.

Glycoprotein V is not involved directly in platelet adhesion and has non-covalent association with the GPIb-IX complex but present in close proximity to it (Gerald et al.,1990). Modderman et al.1992, found that GPV and GPIb-IX form a noncovalent complex in the platelet membrane. It is unknown why in BSS GPV is deficient along with GPIb-IX, but perhaps all three proteins must participate in some or all steps (synthesis,

processing, and assembly) that lead to the expression of the mature complex on the platelet surface (Gerald et al., 1990).

Structural and Functional Defects of Platelets in BSS:

Platelet structure abnormality in BSS:

Large platelets are the most prominent morphological feature of BSS. Mattson et al 1984, studied the size of platelets in two homozygote BSS siblings and their heterozygote parents. Platelets from the two affected siblings had a mean diameter of 3.13 μm and 4.14 μm as measured on Wright Giemsa stained smears, whereas normal controls had platelet diameters ranged from 0.83 - 2.33 μm . In the same study, diameters of spread BSS platelets were larger than fully spread controls as evidenced by both scanning electron microscopy and transmission electron microscopy. Platelets appear to be more spherical and more deformable. As previously indicated the increased deformability may be related to the lack of transmembrane linkages to the cytoskeleton. Platelet survival was markedly shortened in several studies (Najean et al. 1963, Grottum et al. 1969, Cullum et al. 1967).

Platelet function abnormality:

In 1972, it was noted that although BSS platelets aggregated normally in response to adenosine diphosphate (ADP), epinephrine, and collagen, platelets failed to aggregate when stimulated with vWf and ristocetin (Clemetson et al., 1982). Ristocetin induces vWf binding to normal platelets and this is absent or markedly decreased in BSS. *In vitro* normal platelets adhere to immobilized vWf in the absence of ristocetin whereas

BSS platelets do not. This may be analogous to platelet adhesion to subendothelium bound vWf *in vivo*. Von Willebrand factor binding stimulated by ADP or thrombin, which occurs on the GPIIb-IIIa complex rather than GPIb, is normal in BSS platelets, this is consistent with other evidence that the GPIIb-IIIa receptor functions normally (Peerschke et al., 1980). The response of BSS platelets to α -thrombin is decreased, demonstrated by decreased thrombin binding and decreased aggregation. It has been shown that GPIb contains one or more high affinity thrombin binding sites and the diminished response of BSS platelets may be related to the absence of these sites.

Platelet membrane glycoprotein abnormalities:

A plasma membrane abnormality of BSS platelets was first suggested in 1969 by observing a decrease in electrophoretic mobility that was associated with a decrease in sialic acid (Grottum et al., 1969). In 1975, Nurden and Caen demonstrated a decreased amount of the platelet membrane GPIb. These results were confirmed by single and two dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of ^{125}I labeled platelets. These early observations and other studies were important in establishing that GPIb was a platelet receptor for vWf (George et al., 1984). Further studies using H^3 borohidride to radiolabel platelet surface carbohydrate residues demonstrated that, in addition to GPIb, BSS platelets were deficient in GPIX and GPV (Nurden et al., 1983).

GPIb and GPIX form a 1:1 noncovalent complex (Du et al., 1979). This complex is linked to the membrane skeleton by an actin binding protein (Okita et al., 1985). Although GPV is a substrate for thrombin it has no known physiological role.

The deficiency of GPV in the BSS platelets may indicate an association with the GPIb-IX complex (Colman et al., 1994; Modderman et al., 1992).

Molecular Defects Associated with BSS:

The classic form of BSS is characterized by the absence of the GPIb-IX-V complex. However, other variants have been described that show a wide variety of molecular differences. In some cases, relatively normal levels of all four chains (GPIb α , GPIb β , GPIX and GPV) have been detected with the defect eventually being localized to a point mutation in the outer domain of GPIb α (Miller et al., 1992). In other cases, the amounts of all the chains are reduced in parallel suggesting that there is a coordinated expression, or the expression level of one of the chains regulates the amount of the mature complex (Clemetson et al., 1993).

A few rare cases have demonstrated an imbalance between the chains expressed where GPIb was totally absent but low amounts of GPIX have been found (Hourdille et al., 1990). In some cases, at low levels of all the chains, there was clearly less GPIX than the other three (Clemetson et al., 1993). There are rare cases that seem to be due to a double heterozygote defect in GPIb α chain (Ware et al., 1990). Other cases have been produced by a double heterozygote point mutation in the gene encoding GPIX (Wright et al., 1993). A defect in one of the four genes that encodes GPIb, IX or GPV could alter or prevent the assembly and cell surface expression of the entire vWf receptor complex (Finch et al., 1990). A defect in the promoter for one of these genes could lead to a complete lack of expression without there being any problem in the translated, structural region (Clemetson et al., 1993). It was suggested (Lopez et al., 1992) that GPV is not

necessary for the surface expression of the other chains but that all three chains of GPIb-IX are necessary for an efficient surface expression of the complex.

The underlying molecular basis of BSS is currently unclear and may in fact be heterogeneous. As mentioned before, in BSS, deficiencies of membrane GPIb have been associated with comparable deficiencies in GPIX and GPV (Nurden et al., 1993). At the molecular level, different studies support the heterogeneous nature of underlying defects that may lead to an abnormal vWf receptor.

Ware et al. 1990, studied a case in which the GPIb-IX-V complex was absent from the platelet surface, but a truncated fragment could be detected on western blots. Sequencing of GPIb α showed a mutation of TGG to TGA that changed tryptophan 343 to a stop codon. This resulted in a truncated protein missing the membrane-spanning section of GPIb α . Hence it could not be anchored to the membrane and associate with other chains. It may be that the truncated fragment is present in the cytoplasm and is then degraded or secreted. This case is further complicated by the fact that it appears to be a double heterozygote. The Polymerase Chain Reaction (PCR) amplification of the region of the gene containing the mutation gave two sequences, one allele containing the mutation and the other allele being normal. It is proposed that the second allele must contain a defect causing a complete absence of expression of GPIb α , and when combined with the first mutated allele, only the truncated fragment is expressed. The defect in the second allele has still not been demonstrated.

Marco et al. 1990, had identified another BSS variant, termed BSS Bolzano, which leads to the typical symptoms of BSS. In this case, GPIb α appears to be present but in decreased levels. The difference between this variant and the classic form of BSS

lies in the presence of a smaller (105 KD) piece of GPIb α detected on western blots. Binding of thrombin was normal implying that only vWf binding was affected. This could be due to a mutation in the N-terminal region of GPIb α chain that affects the vWf binding site and most likely increases susceptibility to proteolytic degradation. This might occur as a result of the mutation providing a new cleavage site for a protease or by changing the conformation of a domain of the protein, making an already existing cleavage site more accessible.

In 1992 Miller et al. described an autosomal dominant variant of the disease. A point mutation in GPIb α , where a C at position 259 had been substituted by a T, converted a highly conserved leucine at position 57 to phenylalanine within the first leucine rich repeat in one of the alleles. In this case an abnormal rather than absent GPIb α was detected which resulted in abnormal vWf binding and the typical clinical features of BSS. This mutation could also increase the degradation rate of GPIb α . The abnormal GPIb α didn't affect the assembly of GPIb-IX complex on the platelet surface. This case indicates the importance of the integrated LRR..

Salle et al. 1995, reported a case where a three base deletion resulted in the removal of leucine 179, which is located in a highly conserved position of the seventh leucine-rich repeat of GPIb α . Three affected siblings were characterized by absence of ristocetin-induced platelet agglutination, although ADP aggregation was normal. Flow cytometry studies showed detectable amounts of all members of GPIb-IX-V complex on the surface of the patient's platelets, whereas western blot analysis revealed normal levels of GPIX, decreased levels of GPIb β and GPV, and less than 1% of GPIb α . Sequencing of GPIb α revealed the absence of leucine 179 which is believed to cause a conformational

change in the protein that would account for the lack of binding of most of the monoclonal antibodies tested against GPIb α . This mutation could be responsible for the absence of vWf binding . This case indicates the requirement of the leucine-rich region for the correct exposure of the vWf binding site as well as for the correct assembly and stability of the GPIb-IX-V complex on the platelet surface.

Simsek et al. 1994c, used monoclonal antibodies directed against GPIb α and GPIb-IX to show the absence of detectable GPIb α and the presence of small amounts of GP-IX. This indicated a defect in the α subunit of GPIb. Sequence analysis revealed a homozygous single base pair mutation, T to A, this led to the substitution of serine for cysteine at position 209 of the mature protein. Cysteine 209 in GPIb α was found to be involved in intrachain disulphide bond formation with Cys 248. This interaction is important in forming one of the loops that are critical for the ability of the complex to bind vWf. So this mutation had affected the proper folding of GPIb α , and presumably its surface expression as well as its non covalent association with GPIX and GPV which resulted in the disease.

In another case of BSS, Simsek et al. 1994a, analyzed platelet membrane glycoproteins by flow cytometry. This revealed a significant decrease or absence of GPIb α and low levels of GPIX and GPV. Sequence analysis demonstrated a homozygous deletion of T 317 resulting in a frame shift mutation. This deletion causes a shift in the reading frame, predicting a premature stop codon after 19 amino acids leading to a severely truncated nonfunctional protein.

Ware et al. 1993, suggested that the structural integrity of the leucine-rich repeat is necessary for normal function of the GPIb-IX-V complex and possibly for normal

platelet morphology. They studied a patient with BSS Bolzano and found a homozygous mutation which converted Ala 156 to Val within the leucine-rich repeat thus causing a structural abnormality resulting in the loss of conformation-sensitive epitopes. This mutation was responsible for decreased vWf binding. They suggested that the appropriate conformation maintained by the LRR in the extra cytoplasmic domain may also influence the linkage of the intracytoplasmic tail of GPIb α to the cytoskeleton. They concluded that the Ala 156 to Val mutation may be responsible for both the ligand binding defect and the apparent morphology of giant platelets in the Bolzano variant.

Kunishima et al. 1994, studied a novel variant phenotype of BSS in a female patient. Her platelets completely lacked the expression of GPIb α . However, her platelets' lysates contained GPIb α (23 % of normal value), and her plasma contained the glycosialicin part of GPIb α (60% of the normal values). GPIb β and GPIX were expressed in reduced amounts. DNA sequence analysis showed a homozygous single nucleotide substitution from a serine codon (TCA) to a nonsense codon (TAA) at residue 444 in GPIb α gene. The mutant gene generated a truncated GPIb α molecule lacking the transmembrane region and cytoplasmic tail. This mutation resides in the boundary between the macroglycopeptide and the transmembrane coding region which prevented the formation of the disulphide bond between GPIb α and GPIb β . Consequently GPIb α may be secreted from megakaryocytes and platelets as a soluble glycosialicin like molecule. Further the truncated GPIb α may be loosely associated with other subunits in the cytoplasm. When they cross the plasma membrane, the truncated GPIb α with no transmembrane domain is secreted into plasma, while GPIb β and GPIX are inserted into the membrane.

Li et al. 1995, studied two cases of BSS in two related individuals (first cousins) who had the clinical features of the disease. A point mutation was identified in codon 129 in GPIb α , this mutation lies in the first position of the fifth LRR, changing a leucine (CTC) to a proline (CCC). Transcription of GPIb-IX seemed to be normal but its surface expression was decreased (40% of normal), whereas the GPV level was found to be normal. These findings could indicate the critical importance of the LRR of GPIb α , and a defect in this motif could influence surface expression of GPIb-IX-V, hence changing the nature of receptor-ligand interaction.

As mentioned earlier BSS could be due to a defect in GPIb α , GPIb β , GPIX or GPV genes. Double heterozygosity for mutations in the GPIX gene in three siblings with classical BSS have been reported by Wright et al. 1993. Although the expression of GPIb α was reduced on the platelet surface, lesions at its locus had been excluded by restriction fragment length polymorphism analysis, whereas defects in GPIb β gene were excluded by single stranded conformation polymorphism analysis. Analysis of the GPIX gene showed two different missense mutations in the coding region of the GPIX gene. First, an A to a G transition in codon 21 resulted in conversion of a conserved aspartic acid to glycine within the amino terminal flanking sequence, and an A to a G change in codon 45 converted a conserved asparagine to serine within the core of LRR motif. Three affected individuals were doubly heterozygous for these mutations, which alter conserved residues in the GPIX LRR motif. Experiments performed in a Chinese hamster ovary (CHO) cell expression system showed that GPIX is a necessary requirement for membrane expression of the GPIb-IX complex, and the same seems to be true for both GPIb α , and GPIb β but not apparently for GPV. Wright et al. suggested that these

missense mutations cause severe disruption of GPIX structure and it supports evidence that the GPIX molecule, although small, is of critical importance in the formation of the intact, functional complex. The bulk of the GPIX molecule lies outside the plasma membrane, and its extracellular domain is largely composed of a single LRR motif. This study indicates the potential importance of this region.

Sae-Tung et al. 1996, took advantage of the study done by Wright et al. 1993, in which double heterozygote mutations in GPIX resulted in BSS. They studied the effect of these mutations on the biosynthesis of the GPIb-IX complex. Alpha and β CHO cells were transfected with the mutants and wild type GPIX. Wild type GPIX increased the expression of GPIb, whereas both mutants failed to do so. Cotransfection of wild type and mutant GPIX with GPIb β resulted in detection of wild type of GPIX on CHO surface but not the mutants although these mutant polypeptides were detected in low amounts in the intracellular compartments. Immunoprecipitation studies showed that mutant GPIX associates poorly with GPIb β which prevents the stabilization of GPIb on the cell surface thus resulting in BSS. These studies indicate the importance of the conserved leucine-rich repeat of GPIX in the association between GPIX and GPIb- β , and also the importance of GPIX in stabilizing GPIb-IX complex on the platelet surface.

Clemtson et al. 1994, studied another variant of BSS where a homozygous mutation lies in the leucine-rich domain of GPIX. Surface labeling of the platelets and two dimensional gel electrophoresis showed reduced but detectable amounts of GPIb-IX-V. However, there was markedly less GPIX than GPIb α , β , or GPV. This disproportion was confirmed by western blot analysis. Sequence analysis showed no mutations in the GPIb α gene, whereas a point mutation (A to G) was found in GPIX converting Asn 45 to

Ser within the leucine-rich domain. Both alleles of GPIX contained the same defect, which was confirmed by the appearance of a new site for the restriction enzyme *Fnu4H1*. It seemed that this mutation changed the conformation of the leucine rich domain, thus reducing surface expression of the complex by disturbing the association of its parts. Clemetson et al. 1994, suggested that among other possible functions, the leucine-rich domain present on all the components of GPIb-IX-V may play a role in the assembly and surface expression of the complex. Because this mutation is the same as one of the two single base pair substitutions reported by Wright et al. 1993, Clemetson et al. 1994 proposed that codon 45 in GPIX could be vulnerable to mutation because the two families are geographically widely separated. Table 1 summarizes the cases discussed above and shows the different mutations that are related to BSS.

Polymorphism in GPIb-IX

Two major protein polymorphisms of GPIb-IX complex have been described, both of which affect GPIb α . One is a molecular weight polymorphism due to the presence of a variable number of tandem repeats (VNTR) in the macroglycopeptide region. The other is a dimorphism at codon 145 where a Met (ATG), or a Thr (ACG) can be present. This polymorphism is illustrated in Figure 2, which was adapted with modifications from Lopez 1994.

The first polymorphism is molecular weight polymorphism. Size differences in GPIb α were first described by Moroi et al. 1984, who noted four variants (in Japanese donors) that migrated on non-reduced SDS-polyacrylamide gels with molecular masses ranging from 153 KD to 168 KD. They were designated A, B, C and D in order of

Table 1. Mutations found in BSS.

Protein	Mutation	Result	Expression on membrane	Reference
GPIb α	Deletion of Leu 179	BSS	Very low levels of GPIb α .	Salle et al., 1995
GPIb α	Leu 129 to Pro in the fifth LRR	BSS	Low levels of GPIb - IX Normal GP V	Li et al., 1995
GPIb α	Deletion of T 317 resulted in a premature stop codon	BSS	Very low to absent GPIb α . Low levels of GPIX and V	Simsek et al., 1994a
GPIb α	Cys 209 to Ser	BSS	Absence of GPIb α Low levels of GPIX	Simsek et al., 1994c
GPIb α	Ser 444 to stop codon	BSS	Absence of GPIb α Low levels of GPIb β Low levels of GPIX Truncated GPIb α in plasma	Kunishima et al., 1994
GPIb α	Ala 156 to Val in LRR	BSS Type Bolzano	Dysfunctional GPIb-IX-V.	Ware et al., 1993
GPIb α	Leu 57 to Phe in the first LRR	BSS	Abnormal GPIb α	Miller et al., 1992
GPIb α	Trp 343 to stop codon	BSS	Absent GPIb α	Ware et al., 1990
GPIX	Asp 45 to Ser in the LRR	BSS	Low levels of GPIb-IX-V	Clemetson et al., 1994
GPIX	Asp 21 to Gly and Asp 45 to Ser in the LRR	BSS	Low GPIb α . No GPIX	Wright et al., 1993

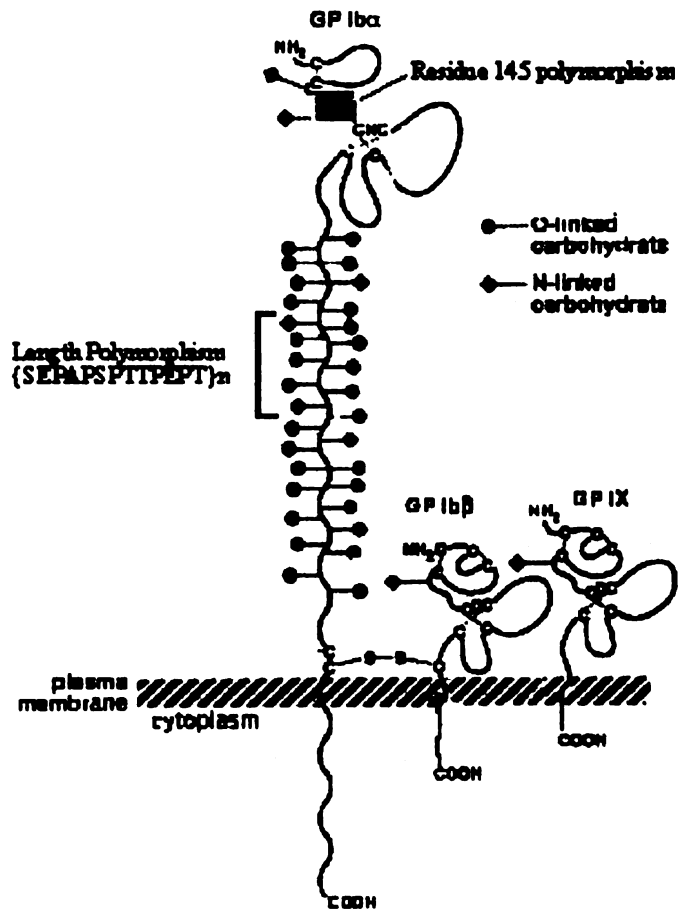


Figure 2. Polymorphism of GPIIb-IX complex. Length polymorphism involves 13 amino acids, starts at Ser 399 and ends at Thr 411, where n represents number of copies of the repeat. n=1, 2, 3, or 4 for variants D, C, B, and A respectively. The other polymorphism is at residue 145 (HPA-2), where a Met or a Thr can be found. Figure is adapted from Lopez 1994.

decreasing molecular mass. The frequency of the variants were quite different between American Caucasian and the Japanese population. In both populations the C variant was the most common, whereas the two largest alleles were uncommon (Table 2).

The region accounting for the polymorphism was later identified as the GPIb α macroglycopeptide. PCR and sequence analysis of genomic DNA performed on 207 individuals from four ethnic groups (Lopez 1994), showed three different PCR fragment lengths and the polymorphism was shown to result from either one or two duplications of the 39 base pair sequence corresponding to Ser 399 through Thr 411. However the fourth length variant was not found in this study.

Each added repeat of 13 amino acids contains five potential sites for glycosylation and thus may add up to 6 KD per repeat to the molecular mass of the polypeptide. Extensive glycosylation coupled with heavy proline content suggested that the repeated sequence has an extended conformation (Lopez 1994). Lopez 1994, also predicted that each repeat would increase the length of the macroglycopeptide and thus the distance of the ligand binding domain from the platelet membrane by about 32 Å. Length polymorphism could be a determinant of platelet sensitivity to shear due to moving vWf-binding domain farther out from the platelet plasma membrane which is likely to affect the fluid forces to which this region is exposed.

Ishida et al. 1996, studied the molecular evolution of GPIb α polymorphism in Eastern Asian population and they indicated that the frequencies of these polymorphisms differ considerably depending on race and the largest variant with four tandem repeats is almost exclusively present in the Japanese population. This study included different races from Eastern Asia, Japan (n= 103), Korea (n= 101), and China (n= 177). The A isoform

(four tandem repeats) was detected in all groups, and the T-4 haplotype, which has Met 145 (ATG) and 4 tandem repeats (TRs), held a majority in these three groups.

Homozygotes for haplotype T-4 were found among both the Japanese and the Korean populations but not in the Chinese Table 3. However, haplotype T-2 which has Thr 145 (ACG) and 2 TRs and was first identified in European Caucasians, was not detected in this study.

Table 2. Gene frequency of molecular weight polymorphism in Japanese and American population. A = highest molecular weight, D = lowest molecular weight

Gene frequency*		
	American	Japanese
A	1%	7%
B	11%	1%
C	80%	56%
D	8%	35%

* Data adapted from Lopez 1994.

So far these variants had no known functional differences, but whether modest variations (30 A per tandem repeat) in the distance by which the active site of GPIIb extend from the surface, influence adhesion and the susceptibility for pathologic thrombi

to form is not known (Nurden 1995). The other type of polymorphism is human platelet alloantigen (HPA-2). This polymorphism is within the LRR, it resulted from the presence of a Thr145 (HPA-2a), or Met 145 (HPA-2b) in the N-terminal globular domain of the human platelet GPIIb/IIIa (Nurden, 1995). Alloantibodies against either dimorphic variant can cause neonatal thrombocytopenia (Bizzaro et al., 1988) or can account for refractoriness to platelet transfusion (Saji et al., 1989).

Table 3. Tandem repeat polymorphism of individuals with ATG 145. A= 4 tandem repeats (TR), B= 3 TR, C= 2 TR, D= 1 TR

Genotype of TR*						
Population	AA	AC	AD	BC	BD	Total
Japanese	3	13	13	1	1	31
Korean	1	8	5	0	1	15
Chinese	0	9	0	1	1	11

*Data adapted from Ishida et al. (1996)

The genotype frequency of HPA-2a in the white population is 92.6%, that of HPA-2b is 7.4% (Kuijpers et al., 1992). The size polymorphism and HPA-2 are in linkage disequilibrium. The D variant (1 TR) or C variant (2 TRs) are associated with HPA-2a. The B variant (3 TRs) or C variant (2TR) are associated with HPA-2b . Ishida

et al. 1991, found that A or B variants are associated with HPA-2b.

Simsek et al. 1994b, studied the genetic association between Met 145 / Thr and the presence of VNTR polymorphism in 106 Caucasian individuals (Table 4). Simsek et al. 1994b, concluded that the two polymorphisms in GPIb α , HPA-2 at the N-terminus and

Table 4. Frequency distribution of GPIb α alleles

Allele	HPA-2/ VNTR	Frequency*
I	HPA-2a / D	0.11
II	HPA-2a / C	0.82
III	HPA-2b / C	<0.01
IV	HPA-2b / B	0.07

*Data adapted from Simsek et al., 1994b.

the size polymorphism of 13 amino acid sequence ³⁹⁹SEPAPSPTTPEPT⁴¹¹ are genetically linked in the Caucasian population. A possible evolutionary model is that allele type I (with D variant and a Thr 145) was the ancestral stage of the protein, then allele type II was the result of tandem duplication of the 13 amino acids sequence. A mutation from Thr 145 to Met 145 had occurred giving rise to type III. A duplication of the 13 amino acids sequence occurred immediately after this mutation leading to the formation of type IV which has Met 145 and 3 copies of the same 13 amino acids. A variant with 4 repeats

had not been found in this group but Simsek hypothesized that A variant would have Met at residue 145

Family History:

The family that is studied in this research is of Middle Eastern origin. Consanguinity has been noticed as the parents are cousins. The patient, a female of 22 years age, has all the clinical features of the disease including bleeding episodes, low platelet count (29,000 cells /uL), prolonged bleeding time (greater than 20 minutes) and giant platelets. Aggregation of the patient's platelets in response to ristocetin showed a value of 16% compared to more than 39% in a normal person. Treatment of patient's platelets with antibodies against GPIb α , showed low reactivity compared to normal. Two dimensional gel electrophoresis indicated the absence of GPIb α from patient's platelets. Other family members show some criteria such as thrombocytopenia and giant platelets, but no bleeding. The family pedigree is shown in Figure 3.

Objectives:

In this research we studied the GPIb α gene in the patient mentioned above and her mother, in an attempt to localize and identify the mutation that could be the cause for an absent GPIb α on the patient's platelets. Understanding the molecular defects in BSS will shed more light on the mechanism by which vWf interacts with its receptor (GPIb-IX) on the platelet surface. Identifying the defect in the patient's gene will also help in screening other members of the family for heterozygosity. This could be done by different approaches, such as synthesizing a probe corresponding to the mutation and

hybridizing it with the questioned gene, or using the restriction fragment length polymorphism (RFLP) technique if the mutation altered a site for a restriction enzyme, or if it generated a new restriction site.

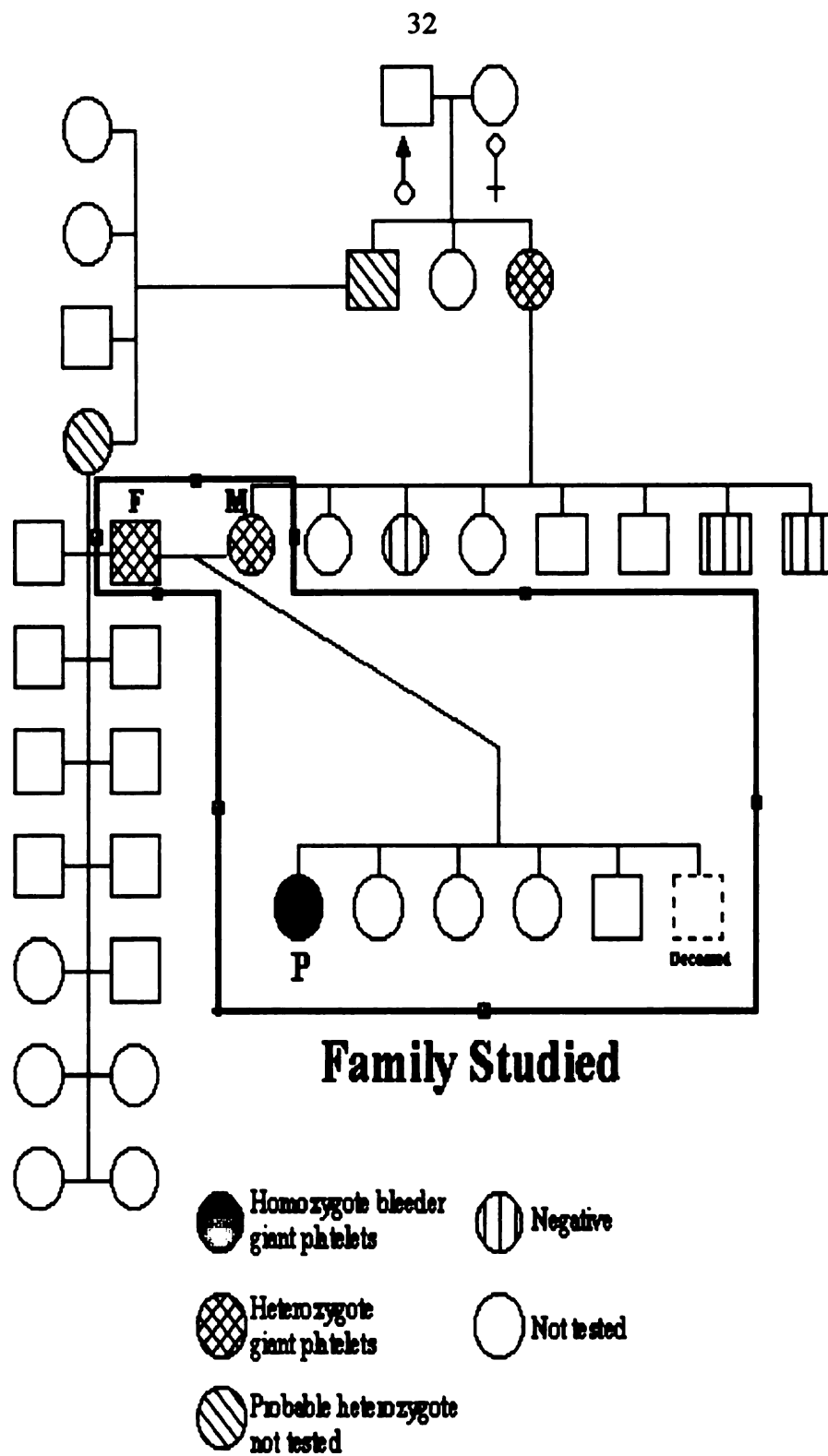


Figure 3. Pedigree of the studied family. A pedigree of the family studied shows the predicted genotypes based on the phenotypic characteristics of the disease.

Materials and Methods

Sample collection and DNA isolation

Whole blood was collected from the propositus, her mother and a normal control, using ethylenediaminetetraacetic acid (EDTA) as the anticoagulant. The samples were centrifuged at 750 x gravity (g) for 20 minutes (centrifugation steps during DNA isolation were performed at room temperature) . Buffy coats were transferred to 15 mL conical polypropylene screw cap tubes and residual red blood cells were lysed by bringing the volume up to 15 mL with Tris ammonium chloride (140 mM NH_4Cl , 6 mM Tris-HCl [pH 7.2]) followed by a 5 minutes incubation at 37 °C. Each tube was centrifuged at 200 xg for 20 minutes and the supernatant was aspirated and discarded without disturbing the pellet. The pellet was resuspended with 10 mL calcium/magnesium free Dulbecco's phosphate buffered saline (DPBS) (Sigma, St. Louis, Missouri) and centrifuged at 200 xg in a microcentrifuge for 10 minutes . The pellet was washed a second time by adding 0.75 mL DPBS per 1 mL original whole blood and centrifuged at 225 xg for 20 minutes. The supernatant was decanted and the pellet was resuspended in the residual liquid. White blood cells were lysed by adding 0.375 mL of lysing solution (10 mM Tris [pH 7.6], 0.25 mM EDTA [pH 8.0], 0.75 mM NaCl) per 1 mL original whole blood and 18.75 μL of 20% sodium dodecyl sulfate per 1mL lysing solution. Cellular proteins were digested by adding 1.5 μL proteinase K (50 mg/mL) (Boehringer Mannheim, Indianapolis,

IN) per 1 mL lysis solution. The RNA was removed by adding 100 µg of DNase free RNase (Behringer Mannheim, Indianapolis, IN) per 1.0 mL lysis buffer and incubating at 37 °C for 1 hour. Cellular debris which contains protein and RNA was precipitated by the addition of 0.125 mL of 6 M saturated NaCl per 1 mL original whole blood followed by vigorous shaking for 10 seconds and centrifugation for 10 minutes at 850 xg. The supernatant, containing the DNA was decanted into a clean tube without disturbing the pellet containing the cell debris. The supernatant was spun for an additional 10 minutes at 850 xg to further remove any remaining proteins. The supernatant was poured into a clean glass tube, and DNA was precipitated by adding cold (-20 °C) absolute ethanol (2X the volume of supernatant). Samples were mixed by gentle inversion. DNA was collected using a wide bore plastic transfer pipet, transferring the DNA to a 1.5 mL microtube followed by washing twice with 90% cold ethanol by filling the tube with 90 % ethanol and centrifuging at 10,000 xg in a microcentrifuge (Microspin 24S, Sorvall Instrument Dupont). The pellet was dried in a speed vac centrifuge under vacuum for 5-7 minutes and resuspended in 100 µL sterile double deionized water (ddH₂O). The concentration of DNA was estimated by preparing a dilution of 1:100 in ddH₂O and measuring the optical density (OD) at 260 nm. Calculation of DNA amount was done by using the formula: OD at 260 X 50 X dilution factor = mg/mL DNA. The OD at 280 nm was also measured in order to verify the purity of the DNA where the ratio of 260/ 280 was calculated and a value of 1.8 - 2.0 indicates a clean DNA.

DNA amplification

The gene encoding GPIb α was amplified using 3 sets (1, 2 and 3) of oligonucleotide primers, Table 5, which generate 3 overlapping fragments.

Oligonucleotide primers were adapted from Simsek et al, 1994a with some modifications and synthesized at the Macromolecular Structure Facility at Michigan State University using the 394 DNA synthesizer (Applied Biosystem, Foster City, California).

Table 5. Sequences of primers used for amplification and sequencing.

Set	Primer	Binding site	Sequence* 5' - 3'	Expected size
Set 1	DE1	554 - 573	GCTGCTCCTGCTGCCAAGCC	704 bp
	DE2	1256 - 1237	AGCATTGTCCTGCAGCCAGC	
Set 2	DE3	1091 - 1111	ATGGGCTGGAGAATCTCGACAC	664 bp
	DE4	1753 - 1730	GGAGTGGGCTCCAGGGTGGTCATG	
Set 3	DE7	1658 - 1681	AACTCCAAAATCCACTACTGAACC	785 bP
	DE6	2442 - 2417	CTCAAGGTCCCCAAACCTCCCACC C	

The first fragment was amplified using oligonucleotide primers DE1 and DE2 (primers set 1), the second fragment was amplified using oligonucleotide primers DE3 and DE4 (primers set 2), and the third fragment was amplified using oligonucleotide primers DE7 and DE6 (primers set 3). Amplification of the first and second fragments was accomplished by mixing 200 nanograms of DNA (or an equivalent amount of sterile ddH₂O for the negative control) with 200 uM of each deoxynucleotide triphosphate (dNTP), 100 uM of each oligonucleotide primer, 10 uL of 10 X PCR reaction buffer (GIBCO BRL), 1.5 mM MgCl₂, and 2.0 U Taq Polymerase (GIBCO, BRL), in a 100 uL reaction volume. The PCR reactions were performed using a GeneAMP 9600 thermal cycler (Perkin Elmer Cetus, Norwalk, CT.) as follows: following an initial denaturation step for 5 minutes at 95 °C, DNA was amplified during 35 cycles of 95 °C for 45 seconds, 68 °C for 45 seconds, and 72 °C for 45 seconds. The PCR reactions were completed by a final elongation step at 72 °C for 10 minutes.

Amplification of the third fragment was accomplished in a manner similar to the previous PCR protocol with the following modifications. Briefly, 200 nanograms of DNA (or equivalent amount of sterile ddH₂O for negative control) was combined with 200 uM of each deoxynucleotide triphosphate (dNTP), 100 uM of each oligonucleotide primer, 10 uL of 10 X PCR reaction buffer (GIBCO BRL), 0.75 mM MgCl₂, and 4.0 U Taq Polymerase (GIBCO BRL), in a 100 uL reaction volume. The PCR reactions were performed using a GeneAMP 9600 thermal cycler (Perkin Elmer Cetus, Norwalk, CT.). Following an initial denaturation step for 5 minutes at 95 °C, DNA was amplified during 30 cycles of 95 °C for 45 seconds, 65 °C for 45 seconds, and 72 °C for 45 seconds. The PCR reactions were completed by a final elongation step at 72 °C for 10 minutes.

Agarose gel electrophoresis

Ten uL of the PCR products were mixed with 1 uL of 10X loading dye (0.25% bromophenol blue, 0.25 xylene cyanol , and 15% (w/v) sucrose in ddH₂O) and run on 1% (W/V) agarose gel (5 x 10 x 1 cm) in 1X Tris acetate EDTA buffer (TAE) (40 mM Tris-2W434Acetate, 1 mM EDTA [pH 8.0]). One ug of the one Kb molecular weight marker (GIBCO, BRL) was loaded on the gel to estimate the sizes of the amplified products. The gel was run at 75 volts for 2-3 hours. Gels were stained with ethidium bromide (5 ug/mL ethidium bromide in ddH₂O) for 20 - 30 minutes with gentle agitation, and destained by soaking in ddH₂O for another 20 - 30 minutes with gentle agitation. Gels were transferred to a trans-illuminator with an ultra violet (UV) light source to visualize the amplified products. The gels were documented by photographing using type 667 Polaroid film (Polaroid, Cambridge, Massachusetts).

PCR products purification

PCR products were purified using Wizard™ PCR Preps DNA Purification System for Rapid Purification of DNA Fragments kit (Promega, Madison, Wisconsin) following manufacturer's recommendations. Briefly, PCR products were run on 0.8% GTG low melting point (LMP) agarose (FMC BioProduct, Rockland, Maine) , gels were electrophoresed, stained and destained under conditions similar to those described above. The appropriate size bands were cut from the gel, transferred to 1.5 mL microtubes, and incubated at 65 °C for 10 - 15 minutes to dissolve the LMP agarose. One mL of resin solution was added to the melted agarose slice and mixed by inversion for 20 second. The mixture was transferred to a 3 mL syringe barrel which was attached to the .

minicolumn assembled at the vacuum manifold. After applying the vacuum to draw the DNA/resin mix into the minicolumn, the column was washed with 2 mL of 80% isopropanol. Columns were dried by centrifugation for 20 seconds at full speed in a microcentrifuge (Microspin 24S, Sorvall Instrument, Dupont). DNA was eluted from the columns by applying 50 μ L of pre-warmed (65 °C) sterile ddH₂O, incubating for 1 minute at room temperature, and centrifugation for 25 seconds at full speed in a microcentrifuge.

Three μ L of the purified PCR product were run on a 1% agarose gel as previously described to check the purity and the yield of the DNA. DNA was stored at -20 °C for further usage.

Heteroduplexing analysis

Using a GeneAMP 9600 thermal cycler (Perkin Elmer Cetus, Norwalk, CT.) purified PCR products were prepared for heteroduplexing analysis as follows: 15 μ L of the PCR products were heated over a period of 4 minutes to 94 °C, tubes were held at 94 °C for 10 minutes, cooled down to 25 °C over a 4 minutes period and held at 25 °C for 30 seconds. Each sample was mixed with 3 μ L 6X loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, and 15% (w/v) sucrose and 3 μ L 8 M urea). Samples were loaded onto 10% urea /12% polyacrylamide (29 :1 acrylamide:N, N-methylenebisacrylamide)gel (20 x 16 x 0.05 cm) in 1X Tris borate EDTA (TBE) (0.089 M Tris base, 0.089 M Boric acid 0.002 M EDTA), and electrophoresed for 20 hours at 100 volts. The gels were maintained cool during electrophoresis by running cold tap water in the special jackets which are part of the electrophoresis apparatus (Protean II, Bio Rad, Hercules, CA).

After electrophoresis the gels were fixed in fixing solution (10% absolute

ethanol, 0.005% glacial acetic acid) for 20 minutes with gentle agitation. The gels were stained with fresh 0.2% silver nitrate solution (0.2 gm silver nitrate in 100 mL ddH₂O) for 30 minutes with agitation, rinsed with ddH₂O for 5 seconds, immersed in fresh developing solution (3% NaOH, 0.008% Formaldehyde) for 8-12 minutes with agitation and washed in ddH₂O for 5 minutes. The gels were transferred to a Whatman filter papers (3 MM) and dried on a vacuum dryer for 1 hour at 80 °C.

Single Stranded Conformation Polymorphism (SSCP)

For SSCP the optimal fragment size is approximately 300 bp. Because the PCR fragments that were generated were bigger than this, the PCR products were digested with different restriction enzymes to generate smaller size fragments. Primer sets 1 and 2 were digested with *Ban* I restriction enzyme (Boehringer Mannheim, Indianapolis, Indiana) as follows: 26 uL purified PCR products were mixed with 3 uL 10X Buffer A (Tris-acetate 33 mM [pH 8.0], Mg-acetate 10 mM, K-acetate 66 mM, Dithiotheitol (DTT) 0.5 mM), 10 units *Ban* I and the mixture was incubated at 37°C over night. Primer set 3 was digested with *Sst* I and *Pst* I restriction enzymes (GIBCO, BRL) as follows: 25 uL of purified PCR products were mixed with 3 uL 10X React 2 buffer (Tris-HCl 50 mM, MgCl₂ 10 mM, NaCl 50 mM), 10 U *Sst* I, 10 U *Pst* I, and the mixture was incubated overnight at 37°C.

To check for complete digestion, ten uL of the restriction enzyme digest were mixed with 1 uL of 10X loading dye and loaded on 8% polyacrylamide gel (16.4 mM acrylamide, 0.2 mM bis(N,N'-methylene-bis-acrylamide)) (8.6 x 8.5 x 0.1 cm), along with 1 Kb molecular weight marker.(GIBCO, BRL). The gel was electrophoresed in 1X TBE buffer at 110 volts for 2 hours, stained with ethidium bromide (0.5 ug/uL ddH₂O) for 10

minutes, destained with ddH₂O for another 10 minutes and visualized using a trans-illumination with a UV light source. The gel was photographed using polaroid type 667 film (Polaroid, Cambridge, Massachusetts).

Single Stranded Conformation Polymorphism (SSCP) analysis was performed (with modifications) as recommended by Ainsworth J. et al 1994,. Briefly, the remaining 20 uL of the restriction digest was mixed with 4 uL of denaturant solution (97.5% deionized formamide, 4.6 M urea, 0.3% bromphenol blue, 0.3% xylene cyanol, 10 mM EDTA) in order to maintain the denatured DNA as single stranded. To denature the DNA, the mixture was heated to 95 °C for 10 minutes in GeneAMP 9600 thermal cycler (Perkin Elmer Cetus, Norwalk, CT.). The mixture was chilled on ice and loaded immediately on 6% polyacrtlamide (29:1 acrylamide : N, N'-methylene bisacrylamide) gel with 5% glycerol (v/v) (8.5 x 8.6 x 0.01 cm), the gel was electrophoresed in 1X TBE at 180 volts for 1 hour at room temperature. After electrophoresis the gel was stained in a way similar to that described in the heteroduplexing section above except that the silver nitrate concentration was reduced to 0.1% in ddH₂O. The gel was transferred to 3 MM Whatmanc filterpaper and dried on vacuum dryer as explained in heteroduplexing

Cloning

The cloning vector pUC19 was used which contains an ampicillin resistance gene that allows for colony selection when grown on a medium with ampicillin. pUC19 also has the Lac Z gene which allows for blue versus white color screening (Figure 5). One ug of pUC19 cloning vector (Boehringer Mannheim, Indianapolis, IN) was linearized by digestion with 20 U *Sma* I restriction enzyme in Buffer A at 37 °C for 2 hours. The

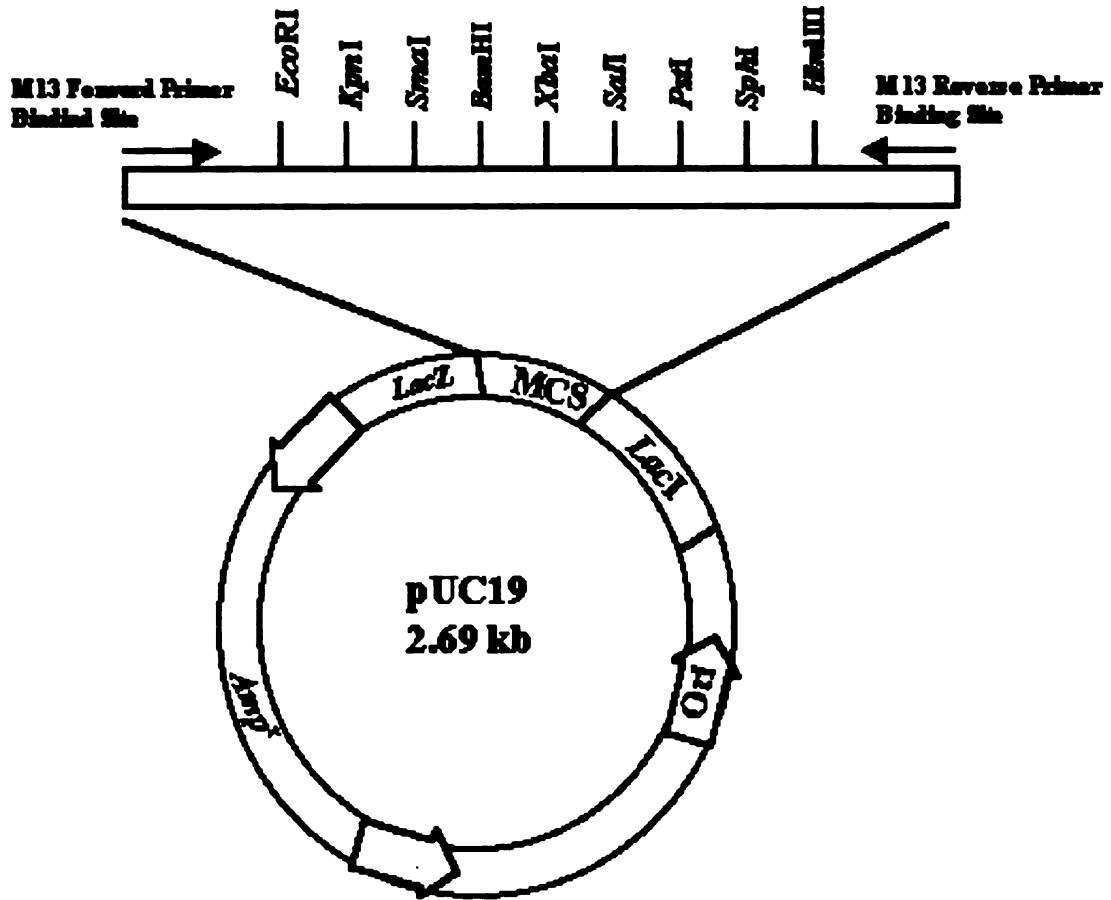


Figure 4. Cloning vector pUC19. The diagram represents pUC19 cloning vector, where the genes encoding Beta-lactamase (*Amp^r*), Beta-galactosidase (*LacZ*), and *lac* repressor (*LacI*), the origin of replication (*ori*) and the multiple cloning sites (*MCS*) where some restriction enzymes recognition sites are illustrated. Also the binding sites for the M13 forward and reverse primers are pointed by an arrow.

linearized vector was dephosphorylated to minimize the vector religation to itself during the ligation reaction. Dephosphorylation was performed by combining 27 μL of the digested product, 35 μL of 10X dephosphorylation buffer (500 mM Tris- HCl [pH 9.0], 10 mM MgCl_2 , 1 mM ZnCl_2 , 10 mM spermidine), 4U Calf Intestinal Alkaline Phosphatase (CIAP) (Boehringer Mannheim, Indianapolis, IN) and 284 μL sterile ddH_2O . The mixture was incubated at 37 °C for 30 minutes followed by addition of another unit of CIAP and continued incubation for another 30 minutes at 37°C. The enzyme was inactivated by heating at 65°C for 10 minutes and the vector was extracted with phenol/ chloroform as follows: 350 μL of phenol/ chloroform (24:1) were added to the mixture, vortexed briefly and centrifuged for 2 minutes in microcentrifuge at full speed. The aqueous phase was transferred to a clean tube and extracted again with phenol/ chloroform as above. The remaining phenol was extracted by adding 350 μL of chloroform/ isoamyl alcohol (24:1) followed by vortexing and centrifugation as above. The aqueous phase was transferred to a clean tube and the vector was precipitated by the addition of 875 μL absolute ethanol, 35 μL 3 M sodium acetate and incubation at -70°C for 30 minutes, followed by centrifugation for 15 minutes at 10,000 $\times g$ in microcentrifuge at 4°C. The pellet was washed by adding 250 μL of 70% ethanol followed by centrifugation for 5 minutes at 10,000 $\times g$ in microcentrifuge. The pellet was dried in a vacuum centrifuge centrifuge at room temperature for 5 minutes and resuspended in 30 μL sterile ddH_2O .

Since oligonucleotide primers lack the phosphate group at their 5' end, PCR products (insert) were phosphorylated using T_4 polynucleotide kinase as follows: 15 μL of purified PCR products were mixed with 28 μL sterile ddH_2O , 5 μL of 10X T_4

polynucleotide kinase buffer (70 mM Tris- HCl [pH 7.6], 100 mM MgCl_2 , 5 mM DTT), 1 μL of 10 mM ATP and 1 μL T_4 polynucleotide kinase (New England Bio Lab, Beverly, MA), the mixture was incubated at 37°C for 15 minutes. The reaction was stopped by adding 2 μL of 0.5 M EDTA. The volume of the mixture was expanded to 100 μL by addition of 50 μL Tris-EDTA (TE) buffer (pH 7.6). The DNA was extracted once with 100 μL of phenol/ chloroform, and once with 100 μL of chloroform:isoamyl alcohol as described above. The DNA was precipitated with 250 μL absolute ethanol and 10 μL 3 M sodium acetate as previously described, the pellet was resuspended in 13 μL sterile ddH_2O .

Ligation of the vector and insert was accomplished in 2 steps, the first step was performed in a small volume to enhance intermolecular ligation between insert and vector. This was accomplished by mixing 7 μL of phosphorylated PCR products, 1 μL dephosphorylated pUC19, 1 μL 10X ligation buffer (660 mM Tris-HCl, 50 mM MgCl_2 , 10 mM DDT, 10 mM ATP) and 1 U T_4 DNA ligase (Boehringer Mannheim, Indianapolis, IN). The mixture was incubated at 15°C for 8 hours. In the second step, the volume was expanded to 50 μL to enhance intramolecular ligation within the same molecule by adding 34 μL sterile ddH_2O , 5 μL 10X ligation buffer and 1 U T_4 DNA ligase enzyme followed by incubation at 15°C overnight. The volume of the reaction was expanded to 100 μL by adding 50 μL of TE buffer (pH 7.6) and the ligated product was precipitated by adding 250 μL absolute ethanol, 10 μL 3 M sodium acetate. This mixture was incubated at -70°C for 30 minutes and centrifuged for 15 minutes at 10,000 $\times g$ in microcentrifuge at 4°C . The pellet was washed once with 70% ethanol and dried in a speed vac centrifuge and resuspended in 10 μL sterile ddH_2O and used for transformation.

Luria-Bertani (LB) media (1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl, 1.5% agar) was used for bacterial growth. Plates were prepared as follows: 5 mg ampicillin/100 mL LB media was added to select for cells that have been transformed by the plasmid (since the plasmid has ampicillin resistance gene), media were poured and allowed to solidify. Forty uL of 0.1 mM isopropylthio-B-D-galactoside (IPTG) and 40 uL of 40 ug/mL 5-bromo-4-chloro-3-indolyl-B-D galactoside (X-gal) were spread on the plate surface to allow for blue /white color screening

For transformation, frozen (- 70 °C) DH5 α competent cells (Stratagene, La Jolla, CA) were thawed slowly on ice over a period of 20 minutes, 5 uL of the ligation product was mixed with cells and incubated on ice for 30 minutes. The DNA was transformed into the cells by heat shock at 42 °C for 90 seconds followed by a cold shock (on ice) for 2 minutes. Nine hundred fifty uL of LB broth was added to the cells and the cells were allowed to multiply by incubation at 37 °C for 1 hour in a shaking incubator. The cells were collected by centrifugation at 2000 xg in a microcentrifuge for 4 minutes. 800 uL of the supernatant was discarded while the cells were resuspended in the remaining 200 uL of broth. To select for transformants, 50 uL of this volume was plated on an LB agar plates, prepared previously, and left at room temperature for 15 minutes to allow the cells to adsorb to the agar surface and incubated overnight at 37 °C.

To analyze the transformants, small-scale plasmid DNA preparations (minipreps) were performed as follows: 5 mL of LB broth with ampicillin was inoculated with selected single white colonies and incubated at 37°C overnight in a shaking incubator. At the same time a replica plate was inoculated with the same colony to double check for selected single white colonies and to be saved for further DNA purification and

preparation of glycerol stocks. Purification of insert DNA was performed using Wizard™ Plus Minipreps DNA Purification System (Promega, Madison, WI) following the manufacturer's recommendations. Briefly, the 5 mL culture was centrifuged at 12,000 xg for 10 minutes. The supernatant was discarded and the pellet was resuspended in 300 uL cell resuspension solution (50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 100 ug/mL Rnase A) and transferred to a 1.5 mL microcentrifuge tube. The cells were lysed with 300 uL of cell lysis solution (0.2 M NaOH, 1% SDS) mixed with 300 uL neutralization solution (1.32 M potassium acetate, pH 4.8). Lysates were centrifuged at 10,000 xg for 5 minutes at room temperature. Meanwhile syringe barrels and minicolumns were assembled at the vacuum manifold. The clear supernatant was transferred to the syringe barrel which contained 1 mL of resuspended resins. After applying the vacuum to draw the DNA/resin mixture into the minicolumn, the column was washed with 2 mL of washing solution (80 mM K-acetate, 8.3 mM Tris-HCl (pH 7.5), 40 uM EDTA, 55% ethanol). The columns were dried by centrifugation for 2 minutes at 10,000 xg in a microcentrifuge. DNA was eluted from the columns by applying 50 uL of pre-warmed (65 °C) sterile ddH₂O, incubated for 1 minute at room temperature, followed by centrifugation for 25 seconds at 10,000 xg in a microcentrifuge.

DNA from the Minipreps was used to analyze for the presence of recombinants by digestion with *EcoRI* and *HindIII* (GIBCO, BRL). One uL of minipreps DNA was mixed with 2.5 uL React 2 buffer (GIBCO, BRL), 10 U *EcoRI*, 1 U *HindIII* and 19.5 uL sterile ddH₂O and incubated at 37 °C for 2 hours. The mixture was mixed with 2.5 uL 10X loading dye and loaded on a 0.8% submerged agarose gel along with a 1 Kb molecular weight marker (GIBCO, BRL). The gel was run at 75 volts for 1.5 hours in 1X

TBE buffer. The gel was stained with ethidium bromide and photographed as previously described.

To check for the orientation of the insert within the plasmid the restriction enzyme *Pst*I (GIBCO, BRL) was used. *Pst*I cuts the vector once just outside the insert as well as within the insert, the reaction mixture consists of 1 uL of purified clones, 2.5 uL React 2 buffer, 20.5 uL sterile ddH₂O and 10 U *Pst*I followed by incubation at 37 °C for 2 hours. 2.5 uL of 10X loading dye was added to the mixture and applied to 0.8% agarose gel along with 1 Kb molecular weight marker (GIBCO, BRL), the gel was run, stained and photographed as previously described.

After obtaining the desired clones, glycerol stocks were prepared as follows: 5 mL of LB broth with ampicillin (0.05 mg/mL) was inoculated from the same colonies that were used to grow the culture for minipreps above. The culture was incubated at 37 °C overnight in a shaking incubator. 800 uL of this culture was transferred into microcentrifuge tube, mixed with 200 uL of sterile glycerol and stored at -70 °C

Preparation of Large scale DNA

A cesium chloride (CsCl) gradient centrifugation method (Sambrook et al 1992) was used to obtain clean DNA for sequencing. Two hundred and fifty mL of LB broth with ampicillin were inoculated with the selected and identified clones and incubated overnight at 37°C in shaking incubator, the culture was centrifuged at 14,000 xg for 10 minutes at 4°C in a Sorvall centrifuge. The supernatant was discarded and the cells were resuspended in 12 mL resuspension solution (50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 100 ug/mL Rnase A) lysed with 12 mL lysing solution (0.2 M NaOH, 1%SDS) and

neutralized with 12 mL neutralization solution (1.32 M potassium acetate, pH 4.8) followed by centrifugation at 14,000 xg for 15 minutes at 4 °C. DNA was precipitated from the clear supernatant by mixing with 0.6 volume isopropanol followed by centrifugation at 14,000 xg for 15 minutes at 4°C. The pellet was washed with 70% ethanol , dried and resuspended in 11.3 mL sterile TE buffer (pH 7.5). Cesium chloride (11.55 gm) and dissolved completely, 8 mg ethidium bromide (800 uL of 10 mg/mL)) was , added and the mixture was centrifuged at 10,000 xg for 5 minutes at 25°C to remove any cell debris which formed a layer at the surface of the tube. The clear solution was transferred to an uLtra centrifuge tube which was , sealed by heat. Supercoiled, nicked, and linear plasmid DNA were separated by ultra centrifugation for 16 hours at 55,000 rpm under vacuum. DNA bands were visualized by UV light. The bands of the supercoiled DNA was aspirated by a syringe (Figure 5). Ethidium bromide was extracted with water saturated butanol, excess CsCl was removed by dialysis against TE buffer. The DNA was , precipitated with 2.5 X volume absolute ethanol and 0.1 X volume sodium acetate followed by incubation at -20°C for 30 minutes and centrifugation for 15 minutes at 4°C at 10,000 rpm. The pellet was washed by adding 5 mL 70% ethanol followed by centrifugation for 10 minutes at 4 °C at 3,000 xg. The pellet was dried in a speed vac and resuspended in sterile ddH₂O. DNA was quantitated as described in the DNA isolation section. Purified DNA was run on a 0.8% agarose gel to confirm its purity prior to sequencing.

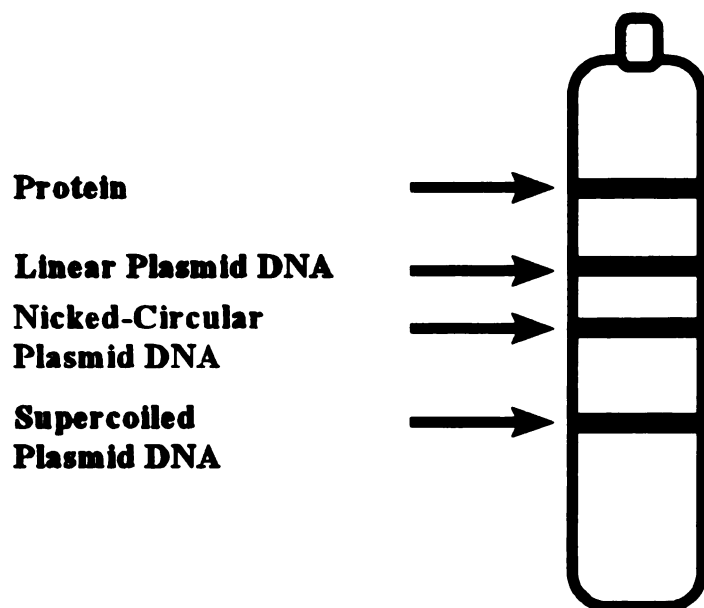


Figure 5. Purification of the supercoiled plasmid DNA. Supercoiled plasmid DNA was prepared by ultracentrifugation using CsCl - ethidium bromide gradient centrifugation method. Arrows indicate the different bands separated as a result of ultracentrifugation.

Sequencing

Sequencing reactions were performed by cyclic sequencing using a dye primer kit where four fluorescent labeled M13 primers, forward (5'TGTAAAACGACGGCCAGT-3') and reverse (5'-CAGGAAACAGCTATGACC-3'), were used. These primers are compatible with the pUC19 sequence. Reactions were performed in four different tubes (A, C, G, T) according to the manufacturer's recommendations (Perkin Elmer, Foster City, CA). The A and C tubes contained 200 ng of cloned template and 4 uL of the T or C mix. The G or T tubes contained 400 ng of the cloned template and 8 uL of the G or T mix. Each mix contains one dideoxynucleotide, the other three nucleotides and one 5' end fluorescently labeled primer, Tris-HCl (pH 9.0), $MgCl_2$, thermal stable phosphatase, and AmpliTaq DNA Polymerase, FS (Perkin Elmer Cetus, Norwalk, CT). Reactions are subjected to 15 cycles of 96°C for 10 seconds, 55°C for 5 seconds, and 70°C for 60 seconds followed by another 15 cycles of 96°C for 10 seconds and 70°C for 60 seconds, using the GeneAMP 9600 thermal cycler (Perkin Elmer Cetus, Norwalk, CT). The contents of the four tubes were combined and the DNA was precipitated with 111 uL of a solution containing 70% ethanol and 0.5 M $MgCl_2$. The supernatant was aspirated, the pellet was dried in a vacuum centrifuge and mixed with 4 uL loading dye (5:1 deionized formamide : 50 mM EDTA). Samples were denatured at 95°C for 3 minutes in a heating block and loaded on 4.75% polyacrylamide (29:1 acrylamide : N, N'-methylene bisacrylamide) / 8.3 M urea sequencing gel (40 x 25 x 0.04 cm). Sequencing gels were run on a 373A automated sequencer (ABI/ Foster City, CA). Gels were run for 14 hours at 32 watt. Raw data generated by the 373A sequencer was analyzed and edited using the Sequencing Analysis Software (ABI/ Foster City, CA).

Sequencing analysis

Sequences generated by Sequencing Analysis software were analyzed using the Genetic Computer Group (GCG) package (Madison, WI). For the third primer set, the 863 bp of interest was assembled using the Assemble program of GCG which makes new sequence constructs from pieces of existing sequences which were obtained from both directions (forward and reverse, where the reverse sequence was reversed using the reverse program of GCG). The Bestfit program which uses the local homology algorithm of Smith and Waterman (1981) to find the best segment of similarity between two sequences was used to compare all the sequences with each other. The Translate program (which creates a peptide sequence by translating the specified nucleic acid sequence) of GCG was used to generate the peptide sequence. Pileup program (which creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments) (Feng D., and Doolittle R. 1987) of GCG was used to align and compare all the generated peptides to each other as well as to the published sequence (Lopez et al., 1987)

Results

Polymerase Chain Reaction

Different PCR conditions had been tested in order to optimize the conditions to amplify the desired fragments. These trials included changing the annealing temperatures (56 °C, 57 °C, 58 °C, 60 °C, 61 °C, 64.5 °C, 65 °C, 67 °C, 68 °C), using different MgCl₂ concentrations (1.5 mM, 1 mM, 0.85 mM, 0.75 mM, 0.65 mM, 0.55 mM, 0.50 mM) and applying touch down PCR (Don et al., 1991). Some PCR conditions resulted in multiple bands in the desired lane along with the desired fragments while some conditions didn't amplify anything at all. Also, another pair of primers DE5 (5'-CCACTACTGAACCAACCCCAAGCCC-3') and DE8 (5'-TCTCAAGGTCCCCAAACCTC-3') was used to amplify the third fragment under different PCR conditions. These primers either amplified non-specifically or would not amplify at all. Primers DE6 and DE7 were selected to amplify this fragment, the final selected conditions are described in the Materials and Methods section.

After optimizing the conditions, the first primer set (DE1 and DE2) successfully amplified the expected 704 bp fragment from the mother, the propositus as well as the normal control and there was no amplification in the negative control (Figure 6). The second set of primers (DE3 and DE4) amplified the expected 664 bp fragment from the mother, the propositus and the control. No products were amplified in the negative

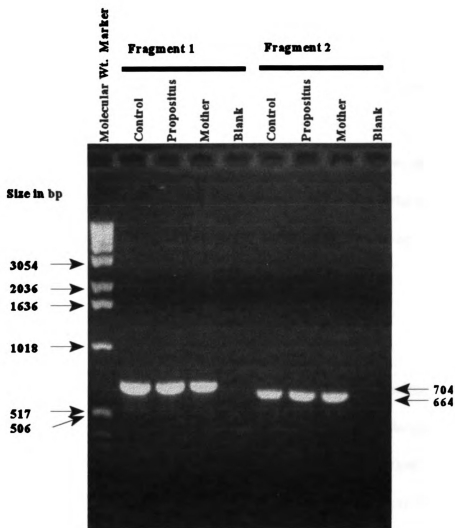


Figure 6. PCR amplification of the first and the second fragments. Agarose gel electrophoresis (0.8%) of amplified PCR products using primers set 1 and 2, respectively. The first lane is one kb marker (GIBCO), second and sixth lanes are normal control DNA, third and seventh lanes are Propositus DNA, fourth and eighth lanes are mother DNA, and fifth and ninth lanes are no template blanks. Sizes of the molecular weight bands in base pairs are indicated at the left.

control (Figure 6). One Kb molecular weight marker (GIBCO) was used to estimate the sizes of the amplified products, sizes are indicated at the left.

Figure 7 shows the amplification results of the third fragment (DE6 and DE7) . The expected size for this fragment was 775, but the amplification gave 2 different sizes. This was likely due to polymorphism resulting in a variable number of tandem repeats in this area of the gene. The control DNA amplified a fragment of 824 bp, whereas the propositus DNA amplified an 863 bp fragment. However, amplification of the mother's DNA of the same region gave two fragments, one 824 bp, and the other 863 bp. No amplification was detected in the negative control.

Heteroduplexing

Purified PCR products were used for heteroduplexing. In case of heterozygosity, there will be a chance for the mutant allele to reanneal to the normal one. The area of mismatch will form a bulb-like structure which will alter its migration in the gel compared to normal DNA. Figure 8 shows results for heteroduplexing analysis on 12% polyacrylamide/ 10% urea for normal controls and the mother. As shown in Figure 8 no obvious differences in the migration pattern were detected between the mother and the normal control after staining the gel with silver nitrate. At the time of performing heteroduplexing analysis, no DNA was available from the propositus , so no data was obtained. This analysis was not sufficient to determine the area of the gene to be sequenced so SSCP was performed.

Single Stranded Conformation Polymorphism (SSCP)

Different combinations of conditions were used to optimize the running conditions for this technique. These included the use of different gel sizes and thickness. Long (40 x 20 x 0.025 cm), 12.5% acrylamide gels had been used, but these were very hard to handle during the staining procedure, so smaller gel sizes (20 x 8 x 0.1 cm and 8.6 x 8.5 x 0.1 cm) using 6% acrylamide with and without 5% glycerol were used. Different concentrations of TBE buffer (1X versus 2X TBE) were also evaluated. In addition the gels were run at room temperature as well as at 4 °C, and different voltages (400 V, 200 V and 180 V) were used. Modifications were also made during the staining step such as 0.1% versus 0.2% silver nitrate and the presence or absence of $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ in the developing solution which was used to reduce the staining of the background compared to the DNA bands. Some of these conditions resulted in dark gels which made it hard to interpret the data, but using 0.1% silver nitrate resulted in better colors. The optimum conditions that are used to run these gels are described in the Materials and Methods section.

This technique is highly affected by the fragment size, with optimum fragment size being less than 300 bp (Ainsworth et al., 1994). Purified PCR products generated earlier were too big for this analysis, thus they were digested with different restriction enzymes to generate smaller size fragments. Digestion of the first fragment with *Ban* I resulted in 2 fragments with sizes of 382 bp and 322 bp. Digestion of the second fragment with *Ban* I enzyme resulted in two fragments with sizes of 392 bp and 272 bp (Figure 9). The third fragment was digested with *Sst* I and *Pst* I (Gibco, BRL), which should have resulted in 3 fragments of 346 bp, 235 bp and 203 bp. But as shown in

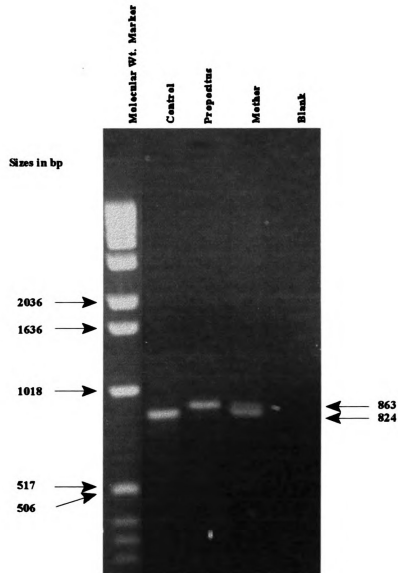


Figure 7. PCR amplification of the third fragment. Agarose gel electrophoresis (0.8%), using primers set 3. First lane is one Kb marker (GIBCO), second lane is normal control, third lane is propositus, fourth lane is mother and fifth lane is no template blank. Sizes of the molecular weight marker are indicated at the left. Arrows at the right indicate the two sizes amplified due to the presence of two tandem repeats (824 bp) and three tandem repeats (863 bp).

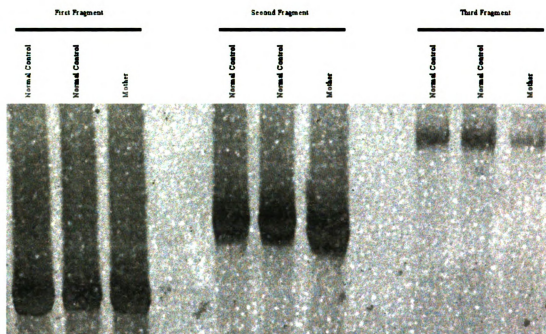


Figure 8. Heteroduplexing analysis gel. Silver Stained 12% acrylamide/ 8% Urea gel which represents heteroduplexing analysis for two normal controls and the mother for the first, second and third fragments of the gene (Primers sets 1, 2 and 3 respectively).

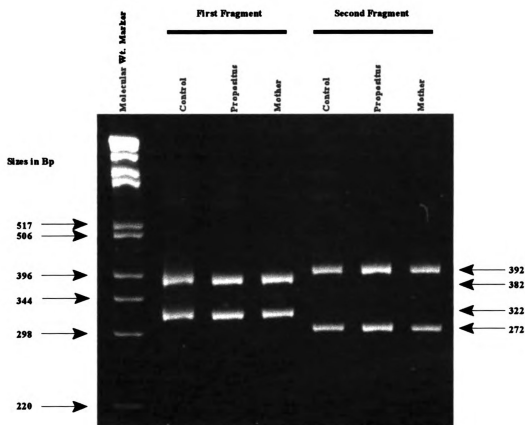


Figure 9. Restriction digest of the first and the second PCR fragments. The first and the second PCR fragment were digested with *BanI* to generate smaller fragments that can be used for SSCP analysis. One kb marker was loaded in the first lane, control DNA in the second and fifth lanes, propositus DNA in the third and sixth lanes, and Mother DNA in the fourth and seventh lanes. Sizes of the molecular weight marker in base pairs are indicated at the left.

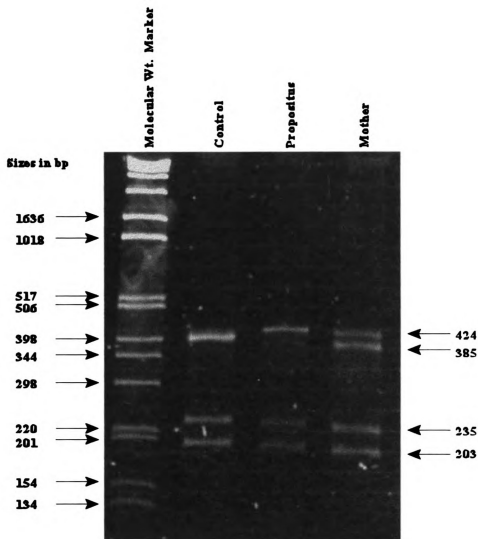


Figure 10. Restriction digest of the third PCR fragment. The third PCR fragment generated using primers set 3 was digested with *Pst*I and *Sst*I to generate smaller fragment to be used in SSCP analysis was run on 8% acrylamide gel. One kb marker was loaded in the first lane, the control DNA in the second lane, the propositus in the third and the mother which gave an extra band is in the fourth lane. Sizes of the molecular weight marker fragments are indicated at the left. The top two arrows at the right indicate the differences in the restriction digest products between the normal control, the propositus and the mother due to the presence of VNTR in this fragment, where the normal control has 2 copies of the 39 bp tandem repeat, the propositus homozygous for 3 copies, whereas, the mother is heterozygous for the repeat with 2 and 3 copies.

Figure 10, the control PCR products gave 3 fragments of 385 bp, 235 bp and 203 bp. The mother PCR products gave four fragments with sizes of 424 bp, 385 bp, 235 bp and 203 bp and the propositus PCR products gave 3 fragments of 424, 235 and 203 bp.

For SSCP analysis, the DNA is single stranded. The migration of the single stranded DNA in the gel will depend on its conformation. Single stranded DNA from normal control, heterozygote and mutant homozygote will show different migration patterns on the gel. Figure 11 shows a typical 6% polyacrylamide/ 5% glycerol gel (8.6 x 8.5 x 0.1 cm) which was stained using silver nitrate. No obvious differences between the control, the mother and the propositus were detected within the first (Figure 11, panel A) or second fragment (Figure 11, panel B) of the gene. However, SSCP gels of the third fragment demonstrated some differences in the migration patterns among the control, mother and propositus products as shown in Figure 12. These differences appear to be localized within two subsets (A+B) of the third fragment. The arrow in panel A represents the absence of this band from the propositus lane. The arrows in panel B indicate two areas of differences among the three lanes

Cloning

The PCR products from the third fragment for the propositus and the mother were cloned into pUC19. Clones generated using propositus PCR products gave the expected size fragment of 923 bp which represent the insert DNA (863 bp) and 60 bp from the vector. Whereas, clones generated from the mother's PCR products resulted in two subfamilies of clones. The first was 923 bp similar to that found in the propositus, and the second was approximately 880 bp. The difference is a result of one allele with 3

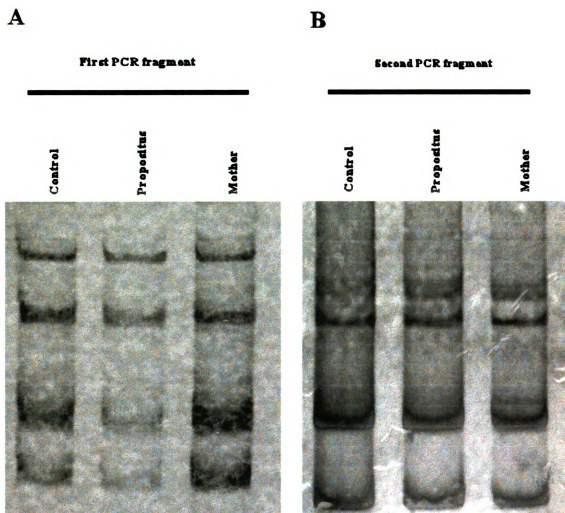


Figure 11. SSCP analysis of the first and second fragments. SSCP analysis of the first (A) and second (B) PCR fragments. Silver stained 6% acrylamide / 5% glycerol gels. No differences were detected between normal control, propositus and the mother.

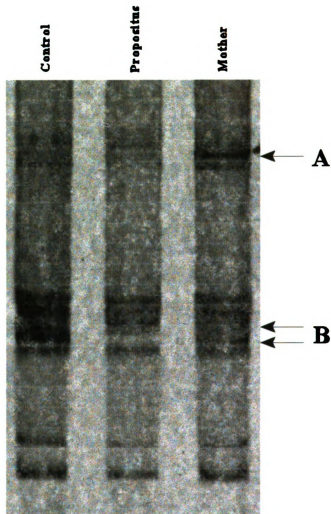


Figure 12. SSCP analysis of the third fragment. Digested PCR products were run on 6% acrylamide / 5% glycerol gel. Comparison between the control in the first lane, the propositus in the second lane and the mother in the third lane showed two main differences in subfragments A and B, respectively. Arrows indicate areas of differences.

VNTR and one with 2 VNTR. Five positive clones from the propositus, 2 positive clones from the mother's first subfamily and 3 clones from the mother's second subfamily were picked and glycerol stocks were prepared as described in the Materials and Methods. Large amounts of DNA were prepared by the CsCl gradient method and DNA was stored at - 20°C prior to sequencing.

Sequencing

Initially, manual sequencing of the third fragment from PCR products was performed. An average of 300 bp were obtained from each run which was not sufficient to accurately determine the entire sequence. Therefore, automated sequencing from PCR products was performed. Initially, data generated using this approach were not conclusive and were hard to manipulate. Based on the PCR and restriction digest results for the mother we assumed that this could be due to the heterozygosity of the tandem repeat in this area of the gene. Thus cloning the PCR products into pUC 19 was determined to be necessary.

A total of 5 clones from the propositus and 5 clones for the mother (3 clones containing 2 tandem repeats and 2 clones containing 3 tandem repeats) were used when sequencing the third fragment. Two hundred ng of cloned PCR products were used for cycle sequencing. This was performed using both M13 forward and reverse primers as described in Materials and Methods. Sequencing reactions were run on a 373 A automated sequencer (ABI) and sequences were analyzed using sequencing analysis 2.1 software (ABI). Each sequencing reaction gave an average of 650 bp. The raw sequence data generated was analyzed by using the GCG program. Sequences for each clone have

been confirmed by sequencing using the M13 forward and reverse primers. The reverse and forward primers sequences were compared to each other. The full length sequences of the third fragment generated using the assemble program was compared to each other as well as to the published sequence (Lopez et al 1987). In addition, the full length nucleic acid sequence was converted to the corresponding amino acid sequence. The generated peptide sequences were compared to each other and to the published sequence.

Sequencing the mother's DNA clones did not reveal any point mutations, insertions, or deletions and gave a correct open reading frame when compared to the published sequence. However sequencing the propositus DNA clones showed one point mutation in two different clones where a C to T at position 1531 (Figure 13) changed the amino acid proline 481 into a serine as shown in Figure 14 (numbering according to Lopez et al., 1987). The sequence of the other 3 clones did not show any differences from the consensus sequence nucleic acid or amino acid.

Sequences from the mother and the propositus clones confirmed the PCR and the restriction enzymes digestion results regarding the presence of a variable number of tandem repeat (VNTR) in this area. The mother's DNA was confirmed to be heterozygote for the repeat with one allele having two copies of the 39 bp repeat while the other allele had 3 copies of the same 39 bp. Sequences from clones that have 2 copies and 3 copies of the VNTR showed a 100% match with the published sequence. Sequencing the propositus DNA supported the PCR and the restriction enzyme digestion results regarding the presence of 3 copies of the VNTR in both alleles.

Figure 13. Comparison of the third fragment sequences. Ten clones of the third fragment of GPIIb gene for the mother and the propositus were sequenced and compared to the GPIIb published sequence using the pileup and lineup program. Glycoprotein Ib alpha published sequence is represented by letter A, mother's clones with 2 copies of the VNTR are designated B and C, whereas the clones with 3 copies of the repeat are designated D, E and F. Five clones for the propositus were designated G through K. Mismatches within the sequences are indicated by an asterisk.

	1				50
A	AACTCCAAAA	TCCACTACTG	AACCAACCCC	AAGCCCGACC	ACCTCAGAGC
B	AACTCCAAAA	TCCACTACTG	AACCAACCCC	AAGCCCGACC	ACCTCAGAGC
C	AACTCCAAAA	TCCACTACTG	AACCAACCCC	AAGCCCGACC	ACCTCAGAGC
D	AACTCCAAAA	TCCACTACTG	AACCAACCCC	AAGCCCGACC	ACCTCAGAGC
E	AACTCCAAAA	TCCACTACTG	AACCAACCCC	AAGCCCGACC	ACCTCAGAGC
F	AACTCCAAAA	TCCACTACTG	AACCAACCCC	AAGCCCGACC	ACCTCAGAGC
G	AACTCCAAAA	TCCACTACTG	AACCAACCCC	AAGCCCGACC	ACCTCAGAGC
H	AACTCCAAAA	TCCACTACTG	AACCAACCCC	AAGCCCGACC	ACCTCAGAGC
I	AACTCCAAAA	TCCACTACTG	AACCAACCCC	AAGCCCGACC	ACCTCAGAGC
J	AACTCCAAAA	TCCACTACTG	AACCAACCCC	AAGCCCGACC	ACCTCAGAGC
K	AACTCCAAAA	TCCACTACTG	AACCAACCCC	AAGCCCGACC	ACCTCAGAGC
	51				100
A	CCGTCCCGGA	GCCCGCCCCA	AACATGACCA	CCCTGGAGCC	CACTCCAAGC
B	CCGTCCCGGA	GCCCGCCCCA	AACATGACCA	CCCTGGAGCC	CACTCCAAGC
C	CCGTCCCGGA	GCCCGCCCCA	AACATGACCA	CCCTGGAGCC	CACTCCAAGC
D	CCGTCCCGGA	GCCCGCCCCA	AACATGACCA	CCCTGGAGCC	CACTCCAAGC
E	CCGTCCCGGA	GCCCGCCCCA	AACATGACCA	CCCTGGAGCC	CACTCCAAGC
F	CCGTCCCGGA	GCCCGCCCCA	AACATGACCA	CCCTGGAGCC	CACTCCAAGC
G	CCGTCCCGGA	GCCCGCCCCA	AACATGACCA	CCCTGGAGCC	CACTCCAAGC
H	CCGTCCCGGA	GCCCGCCCCA	AACATGACCA	CCCTGGAGCC	CACTCCAAGC
I	CCGTCCCGGA	GCCCGCCCCA	AACATGACCA	CCCTGGAGCC	CACTCCAAGC
J	CCGTCCCGGA	GCCCGCCCCA	AACATGACCA	CCCTGGAGCC	CACTCCAAGC
K	CCGTCCCGGA	GCCCGCCCCA	AACATGACCA	CCCTGGAGCC	CACTCCAAGC
	101				150
A	CCGACCACCC	CAGAGCCCAC	C.....		
B	CCGACCACCC	CAGAGCCCAC	C.....		
C	CCGACCACCC	CAGAGCCCAC	C.....		
D	CCGACCACCC	CAGAGCCCAC	C.....		
E	CCGACCACCC	CAGAGCCCAC	CTCAGAGCCC	GCCCCCAGCC	CGACCACCCC
F	CCGACCACCC	CAGAGCCCAC	CTCAGAGCCC	GCCCCCAGCC	CGACCACCCC
G	CCGACCACCC	CAGAGCCCAC	CTCAGAGCCC	GCCCCCAGCC	CGACCACCCC
H	CCGACCACCC	CAGAGCCCAC	CTCAGAGCCC	GCCCCCAGCC	CGACCACCCC
I	CCGACCACCC	CAGAGCCCAC	CTCAGAGCCC	GCCCCCAGCC	CGACCACCCC
J	CCGACCACCC	CAGAGCCCAC	CTCAGAGCCC	GCCCCCAGCC	CGACCACCCC
K	CCGACCACCC	CAGAGCCCAC	CTCAGAGCCC	GCCCCCAGCC	CGACCACCCC

	151				200
A					T
B		TCAGAGCCCG	CCCCCAGCCC	GACCACCCCG	GAGCCCACCT
C		TCAGAGCCCG	CCCCCAGCCC	GACCACCCCG	GAGCCCACCT
D		TCAGAGCCCG	CCCCCAGCCC	GACCACCCCG	GAGCCCACCT
E	GGAGCCCACC	TCAGAGCCCG	CCCCCAGCCC	GACCACCCCG	GAGCCCACCT
F	GGAGCCCACC	TCAGAGCCCG	CCCCCAGCCC	GACCACCCCG	GAGCCCACCT
G	GGAGCCCACC	TCAGAGCCCG	CCCCCAGCCC	GACCACCCCG	GAGCCCACCT
H	GGAGCCCACC	TCAGAGCCCG	CCCCCAGCCC	GACCACCCCG	GAGCCCACCT
I	GGAGCCCACC	TCAGAGCCCG	CCCCCAGCCC	GACCACCCCG	GAGCCCACCT
J	GGAGCCCACC	TCAGAGCCCG	CCCCCAGCCC	GACCACCCCG	GAGCCCACCT
K	GGAGCCCACC	TCAGAGCCCG	CCCCCAGCCC	GACCACCCCG	GAGCCCACCT
	201				250
A	CAGAGCCCGC	CCCCAGCCCG	ACCACCCCGG	AGCCCACCCC	AATCCCGACC
B	CAGAGCCCGC	CCCCAGCCCG	ACCACCCCGG	AGCCCACCCC	AATCCCGACC
C	CAGAGCCCGC	CCCCAGCCCG	ACCACCCCGG	AGCCCACCCC	AATCCCGACC
D	CAGAGCCCGC	CCCCAGCCCG	ACCACCCCGG	AGCCCACCCC	AATCCCGACC
E	CAGAGCCCGC	CCCCAGCCCG	ACCACCCCGG	AGCCCACCCC	AATCCCGACC
F	CAGAGCCCGC	CCCCAGCCCG	ACCACCCCGG	AGCCCACCCC	AATCCCGACC
G	CAGAGCCCGC	CCCCAGCCCG	ACCACCCCGG	AGCCCACCCC	AATCCCGACC
H	CAGAGCCCGC	CCCCAGCCCG	ACCACCCCGG	AGCCCACCCC	AATCCCGACC
I	CAGAGCCCGC	CCCCAGCCCG	ACCACCCCGG	AGCCCACCCC	AATCCCGACC
J	CAGAGCCCGC	CCCCAGCCCG	ACCACCCCGG	AGCCCACCCC	AATCCCGACC
K	CAGAGCCCGC	CCCCAGCCCG	ACCACCCCGG	AGCCCACCCC	AATCCCGACC
	251				300
A	ATCGCCACAA	GCCCGACCAT	CCTGGTGTCT	GCCACAAGCC	TGATCACTCC
B	ATCGCCACAA	GCCCGACCAT	CCTGGTGTCT	GCCACAAGCC	TGATCACTCC
C	ATCGCCACAA	GCCCGACCAT	CCTGGTGTCT	GCCACAAGCC	TGATCACTCC
D	ATCGCCACAA	GCCCGACCAT	CCTGGTGTCT	GCCACAAGCC	TGATCACTCC
E	ATCGCCACAA	GCCCGACCAT	CCTGGTGTCT	GCCACAAGCC	TGATCACTCC
F	ATCGCCACAA	GCCCGACCAT	CCTGGTGTCT	GCCACAAGCC	TGATCACTCC
G	ATCGCCACAA	GCCCGACCAT	CCTGGTGTCT	GCCACAAGCC	TGATCACTCC
H	ATCGCCACAA	GCCCGACCAT	CCTGGTGTCT	GCCACAAGCC	TGATCACTCC
I	ATCGCCACAA	GCCCGACCAT	CCTGGTGTCT	GCCACAAGCC	TGATCACTCC
J	ATCGCCACAA	GCCCGACCAT	CCTGGTGTCT	GCCACAAGCC	TGATCACTCC
K	ATCGCCACAA	GCCCGACCAT	CCTGGTGTCT	GCCACAAGCC	TGATCACTCC

	301				350
A	AAAAAGCACA	TTTTTAACTA	CCACAAAACC	CGTATCACTC	TTAGAATCCA
B	AAAAAGCACA	TTTTTAACTA	CCACAAAACC	CGTATCACTC	TTAGAATCCA
C	AAAAAGCACA	TTTTTAACTA	CCACAAAACC	CGTATCACTC	TTAGAATCCA
D	AAAAAGCACA	TTTTTAACTA	CCACAAAACC	CGTATCACTC	TTAGAATCCA
E	AAAAAGCACA	TTTTTAACTA	CCACAAAACC	CGTATCACTC	TTAGAATCCA
F	AAAAAGCACA	TTTTTAACTA	CCACAAAACC	CGTATCACTC	TTAGAATCCA
G	AAAAAGCACA	TTTTTAACTA	CCACAAAACC	CGTATCACTC	TTAGAATCCA
H	AAAAAGCACA	TTTTTAACTA	CCACAAAACC	CGTATCACTC	TTAGAATCCA
I	AAAAAGCACA	TTTTTAACTA	CCACAAAACC	CGTATCACTC	TTAGAATCCA
J	AAAAAGCACA	TTTTTAACTA	CCACAAAACC	CGTATCACTC	TTAGAATCCA
K	AAAAAGCACA	TTTTTAACTA	CCACAAAACC	CGTATCACTC	TTAGAATCCA
	351				400
A	CCAAAAAAAC	CATCCCTGAA	CTTGATCAGC	CACCAAAGCT	CCGTGGGGTG
B	CCAAAAAAAC	CATCCCTGAA	CTTGATCAGC	CACCAAAGCT	CCGTGGGGTG
C	CCAAAAAAAC	CATCCCTGAA	CTTGATCAGC	CACCAAAGCT	CCGTGGGGTG
D	CCAAAAAAAC	CATCCCTGAA	CTTGATCAGC	CACCAAAGCT	CCGTGGGGTG
E	CCAAAAAAAC	CATCCCTGAA	CTTGATCAGC	CACCAAAGCT	CCGTGGGGTG
F	CCAAAAAAAC	CATCCCTGAA	CTTGATCAGC	CACCAAAGCT	CCGTGGGGTG
G	CCAAAAAAAC	CATCCCTGAA	CTTGATCAGC	CACCAAAGCT	CCGTGGGGTG
H	CCAAAAAAAC	CATCCCTGAA	CTTGATCAGC	CACCAAAGCT	CCGTGGGGTG
I	CCAAAAAAAC	CATCCCTGAA	CTTGATCAGC	CACCAAAGCT	CCGTGGGGTG
J	CCAAAAAAAC	CATCCCTGAA	CTTGATCAGC	CACCAAAGCT	CCGTGGGGTG
K	CCAAAAAAAC	CATCCCTGAA	CTTGATCAGC	CACCAAAGCT	CCGTGGGGTG
	401				450
A	CTCCAAGGGC	ATTTGGAGAG	CTCCAGAAAT	GACCCTTTTC	TCCACCCCGA
B	CTCCAAGGGC	ATTTGGAGAG	CTCCAGAAAT	GACCCTTTTC	TCCACCCCGA
C	CTCCAAGGGC	ATTTGGAGAG	CTCCAGAAAT	GACCCTTTTC	TCCACCCCGA
D	CTCCAAGGGC	ATTTGGAGAG	CTCCAGAAAT	GACCCTTTTC	TCCACCCCGA
E	CTCCAAGGGC	ATTTGGAGAG	CTCCAGAAAT	GACCCTTTTC	TCCACCCCGA
F	CTCCAAGGGC	ATTTGGAGAG	CTCCAGAAAT	GACCCTTTTC	TCCACCCCGA
G	CTCCAAGGGC	ATTTGGAGAG	CTCCAGAAAT	GACCCTTTTC	TCCACTCCGA
H	CTCCAAGGGC	ATTTGGAGAG	CTCCAGAAAT	GACCCTTTTC	TCCACTCCGA
I	CTCCAAGGGC	ATTTGGAGAG	CTCCAGAAAT	GACCCTTTTC	TCCACCCCGA
J	CTCCAAGGGC	ATTTGGAGAG	CTCCAGAAAT	GACCCTTTTC	TCCACCCCGA
K	CTCCAAGGGC	ATTTGGAGAG	CTCCAGAAAT	GACCCTTTTC	TCCACCCCGA

★

	451				500
A	CTTTTGCTGC	CTCCTCCCCC	TGGGCTTCTA	TGTCTTGGGT	CTCTTCTGGC
B	CTTTTGCTGC	CTCCTCCCCC	TGGGCTTCTA	TGTCTTGGGT	CTCTTCTGGC
C	CTTTTGCTGC	CTCCTCCCCC	TGGGCTTCTA	TGTCTTGGGT	CTCTTCTGGC
D	CTTTTGCTGC	CTCCTCCCCC	TGGGCTTCTA	TGTCTTGGGT	CTCTTCTGGC
E	CTTTTGCTGC	CTCCTCCCCC	TGGGCTTCTA	TGTCTTGGGT	CTCTTCTGGC
F	CTTTTGCTGC	CTCCTCCCCC	TGGGCTTCTA	TGTCTTGGGT	CTCTTCTGGC
G	CTTTTGCTGC	CTCCTCCCCC	TGGGCTTCTA	TGTCTTGGGT	CTCTTCTGGC
H	CTTTTGCTGC	CTCCTCCCCC	TGGGCTTCTA	TGTCTTGGGT	CTCTTCTGGC
I	CTTTTGCTGC	CTCCTCCCCC	TGGGCTTCTA	TGTCTTGGGT	CTCTTCTGGC
J	CTTTTGCTGC	CTCCTCCCCC	TGGGCTTCTA	TGTCTTGGGT	CTCTTCTGGC
K	CTTTTGCTGC	CTCCTCCCCC	TGGGCTTCTA	TGTCTTGGGT	CTCTTCTGGC
	501				550
A	TGCTCTTTGC	CTCTGTGGTC	CTCATCCTGC	TGCTGAGCTG	GGTTGGGCAT
B	TGCTCTTTGC	CTCTGTGGTC	CTCATCCTGC	TGCTGAGCTG	GGTTGGGCAT
C	TGCTCTTTGC	CTCTGTGGTC	CTCATCCTGC	TGCTGAGCTG	GGTTGGGCAT
D	TGCTCTTTGC	CTCTGTGGTC	CTCATCCTGC	TGCTGAGCTG	GGTTGGGCAT
E	TGCTCTTTGC	CTCTGTGGTC	CTCATCCTGC	TGCTGAGCTG	GGTTGGGCAT
F	TGCTCTTTGC	CTCTGTGGTC	CTCATCCTGC	TGCTGAGCTG	GGTTGGGCAT
G	TGCTCTTTGC	CTCTGTGGTC	CTCATCCTGC	TGCTGAGCTG	GGTTGGGCAT
H	TGCTCTTTGC	CTCTGTGGTC	CTCATCCTGC	TGCTGAGCTG	GGTTGGGCAT
I	TGCTCTTTGC	CTCTGTGGTC	CTCATCCTGC	TGCTGAGCTG	GGTTGGGCAT
J	TGCTCTTTGC	CTCTGTGGTC	CTCATCCTGC	TGCTGAGCTG	GGTTGGGCAT
K	TGCTCTTTGC	CTCTGTGGTC	CTCATCCTGC	TGCTGAGCTG	GGTTGGGCAT
	551				600
A	GTGAAACCAC	AGGCCCTGGA	CTCTGGCCAA	GGTGCTGCTC	TGACCACAGC
B	GTGAAACCAC	AGGCCCTGGA	CTCTGGCCAA	GGTGCTGCTC	TGACCACAGC
C	GTGAAACCAC	AGGCCCTGGA	CTCTGGCCAA	GGTGCTGCTC	TGACCACAGC
D	GTGAAACCAC	AGGCCCTGGA	CTCTGGCCAA	GGTGCTGCTC	TGACCACAGC
E	GTGAAACCAC	AGGCCCTGGA	CTCTGGCCAA	GGTGCTGCTC	TGACCACAGC
F	GTGAAACCAC	AGGCCCTGGA	CTCTGGCCAA	GGTGCTGCTC	TGACCACAGC
G	GTGAAACCAC	AGGCCCTGGA	CTCTGGCCAA	GGTGCTGCTC	TGACCACAGC
H	GTGAAACCAC	AGGCCCTGGA	CTCTGGCCAA	GGTGCTGCTC	TGACCACAGC
I	GTGAAACCAC	AGGCCCTGGA	CTCTGGCCAA	GGTGCTGCTC	TGACCACAGC
J	GTGAAACCAC	AGGCCCTGGA	CTCTGGCCAA	GGTGCTGCTC	TGACCACAGC
K	GTGAAACCAC	AGGCCCTGGA	CTCTGGCCAA	GGTGCTGCTC	TGACCACAGC

	601				650
A	CACACAAACC	ACACACCTGG	AGCTGCAGAG	GGGACGGCAA	GTGACAGTGC
B	CACACAAACC	ACACACCTGG	AGCTGCAGAG	GGGACGGCAA	GTGACAGTGC
C	CACACAAACC	ACACACCTGG	AGCTGCAGAG	GGGACGGCAA	GTGACAGTGC
D	CACACAAACC	ACACACCTGG	AGCTGCAGAG	GGGACGGCAA	GTGACAGTGC
E	CACACAAACC	ACACACCTGG	AGCTGCAGAG	GGGACGGCAA	GTGACAGTGC
F	CACACAAACC	ACACACCTGG	AGCTGCAGAG	GGGACGGCAA	GTGACAGTGC
G	CACACAAACC	ACACACCTGG	AGCTGCAGAG	GGGACGGCAA	GTGACAGTGC
H	CACACAAACC	ACACACCTGG	AGCTGCAGAG	GGGACGGCAA	GTGACAGTGC
I	CACACAAACC	ACACACCTGG	AGCTGCAGAG	GGGACGGCAA	GTGACAGTGC
J	CACACAAACC	ACACACCTGG	AGCTGCAGAG	GGGACGGCAA	GTGACAGTGC
K	CACACAAACC	ACACACCTGG	AGCTGCAGAG	GGGACGGCAA	GTGACAGTGC
	651				700
A	CCCGGGCCTG	GCTGCTCTTC	CTTCGAGGTT	CGCTTCCCAC	TTTCCGCTCC
B	CCCGGGCCTG	GCTGCTCTTC	CTTCGAGGTT	CGCTTCCCAC	TTTCCGCTCC
C	CCCGGGCCTG	GCTGCTCTTC	CTTCGAGGTT	CGCTTCCCAC	TTTCCGCTCC
D	CCCGGGCCTG	GCTGCTCTTC	CTTCGAGGTT	CGCTTCCCAC	TTTCCGCTCC
E	CCCGGGCCTG	GCTGCTCTTC	CTTCGAGGTT	CGCTTCCCAC	TTTCCGCTCC
F	CCCGGGCCTG	GCTGCTCTTC	CTTCGAGGTT	CGCTTCCCAC	TTTCCGCTCC
G	CCCGGGCCTG	GCTGCTCTTC	CTTCGAGGTT	CGCTTCCCAC	TTTCCGCTCC
H	CCCGGGCCTG	GCTGCTCTTC	CTTCGAGGTT	CGCTTCCCAC	TTTCCGCTCC
I	CCCGGGCCTG	GCTGCTCTTC	CTTCGAGGTT	CGCTTCCCAC	TTTCCGCTCC
J	CCCGGGCCTG	GCTGCTCTTC	CTTCGAGGTT	CGCTTCCCAC	TTTCCGCTCC
K	CCCGGGCCTG	GCTGCTCTTC	CTTCGAGGTT	CGCTTCCCAC	TTTCCGCTCC
	701				750
A	AGCCTCTTCC	TGTGGGTACG	GCCTAATGGC	CGTGTGGGGC	CTCTAGTGGC
B	AGCCTCTTCC	TGTGGGTACG	GCCTAATGGC	CGTGTGGGGC	CTCTAGTGGC
C	AGCCTCTTCC	TGTGGGTACG	GCCTAATGGC	CGTGTGGGGC	CTCTAGTGGC
D	AGCCTCTTCC	TGTGGGTACG	GCCTAATGGC	CGTGTGGGGC	CTCTAGTGGC
E	AGCCTCTTCC	TGTGGGTACG	GCCTAATGGC	CGTGTGGGGC	CTCTAGTGGC
F	AGCCTCTTCC	TGTGGGTACG	GCCTAATGGC	CGTGTGGGGC	CTCTAGTGGC
G	AGCCTCTTCC	TGTGGGTACG	GCCTAATGGC	CGTGTGGGGC	CTCTAGTGGC
H	AGCCTCTTCC	TGTGGGTACG	GCCTAATGGC	CGTGTGGGGC	CTCTAGTGGC
I	AGCCTCTTCC	TGTGGGTACG	GCCTAATGGC	CGTGTGGGGC	CTCTAGTGGC
J	AGCCTCTTCC	TGTGGGTACG	GCCTAATGGC	CGTGTGGGGC	CTCTAGTGGC
K	AGCCTCTTCC	TGTGGGTACG	GCCTAATGGC	CGTGTGGGGC	CTCTAGTGGC

	751				800
A	AGGAAGGAGG	CCCTCAGCTC	TGAGTCAGGG	TCGTGGTCAG	GACCTGCTGA
B	AGGAAGGAGG	CCCTCAGCTC	TGAGTCAGGG	TCGTGGTCAG	GACCTGCTGA
C	AGGAAGGAGG	CCCTCAGCTC	TGAGTCAGGG	TCGTGGTCAG	GACCTGCTGA
D	AGGAAGGAGG	CCCTCAGCTC	TGAGTCAGGG	TCGTGGTCAG	GACCTGCTGA
E	AGGAAGGAGG	CCCTCAGCTC	TGAGTCAGGG	TCGTGGTCAG	GACCTGCTGA
F	AGGAAGGAGG	CCCTCAGCTC	TGAGTCAGGG	TCGTGGTCAG	GACCTGCTGA
G	AGGAAGGAGG	CCCTCAGCTC	TGAGTCAGGG	TCGTGGTCAG	GACCTGCTGA
H	AGGAAGGAGG	CCCTCAGCTC	TGAGTCAGGG	TCGTGGTCAG	GACCTGCTGA
I	AGGAAGGAGG	CCCTCAGCTC	TGAGTCAGGG	TCGTGGTCAG	GACCTGCTGA
J	AGGAAGGAGG	CCCTCAGCTC	TGAGTCAGGG	TCGTGGTCAG	GACCTGCTGA
K	AGGAAGGAGG	CCCTCAGCTC	TGAGTCAGGG	TCGTGGTCAG	GACCTGCTGA

	801				850
A	GCACAGTGAG	CATTAGGTAC	TCTGGCCACA	GCCTCTGAGG	GTGGGAGGTT
B	GCACAGTGAG	CATTAGGTAC	TCTGGCCACA	GCCTCTGAGG	GTGGGAGGTT
C	GCACAGTGAG	CATTAGGTAC	TCTGGCCACA	GCCTCTGAGG	GTGGGAGGTT
D	GCACAGTGAG	CATTAGGTAC	TCTGGCCACA	GCCTCTGAGG	GTGGGAGGTT
E	GCACAGTGAG	CATTAGGTAC	TCTGGCCACA	GCCTCTGAGG	GTGGGAGGTT
F	GCACAGTGAG	CATTAGGTAC	TCTGGCCACA	GCCTCTGAGG	GTGGGAGGTT
G	GCACAGTGAG	CATTAGGTAC	TCTGGCCACA	GCCTCTGAGG	GTGGGAGGTT
H	GCACAGTGAG	CATTAGGTAC	TCTGGCCACA	GCCTCTGAGG	GTGGGAGGTT
I	GCACAGTGAG	CATTAGGTAC	TCTGGCCACA	GCCTCTGAGG	GTGGGAGGTT
J	GCACAGTGAG	CATTAGGTAC	TCTGGCCACA	GCCTCTGAGG	GTGGGAGGTT
K	GCACAGTGAG	CATTAGGTAC	TCTGGCCACA	GCCTCTGAGG	GTGGGAGGTT

	851	863
A	TGGGGACCTT	GAG
B	TGGGGACCTT	GAG
C	TGGGGACCTT	GAG
D	TGGGGACCTT	GAG
E	TGGGGACCTT	GAG
F	TGGGGACCTT	GAG
G	TGGGGACCTT	GAG
H	TGGGGACCTT	GAG
I	TGGGGACCTT	GAG
J	TGGGGACCTT	GAG
K	TGGGGACCTT	GAG

Figure 14. Comparison of the third fragment peptide sequences. Ten clones of the third fragment of GPIba gene for the mother and the propositus were sequenced, translated and compared to the GPIba published sequence using the pileup and lineup program. Glycoprotein Iba published sequence is represented by letter A, mother's clones with 2 copies of the VNTR are designated B and C, whereas the clones with 3 copies of the repeat are designated D, E and F. Five clones for the propositus were designated G through K. Mismatches within the peptide sequences are indicated by an asterisk.

	1				50
A	TPKSTTEPTP	SPTTSEPVPE	PAPNMTTLEP	TPSPTTPEPT	
B	TPKSTTEPTP	SPTTSEPVPE	PAPNMTTLEP	TPSPTTPEPT	
C	TPKSTTEPTP	SPTTSEPVPE	PAPNMTTLEP	TPSPTTPEPT	
D	TPKSTTEPTP	SPTTSEPVPE	PAPNMTTLEP	TPSPTTPEPT	
E	TPKSTTEPTP	SPTTSEPVPE	PAPNMTTLEP	TPSPTTPEPT	SEPAPSPTTP
F	TPKSTTEPTP	SPTTSEPVPE	PAPNMTTLEP	TPSPTTPEPT	SEPAPSPTTP
G	TPKSTTEPTP	SPTTSEPVPE	PAPNMTTLEP	TPSPTTPEPT	SEPAPSPTTP
H	TPKSTTEPTP	SPTTSEPVPE	PAPNMTTLEP	TPSPTTPEPT	SEPAPSPTTP
I	TPKSTTEPTP	SPTTSEPVPE	PAPNMTTLEP	TPSPTTPEPT	SEPAPSPTTP
J	TPKSTTEPTP	SPTTSEPVPE	PAPNMTTLEP	TPSPTTPEPT	SEPAPSPTTP
K	TPKSTTEPTP	SPTTSEPVPE	PAPNMTTLEP	TPSPTTPEPT	SEPAPSPTTP
	51				100
A		SEPA	PSPTTPEPTP	IPTIATSPTI	LVSATSLITP
B	...SEPAPSP	TTPEPTSEPA	PSPTTPEPTP	IPTIATSPTI	LVSATSLITP
C	...SEPAPSP	TTPEPTSEPA	PSPTTPEPTP	IPTIATSPTI	LVSATSLITP
D	...SEPAPSP	TTPEPTSEPA	PSPTTPEPTP	IPTIATSPTI	LVSATSLITP
E	EPTSEPAPSP	TTPEPTSEPA	PSPTTPEPTP	IPTIATSPTI	LVSATSLITP
F	EPTSEPAPSP	TTPEPTSEPA	PSPTTPEPTP	IPTIATSPTI	LVSATSLITP
G	EPTSEPAPSP	TTPEPTSEPA	PSPTTPEPTP	IPTIATSPTI	LVSATSLITP
H	EPTSEPAPSP	TTPEPTSEPA	PSPTTPEPTP	IPTIATSPTI	LVSATSLITP
I	EPTSEPAPSP	TTPEPTSEPA	PSPTTPEPTP	IPTIATSPTI	LVSATSLITP
J	EPTSEPAPSP	TTPEPTSEPA	PSPTTPEPTP	IPTIATSPTI	LVSATSLITP
K	EPTSEPAPSP	TTPEPTSEPA	PSPTTPEPTP	IPTIATSPTI	LVSATSLITP
	101				150
A	KSTFLTTTKP	VSLESTKKT	IPELDQPPKL	RGVLQGHLES	SRNDPFLHPD
B	KSTFLTTTKP	VSLESTKKT	IPELDQPPKL	RGVLQGHLES	SRNDPFLHPD
C	KSTFLTTTKP	VSLESTKKT	IPELDQPPKL	RGVLQGHLES	SRNDPFLHPD
D	KSTFLTTTKP	VSLESTKKT	IPELDQPPKL	RGVLQGHLES	SRNDPFLHPD
E	KSTFLTTTKP	VSLESTKKT	IPELDQPPKL	RGVLQGHLES	SRNDPFLHPD
F	KSTFLTTTKP	VSLESTKKT	IPELDQPPKL	RGVLQGHLES	SRNDPFLHPD
G	KSTFLTTTKP	VSLESTKKT	IPELDQPPKL	RGVLQGHLES	SRNDPFLHSD
H	KSTFLTTTKP	VSLESTKKT	IPELDQPPKL	RGVLQGHLES	SRNDPFLHSD
I	KSTFLTTTKP	VSLESTKKT	IPELDQPPKL	RGVLQGHLES	SRNDPFLHPD
J	KSTFLTTTKP	VSLESTKKT	IPELDQPPKL	RGVLQGHLES	SRNDPFLHPD
K	KSTFLTTTKP	VSLESTKKT	IPELDQPPKL	RGVLQGHLES	SRNDPFLHPD

★

	151				200
A	FCCLLPPLGFY	VLGLFWLLFA	SVVLILLLSW	VGHVKPQALD	SGQGAALTTA
B	FCCLLPPLGFY	VLGLFWLLFA	SVVLILLLSW	VGHVKPQALD	SGQGAALTTA
C	FCCLLPPLGFY	VLGLFWLLFA	SVVLILLLSW	VGHVKPQALD	SGQGAALTTA
D	FCCLLPPLGFY	VLGLFWLLFA	SVVLILLLSW	VGHVKPQALD	SGQGAALTTA
E	FCCLLPPLGFY	VLGLFWLLFA	SVVLILLLSW	VGHVKPQALD	SGQGAALTTA
F	FCCLLPPLGFY	VLGLFWLLFA	SVVLILLLSW	VGHVKPQALD	SGQGAALTTA
G	FCCLLPPLGFY	VLGLFWLLFA	SVVLILLLSW	VGHVKPQALD	SGQGAALTTA
H	FCCLLPPLGFY	VLGLFWLLFA	SVVLILLLSW	VGHVKPQALD	SGQGAALTTA
I	FCCLLPPLGFY	VLGLFWLLFA	SVVLILLLSW	VGHVKPQALD	SGQGAALTTA
J	FCCLLPPLGFY	VLGLFWLLFA	SVVLILLLSW	VGHVKPQALD	SGQGAALTTA
K	FCCLLPPLGFY	VLGLFWLLFA	SVVLILLLSW	VGHVKPQALD	SGQGAALTTA
	201				250
A	TQTTHLELQR	GRQVTVPRAW	LLFLRGS�PT	FRSSLFLWVR	PNGRVGPLVA
B	TQTTHLELQR	GRQVTVPRAW	LLFLRGS�PT	FRSSLFLWVR	PNGRVGPLVA
C	TQTTHLELQR	GRQVTVPRAW	LLFLRGS�PT	FRSSLFLWVR	PNGRVGPLVA
D	TQTTHLELQR	GRQVTVPRAW	LLFLRGS�PT	FRSSLFLWVR	PNGRVGPLVA
E	TQTTHLELQR	GRQVTVPRAW	LLFLRGS�PT	FRSSLFLWVR	PNGRVGPLVA
F	TQTTHLELQR	GRQVTVPRAW	LLFLRGS�PT	FRSSLFLWVR	PNGRVGPLVA
G	TQTTHLELQR	GRQVTVPRAW	LLFLRGS�PT	FRSSLFLWVR	PNGRVGPLVA
H	TQTTHLELQR	GRQVTVPRAW	LLFLRGS�PT	FRSSLFLWVR	PNGRVGPLVA
I	TQTTHLELQR	GRQVTVPRAW	LLFLRGS�PT	FRSSLFLWVR	PNGRVGPLVA
J	TQTTHLELQR	GRQVTVPRAW	LLFLRGS�PT	FRSSLFLWVR	PNGRVGPLVA
K	TQTTHLELQR	GRQVTVPRAW	LLFLRGS�PT	FRSSLFLWVR	PNGRVGPLVA
	251		279		
A	GRRPSALSQG	RGQDLLSTVS	IRYSGHSL*		
B	GRRPSALSQG	RGQDLLSTVS	IRYSGHSL*		
C	GRRPSALSQG	RGQDLLSTVS	IRYSGHSL*		
D	GRRPSALSQG	RGQDLLSTVS	IRYSGHSL*		
E	GRRPSALSQG	RGQDLLSTVS	IRYSGHSL*		
F	GRRPSALSQG	RGQDLLSTVS	IRYSGHSL*		
G	GRRPSALSQG	RGQDLLSTVS	IRYSGHSL*		
H	GRRPSALSQG	RGQDLLSTVS	IRYSGHSL*		
I	GRRPSALSQG	RGQDLLSTVS	IRYSGHSL*		
J	GRRPSALSQG	RGQDLLSTVS	IRYSGHSL*		
K	GRRPSALSQG	RGQDLLSTVS	IRYSGHSL*		

Discussion

Bernard-Soulier syndrome, an autosomal recessive disease, was first described in 1948 (Bernard et al., 1948), since then many cases have been studied and the diagnostic criteria of the disease had been established. The molecular hallmark of the disease is an absent or dysfunctional GPIb-IX-V complex (Nurden et al. 1981, Ware et al. 1993) which serves as a receptor for the adhesive protein von Willebrand factor (vWf) (George et al., 1984). The familial cases of BSS that were studied at the molecular level showed that a defect in the genes encoding GPIb α and GPIX could alter the conformation and prevent the formation of the complex (Clemetson et al., 1993).

In this research a family with BSS was investigated. Consanguinity had been noticed as the parents are cousins (Figure 3). The mother expressed some clinical features characteristic of BSS including giant platelets and mild thrombocytopenia, whereas the propositus has episodically severe thrombocytopenia, bleeding episodes and her platelets were found to lack GPIb α as documented by two dimensional gel electrophoresis (data not shown, personal communications with Dr.Mattson). Based on preliminary results, the assumption was made that the mother was a carrier heterozygote for the disease while the propositus was homozygote.

Glycoprotein Ib α gene was amplified into three overlapping fragments using three sets of primers (**Table 5**). Amplifying the gene into three fragments enabled the

generation of small fragments suitable for heteroduplexing and single stranded conformation polymorphism analyses. The PCR products of the first and second fragments for the normal control, the propositus and the mother showed no gross rearrangements (insertion or deletion) within that part of the gene. However, results from the PCR products of the third fragment (Figure 7) showed differences in the sizes between the normal control, the mother and the propositus. It was believed that this was due to the presence of VNTR polymorphism that is present at the 3' end of the gene (Figure 2). Based on the calculated sizes of the PCR products, it was concluded that the mother was heterozygote for the 39 bp repeat, one allele having two copies of the repeat and the other allele having three copies of the repeat. However, amplification products from the propositus yielded one fragment of 860 bp which indicated homozygosity for a three copy repeat.

Digestion of the third fragment with the restriction enzymes *Pst*I and *Sst*I (Figure 10) generated smaller fragments which further confirmed that differences in fragment sizes between the normal control, the mother and the propositus were due to a variable number of tandem repeats. The digest of the mother's PCR products gave one subfragment that matched the result of the normal control and another subfragment that matched the result of the propositus. This confirmed that the mother is heterozygous for this repeat and the propositus is homozygous.

Localization of mutation(s) by heteroduplexing and Single Stranded Conformation Polymorphism

Since no size differences, which might indicate gross insertion or deletion, had been detected using PCR amplification, heteroduplexing analysis, which helps to identify heterogeneous PCR products was used to screen and localize the mutation within one of the three PCR fragments. When the double stranded DNA is denatured and allowed to reanneal again, there is a chance for the mutated and the normal alleles to anneal. If this occurs, it alters the structure of double stranded DNA (heteroduplexes) retarding its gel migration when compared to the normal DNA (homoduplexes). Based on the clinical data presented it was hypothesized that the mother was heterozygous and therefore heteroduplexing analysis might identify the region of GPIIb α that has the defect. Unfortunately the data generated from heteroduplexing did not reveal any differences between the mother and the normal control. A possible reason why heteroduplexing did not resolve any differences could be due to the large size of the amplified fragment. Even if heteroduplexes were formed, the differences in migration patterns between homoduplexes and heteroduplexes might not have been resolved by heteroduplexing gels.

Because the data generated by heteroduplexing analyses was not useful in detecting differences in any of the fragments, a different approach SSCP, was used. In this technique the DNA is denatured at 95 °C and cooled on ice immediately before it is loaded on the gel. The single stranded DNA takes certain conformations based on its sequence. The mutated sequence is detected as a change in mobility in polyacrylamide gel electrophoresis under non-denaturing conditions (Hayashi 1991). However, SSCP can

be affected by different parameters and conditions. Shorter fragments (less than 300 bp) are better suited for detection of mutations. As the detection of mutations depends on the conformation of the single stranded DNA. The physical environment of the gel will affect this detection. This includes temperature, percentage of acrylamide, the ionic strength of the buffer and glycerol concentration in the gel (Spinardi et al., 1991). Rising temperatures during the run may affect the reproducibility of the results. Therefore, efficient cooling is important. Hayashi 1991, indicated that running a gel at room temperature (20 -26 °C) versus 4 °C can dramatically affect the mobility of the single-stranded DNA. Spinardi et al. 1994, used a combination of different conditions to detect known mutations. They concluded that an increase in the ionic strength of the running buffer (TBE) over 1X, raising the glycerol concentration over 10% or increasing the polyacrylamide concentration above 6% did not result in any improvement. It has been found that a low concentration of glycerol (5- 10%) in the gel usually improves separation of the mutated single-stranded DNA. This could be due to the weak denaturing action of glycerol on nucleic acid, that it partially opening the folded structure of single-stranded DNA so that more surface area of the molecule is exposed. Therefore there is more chance for the acrylamide fibers to detect locally confined structural differences caused by mutation (Hayashi 1991).

To obtain accurate data using SSCP and, since PCR products were bigger than the optimum 300 bp, digestion with restriction enzymes was used to obtain smaller fragments in an attempt to optimize the conditions for this technique. Digestion of the PCR products of the first and second fragments with *Ban I* gave fragments ranging in size from 272 - 392 bp which are suitable for SSCP analysis. Also restriction digest of PCR

products of the third fragment with *Pst*I and *Sst*I gave fragments ranging in size from 203 bp - 424 bp which are also good for SSCP analysis.

The completely digested PCR products were loaded on 6% polyacrylamide/ 5% glycerol gel which gave good resolution of the smaller fragments. The results that were obtained for PCR products for the first and second fragments did not show any differences in migration patterns between the control the mother and the propositus (Figure 11). These results might indicate that there is no mutation present in the first or second fragment of the gene. On the other hand, this may indicate that a mutation was not detected in this analysis because the conditions used were not the right combinations to detect mutations in this sequence.

Results of SSCP analysis of the PCR products for the third fragment showed some significance differences in the migration patterns between the normal control the mother and the propositus. These differences have been localized to two subfragments within the 3' end of the gene. The first subfragment (A) in Figure 12, could be due to the presence of different numbers of the 39 bp repeats within this part of the gene where as the second subfragment (B) in Figure 12, is most probably due to a mutation which changed the migration pattern for the propositus in this region. These preliminary results led us to assume that the defect was within the 3' end of the gene. This part of the gene encodes the region of GPIb α that associates with GPIb β , and has the transmembrane and cytoplasmic domain which helps in stabilizing the platelet plasma membrane by its association with the cytoskeleton through an actin binding protein. Although complete digestion of PCR products was examined before running the SSCP gels, any incomplete digestion that was not picked up by ethidium bromide staining will be detected by silver

staining because it is more sensitive. This could give false positive results and lead to misinterpretation of the results. To confirm that a mutation is indeed located within the 3' end of the gene and to identify the mutation we cloned and sequenced the 3' end of the gene for the mother and the propositus.

Sequencing and Sequence Analysis

Since the 3' end of GPIIb gene is GC rich and has multiple copies of the 39 bp repeat, sequencing this region directly from PCR products was very difficult and false termination was detected within this fragment which made it impossible to interpret the data. Because of this the PCR product of this fragment was cloned into the pUC19 vector for sequencing. pUC19 is 2.8 Kb circular plasmid with a high copy number, it has the ampicillin resistance gene which allows for selection. After transformation only colonies that contain the plasmid will grow on a media with ampicillin. This vector has a multiple cloning site within the Lac Z gene which is responsible for producing the N terminus (alpha peptide) of the enzyme B-galactosidase, where as the host strain provide the C-terminus (w- peptide) of this enzyme. X-gal is a colorless chemical substrate for B-galactosidase, when it is cleaved by the enzyme it gives a blue product. The insertion of a DNA fragment in the multiple cloning site will disrupt the alpha region of the lac-Z gene and it will not produce the alpha peptide of B-galactosidase. As a result the enzyme is not produced, X-gal is not cleaved and no blue color is developed. This characteristic of pUC19 allows for screening where white colonies indicate the presence of an insert. The purified PCR products of the third fragment were successfully cloned into pUC19 vector and purified using the CsCl gradient centrifugation method (Sambrook et al., 1992).

Since PCR primers can not differentially amplify maternal or paternal alleles, the amplified product represents a mixture of DNA from both the maternally and paternally inherited DNA. In an effort to determine the mutation responsible for the disease and the origin of that mutation, multiple clones have been sequenced. Five clones for the proband DNA were successfully sequenced, two of which had a single point mutation, a C to a T, that changed the amino acid proline 481 to a serine. Sequences from the other three clones demonstrated no mutations and the generated sequences completely matched the consensus sequence (Lopez et al., 1987). For the mother a total of 5 clones were chosen for sequencing. Three of the 5 sequenced clones, as expected, had 3 copies of the 39 bp repeat and the remaining two had 2 copies of the repeat. No mutations were detected in any of the clones and the sequence had a 100% match with the published sequence (Lopez et al., 1987). The sequence data proved that the mother is heterozygous for the repeat, but since the proband is homozygote for this repeat, we are mainly concerned about the copy of the gene that has 3 copies of the VNTR.

Bernard-Soulier syndrome is inherited in an autosomal recessive manner. An affected individual should have inherited two defective alleles, one from each parent. From the heterozygous mother we expected to identify a mutation in one of the alleles, specifically, the one with the 3 copies of VNTR because as was demonstrated this is the allele that the proband inherited from her mother. For this part of the gene, no mutation was identified in the mother's DNA sequence indicating that the defect may reside in another part of the gene.

The mutation that was identified in the proband DNA sequence was not identified in the mother's sequence which means that she inherited the allele that has the

mutation from her father. The propositus is homozygous, because she has all the clinical symptoms of the disease including bleeding episodes, so the mutation identified could not be the only cause of the disease. There are rare cases that seem to be due to a double heterozygous defect in GPIb α chain (Ware et al., 1990), but other cases of BSS are produced by double heterozygote point mutation in the gene encoding GPIX (Clemetson et al. 1993; Wright et al. 1993). Therefore, there is a possibility that another mutation could be present in the maternal allele that, when coupled with the paternally inherited defect results in BSS.. The sequenced part of the maternal gene (3' end) did not demonstrated any mutations. Therefore, the presence of a mutation in the 5' end of the gene is possible. However this does not exclude that the propositus could be homozygous for another mutation (at the 5' end of the gene) which she inherited from both parents.

As mentioned earlier, a defect in one of the genes encoding for any member of GP-Ib-IX complex could prevent the assembly and cell surface expression of the complex (Clemetson et al., 1993). Although GPIb α was not detected by two dimensional gel electrophoresis and showed low reactivity when treated with monoclonal antibodies, this does not exclude that a defect could be in one of the other genes of GPIb β , GPIX or GPV. On the other hand, a defect in the promoter for one of these genes could also lead to complete lack of expression of the designated glycoprotein without there being any problem in the translated region. This in turn could affect the formation and surface expression of the whole complex. In conclusion, the mother was found to be heterozygote for the 39 bp repeat, while the propositus is homozygote for this repeat. The point mutation identified here, Pro 481 to Ser, could not be the sole cause of the disease. Therefore, other parts of the gene and the GPIb-IX-V complex should be investigated.

Future Research

Since a point mutation had been identified, allele specific PCR can be used to type other family members for this specific mutation. This can be approached by synthesizing two oligonucleotide primers, one primer (X) has this point mutation at its 3' end (5' CCAGAAATGACCCTTTTCTCCACT 3'), the other primer (Y) is a normal one (5' CCAGAAATGACCCTTTTCTCCACC 3'). These two primers can be used with DE6 primer (Table 5) to amplify a 440 bp fragments in two separate PCR reactions. DNA from a normal individual will amplify a fragment in the PCR that has primers Y and DE6 only, DNA from a person homozygote for this mutation will amplify a fragment in the PCR with primers X and DE6, while DNA from a person heterozygote for this mutation will amplify fragments from both PCR reactions using primers X / DE6 and Y / DE6.

Since only the 3' end of GPIIb α gene was sequenced and a point mutation that could explain the propositus clinical symptoms of the disease was not found, it will be necessary to sequence the remaining part of the gene that is represented by the first two fragments. In case a mutation is identified, allele specific PCR can be used to screen other family members for this mutation using the approach just explained above. Also screening other members of the family can be approached by restriction fragment length polymorphism analysis (RFLP) if the mutation destroys a known restriction site or if it creates a new one. This approach does not apply for the mutation identified here because

this mutation did not destroy or create a restriction site.

If no mutation (s) has been identified within the open reading frame of the gene, the 5' end which has the promoter sequence has to be investigated for mutations that might inactivate the promoter. After identifying the transcription starting site either by S1 nuclease mapping or by primer extension the promoter region can be sequenced using dye terminator sequencing reagent and the sequence analyzed by GCG and Genepro program to identify sequences within that region which might represent binding site for genes as transcription factors.

After defining the promoter region, oligonucleotide primers should be designed to specifically amplify the potential promoter, which will be cloned upstream of a reporter gene (either CAT or Luciferase) in such a way that the expression of the gene is driven by the promoter of interest. The promoter region from the mother, the propositus and a normal control should be cloned. All recombinants can be transfected into a eukaryotic cell line (Hela cells or NIH3 cells) by any transfection method. Cells can be harvested and protein purified and assayed for enzyme activity. Enzymatic activity for the mother, propositus and normal control can be compared and if comparable results are obtained, the possibilities that the defect causing the disease is present in the other components of the complex, GPIIb β , GPIX or GPV should be investigated. Western blot analysis can be used to check for the presence or absence of these glycoproteins and , the molecular defect can be investigated in a manner similar to what was done in this project.

LIST OF REFERENCES

List of references

- Ainsworth J., D. I. Rodenhiser** (1994). Methods in molecular Biology (A.J. Harwood Totowa, NJ) **31**: pp 205-210
- Alagille D., F. Josso, J. L. Binet, M. L. Blin** (1964). La dystrophie thrombocytaire hemorragipare : Discussion nosologique. *Nouv Rev Fr Hematol* **4**: 755
- Andrews R., J. Fox** (1991). Interaction of purified actin binding protein with the platelet membrane glycoprotein Ib-IX complex. *J Biol Chem* **266**: 7144- 7147
- Bernard J., J. P. Soulier** (1948). Sur une nouvelle variete de dystrophie thrombocytaire hemorragipare congenitale. *Sem Hop Paris* **24**: 3217
- Bernard J.** (1983). History of congenital hemorrhagic thrombocytopathic distrophy. *Blood Cells* **9**: 179-193
- Bizzaro N., G. Dianese** (1988). Neonatal alloimmune amegakaryocytosis. Case report. *Vox Sang* **54**: 112-114
- Brass L.F., C. C. Shaller, E. J. Belmonte** (1987). Inositol 1,4,5, triphosphate induced granule secretion in platelets. Evidence that the activation of phospholipase C mediated by platelet thromboxane receptors involves a guanine nucleotide binding protein-dependent mechanism distinct from that of thrombin. *J Clin Invest* **79**: 1269
- Brown A. B.** (1984). Hematology: Principles and Procedures. (Lea & Febiger Philadelphia, PA) pp 179 - 186.
- Charo I. F., R. D. Feinman, T. C. Detwiler** (1977). Interrelations of platelet aggregation and secretion. *J Clin Invest* **60**: 866
- Clemetson K. J. and J. Clemetson** (1993). Molecular pathology of Bernard-Soulier Syndrome, Platelet-type von Willebrand's disease and Glanzman's thrombosthania. *Beiter Infusionsther* **31**:168-173
- Clemetson J. M., P. A. Kyrle, B. Brener, K. J. Clemetson** (1994). Variant Bernard-Soulier syndrome associated with a homozygous mutation in the leucine-rich domain of

glycoprotein IX. *Blood* **84** (4): 1124-1131

Clemetson, K., J. McGregor, E. James, M. Dechavanne, and E. Luscher (1982). Characterization of the platelet Membrane glycoprotein abnormalities in Bernard Soulier Syndrome and comparison with normal by surface-labeling techniques and high-resolution two dimensional gel electrophoresis. *J.Clin.Invet.* **70**:304-311

Coller B S. (1978). The effects of ristocetin and von Willebrand factor on platelet electrophoretic mobility. *J Clin Invest* **61**: 1168-1175

Coller B. S. (1982). Effects of tertiary amine local anesthetics on von Willebrand factor-dependent platelet function: alteration of membrane reactivity and degradation of GPIb by a calcium-dependent protease(s). *Blood* **60**: 731-743

Coller B. S., H. Gralnick (1977). Studies on the mechanism of ristocetin-induced platelet agglutination. Effects of structural modification of ristocetin and vancomycin. *J Clin Invest* **60**: 302-312

Coller B. S. (1986). Activation affects access to the platelet receptor for adhesive glycoproteins. *J Cell Biol* **103**: 451

Corriveau M. D., G. A. Fritsma (1988). Haemostasis and Thrombosis in the Clinical Laboratory. (J.B.Lippincott co., Philadelphia, PA), pp 8, 14-17, 23-28

Cruz M. A., E. Peterson, S. M. Turci (1992). Functional analysis of a recombinant glycoprotein Ib A polypeptide which inhibits von Willebrand factor binding to the platelet glycoprotein Ib-IX complex and to Collagen. *J Biol Chem* **267**: 1303-1309

Cullum C., D. P. Cooney, S. L. Schrier (1967). Familial Thrombocytopenic thrombocytopathy. *Br J Haematol* **13**: 147

Don R. H., P. T. Cox, B. J. Wainwright, K. Baker, J. S. Mattick (1991). 'Touchdown' PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Research* **19**: 4008

Du, X., L. Beutler, C. Ruan, P. A. Castaldi, M. C. Berndt (1987). Glycoprotein Ib and glycoprotein IX are fully complexed in the intact platelet membrane. *Blood* **69**:1524-1527

Federici A., R. Bader, S. Pagani (1989). Binding of von Willebrand factor to glycoprotein Ib and IIb-IIIa complex: affinity is related to multimeric size. *Br J Haematol* **73**: 93-99

Feng D-F., R. F. Doolittle (1987). Progressive sequence alignment as a prerequisite to correct phylogenetic trees. *Journal of molecular evolution* **25**: 235-260

Finch, C., L. Miller, R. Handin, V. Lyle (1990). Evidence that an abnormality in the Glycoprotein Ib α gene is not the cause of abnormal platelet function in a family with classic Bernard-Soulier disease. *Blood* **75**:2357-2362.

Fontento J. D., N. Tjandra, D. Bu, C. Ho, R. C. Montelaro, O. J. Finn (1993). Biophysical characterization of one-, two-, and three-tandem repeats on human mucin (muc-1) protein core. *Cancer Res.* **53**: 5386-5394

fox J., M. Berndt (1989). Cyclic AMP-dependent phosphorylation of glycoprotein Ib inhibits collagen induced polymerization of actin in platelets. *J Biol Chem* **264**: 9520-9526

Fox J., L. Aggerbeck, M. Berndt (1988). Structure of the Glycoprotein Ib-IX complex from platelet membranes. *J Biol Chem* **263**: 4882- 4890

Fox J., K. Boyles, M. Berndt (1988). Identification of a membrane skeleton in platelets. *J Cell Biol* **106**: 1525

George J. N., A. T. Nurden, D. R. Phillips (1984). Molecular defects in interaction of platelets with the vessel wall. *N Eng J Med* **311**: 1084

Gerald, R., T. Church, B. McMullen, and S. Williams (1990). Human platelet glycoprotein V: A surface leucine-rich glycoprotein related to adhesion. *Biochemical and Biophysical Research Communications* **170**(1):153-161.

Gerrard J. M., R. C. Carroll (1981). Stimulation of protein phosphorylation by arachidonic acid and endoperoxide analog. *Prostaglandins* **22**: 81

Golden A., S. J. Brugge, S. J. Shattil (1990). Role of platelet glycoprotein Iib-IIIa in agonist-induced tyrosine phosphorylation of platelet proteins. *J Cell Biol* **111**: 3117

Greensburg S., F. Divirgilio, T. H. Steinberg, S. C. Silverstein (1988). Extracellular nucleotides mediate Ca⁺² fluxes in J774 macrophages by two distinct mechanisms. *J Biol Chem* **263**: 10337

Grottum K. A., N. O. Solum (1964). Congenital thrombocytopenia with giant platelets : A defect in the platelet membrane. *Br J Haematol* **16**: 277-290

Grottum K. A., N. O. Solum (1969). Congenital thrombocytopenia with giant platelets: a defect in the platelet membrane. *Br J Haematol* **16**: 277

Handin R. I., D. D. Wagner (1989). Molecular and cellular biology of von Willebrand factor. *Prog Hemost Thromb* **9**: 233

Hartwig J., M. DeSisto (1991). The cytoskeleton of the resting human blood platelets: structure of the membrane skeleton and its attachment to actin filaments. *J Cell Biol* **112**: 407-425

Hayashi, K. (1991). PCR-SSCP: A simple and sensitive method for detection of mutations in the genomic DNA. *PCR Methods and Applications* **1**:34-38.

He R., D. M. Reid, C. E. Jones, N. R. Shulman (1995). Extracellular epitopes of platelet glycoprotein Ib-a reactive with serum antibodies from patients with chronic idiopathic thrombocytopenic purpura. *Blood* **86**: 3789-3796

Hession C., C. Osborn, D. Goff (1990). Endothelial leukocyte adhesion molecule 1: Direct expression, cloning and functional interactions. *Proc Natl Acad Sci* **87**: 1673

Hickey M. J., G. J. Roth (1993). Characterization of the gene encoding human platelet glycoprotein IX. *J Biol Chem* **268**: 3438- 3443

Hickey, J. , S. Williams, and G. Roth (1989). Human platelet glycoprotein IX: An adhesive prototype of leucine-rich glycoproteins flank-center-flank structures. *Proc. Natl. Acad. Sci (USA)*. **86**:6773-6777.

Hoffman R., E. Benz, D. J. Shattil, B. Furie, H. J. Cohen (1991). Hematology Basic Principles and practice. (Churchill Livingstone Inc. New York, NY), pp 1167

Ishida F., K. Furihata, K. Ishida, H. Kodaira, K. S.Han, L. Da-Zhuang, K. Kitano, K. Kiyosawa (1996). The largest isoform of platelet membrane glycoprotein Ib- α is commonly distributed in Eastern Asian populations. *Thrombosis and Haemostasis* **76**(2): 245-247

Jenkins C., K. J. Clemetson, E. F. Luscher (1979). Studies on the mechanism of ristocetin-induced platelet agglutination: binding of ristocetin to platelets. *J Lab Clin Med* **93**: 220-231

Judson P. A., D. J. Anstee, J. R. Clamp (1982). Isolation and characterization of the major oligosaccharide of human platelet membrane glycoprotein GPIb. *Biochem J* **205**:81-90

Kertz, K. W. Callen, and V. Hedden (1994). Cycle Sequencing. *PCR Methods and Applications* **3**:107-112.

Korrel S. A. M., K. J. Clemetson, H. Van Halbeek. (1984). Structural studies on the O-linked carbohydrate chains of human platelet glycolalacin. *Eur J Biochem* **140**: 571-576

Kroll M. H., A. I. Schafer (1989). Biochemical mechanisms of platelet activation.

Blood 74: 1181

Kuijpers R., W. Ouwehand, P. Bleeker, D. Christie, A. Kr. Von dem Borne (1992). Localization of the platelet-specific HPA-2 (Ko) alloantigens on the N-terminal globular fragment of platelet glycoprotein Ib alpha. *Blood* 79(1): 283-288

Kunishima S., H. Miura, H. Fukutani, H. Yoshida, K. Osumi, S. Kobayashi, R. Ohno, T. Naoe (1994). Bernard-Soulier syndrome Kagoshima: Ser 444→ stop mutation of glycoprotein (GP) Ib α resulting in circulating truncated GPIb- α and surface expression of GPIb- β and GPIX. *Blood* 84(10): 3356-3362

Lanza, F., M. Morales, C. de-La-Salle, J. P. Gazenave, K. J. Clemetson, T. Shimimura, and D. R. Phillip (1993). Cloning and characterization of the gene encoding the human platelet glycoprotein V. A member of the leucine-rich glycoprotein family cleaved during thrombin-induced platelet activation. *JBC* 268(28):20801-7

Lee, J. (1991). Alternative dideoxy sequencing of double stranded DNA by cyclic reaction using Taq polymerase. *DNA and Cell Biology* 10:67-73.

Leren, T., K. Solberg, O.K. Rodningen, L. Ose, S. Tonstand, K. Berg (1993). Evaluation of running conditions for SSCP analysis: Application of SSCP for detection of point mutation in the LDL receptor gene. *PCR Methods and Applications* 3:159-162.

Li C., S. E. Martin, G. J. Roth (1995). The genetic defect in two well-studied cases of Bernard-Soulier syndrome: a point mutation in the fifth leucine-rich repeat of platelet glycoprotein GPIb α . *Blood* 86(10): 3805-3814.

Lopes, J. A. (1994). The platelet glycoprotein Ib-IX complex. *Blood Coagulation and Fibrinolysis*. 5:97-119

Lopez, J. , D. Chung, K. Fujikawa, F. Hagen, T. Papayannopoulou, and G. Roth. (1987). Cloning of the alpha chain of human platelet glycoprotein Ib: A transmembrane protein with homology to leucine -rich alpha2-glycoprotein. *Proc. Nat. Acad. Sci (USA)* 84:5615-5619

Lopez J. A., D. W. Chung, K. Fujikawa, F. S. Hagen, E. W. Davie, G. J. Roth (1988 b). *Proc. Natl. Acad. Sci. USA* 85: 2135-2139.

Lopez A. J., B. Leung, C. Reynoldst, Q. Chester, and J. Fox (1992). Efficient plasma membrane expression of a functional platelet glycoprotein Ib-IX complex requires the presence of its three subunits. *JBC* 267(18) 12851-12859

Lopez J. A., D. W. Chung, K. Fujikawa (1988). The alpha and beta chains of human platelet glycoprotein Ib are both membrane proteins containing a leucine-rich amino acid sequence. *Pro Natl Acad Sci (USA)* 85: 2135-2139

Madoff M. A., S. Ebbe, M. Baldini (1964). Sialic acid of human blood platelets. *J Clin Invest* **43**: 870-877

Maoke J., N. Turner, N. Stathopoulos (1988). Shear induced platelet aggregation can be mediated by vWF released from platelets, as well as by exogenous large or unusually large vWF multimers, requires adenosine diphosphate, and is resistant to aspirin. *Blood* **71**: 1366- 1374

Marco L. D., M. Mazzucato, F. Fabris, D. Roia, P. Coser, A. Girolami (1990). Variant Bernard-Soulier syndrome type bolzano a congenital bleeding disorder due to a structural and functional abnormality of the platelet glycoprotein Ib-IX complex. *J.Clin.Invest.* **86**: 25-30

Mattson J.C., D. M. Peterson, S. McCarron, N. Stathopoulos (1984). The Bernard-Soulier platelets: II. A comparative study of changes in platelet morphology and cytoskeletal architecture following contact activation. *Scanning Electron Microscopy IV*: 1941-1950

McGill M., G. Jamieson, J. Drouin, M. Cho, G. Rock (1984). Morphometric analysis of platelets in Bernard-Soulier Syndrome: size and configuration in patients and carriers. *Thromb Haemost* **52**: 37

Miller, J., V. Lyle, and D. Cunningham (1992). Mutation of Leucine -57to phenylalanine in a platelet glycoprotein Ib α leucine tandem repeat occurring in patients with an autosomal dominant variant of Bernard Soulier disease. *Blood* **79**:439-446.

Modderman, P. L. Admiral, A. Sonnenberg, and A. Von dem Borne (1992). Glycoproteins V and Ib-IX form a non covalent complex in the platelet membrane. *JBC* **267**(1),364-369.

Moroi, M., S. M. Jung, and N. Yoshida (1984). Genetic polymorphism of platelet glycoprotein Ib. *Blood* **64**:622-629.

Najean Y., N. Aradaillou, J. P. Cean (1963). Survival of radiochromium -labeled platelets in thrombocytopenias. *Blood* **22**: 718

Neame P. J., H. U. Choi, L. C. Rosenberg (1989). The primary structure of the core protein of the small, leucine -rich proteoglycan (PG I) from bovine articular cartilage. *J Biol Chem* **264**: 8653-8661

Nurden T. A. (1995). Polymorphism of human platelet membrane glycoproteins: structure and clinical significance. *Thrombosis and Haemostasis* **74**(1): 345-351

Nurden A. T., J. P. Cean (1974). An abnormal platelet glycoprotein pattern in three cases of Glanzman's thrombasthania. *Br J Haematol* **28**: 233

Nurden A. T., J. P. Caen (1975). Specific roles for platelet surface glycoproteins in platelet function. *Nature (Lond)* **255**: 720

Nurden, A. , D. Didry, and J-P. Rosa (1983). Molecular Defects of Platelets in Bernard Soulier Syndrome. *Blood Cells* **9**:333-358.

Nurden, A. , D. Dupuis, T. Kunicki, and J. Caen (1981). Analysis of Glycoprotein and Protein Composition of Bernard- Soulier Platelets by Single and Two Dimensional Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. *J. Clin. Invest.* **67**:1431-1440.

Okita, JR. ,D. Pidard, P. J. Newman, R. R. Montgomery, T. J. Kuinki (1985). On the association of glycoprotein Ib and actin-binding protein in human platelets. *J Cell biol.* **100**:317-321

Okita J., D. Pidard, P. J. Newman (1985). On the association of glycoprotein Ib and actin- binding protein in human platelet. *J Cell Biol* **100**: 317

Peerschke E. I. (1985). The platelet fibrinogen receptor. *Semin Hematol* **22**: 241

Peerschke E. I., M. B. Zucker, R. A. Grant, J. J. Egan, M. M. Johnson (1980). Correlation between fibrinogen binding to human platelets and platelet aggregability. *Blood* **55**: 841

Peterson D. M., N. Stathopoulos, T. Giorgio (1987). Shear induced platelet aggregation requires von Willebrand factor and platelet membrane glycoprotein Ib and IIb- IIIa. *Blood* **69**: 625-628

Phillips D.R., I. F. Charo, L. V. Parise, L. A. Fitzgerald (1988). The platelet membrane Glycoprotein Iib-IIIa complex. *Blood* **71**: 831

Ratnoff O., C. D. Forbes (1984). The evolution of knowledge of hemostasis. In Disorders of hemostasis, (Grune & Stratton New York, NY) pp 1- 20

Sae-Tung, G., J-F. Dong, and J. A. Lopez.(1996). Biosynthetic defect in platelet glycoprotein IX mutants associated with Bernard-Soulier Syndrome. *Blood* **87** (4):1361-1367.

Saji H., E. Maruya, H. Fujii (1989). New platelet antigen, Sib^a, involved in platelet transfusion refractoriness in a Japanese man. *Vox Sang* **56**: 283-287

Salle C. D. L., M-J. Baas, F. Lanza, A. Schwartz, D. Hanau (1995). A three-base deletion removing a leucine residue in a leucine-rich repeat of a platelet glycoprotein Ib α associated with a variant of Bernard-Soulier syndrome. *British Journal of Haematology* **89**: 386-396

Sambrook J., T. Maniatis, E. Fritish, (1992). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor Laboratory, New York, NY) pp 280

Shattil S.J., L. F. Brass (1987). Induction of fibrinogen receptor on human platelets by intracellular mediators. *J Biol Chem* **262**: 992.

Shogren R., T. A. Gerken, N. Jentoft (1989). Role of glycosylation on the conformation and chain dimensions of O-linked glycoproteins: light-scattering studies of ovine submaxillary mucin. *Biochemistry* **28**: 5525-553

Simsek S., P. Noris, M. Lozano, M. Pico, A. E. G. Kr.Von Dem Borne, A. Ribera, D.Gallardo (1994c). Cys209 Ser mutation in the platelet membrane glycoprotein Ib α gene is associated with Bernard-Soulier syndrome. *British Journal of Haematology* **88**: 839-844

Simsek, S., L. G. Admiral, P. W. Modderman, C. E. Van der Schoot, and A. E. G. Kr. von der Borne (1994a). Identification of a homozygous single base pair deletion in the gene coding for the human platelet glycoprotein Ib α causing Bernard-Soulier syndrome. *Thromb Haemost* **72** (3):444-449.

Simsek S., P. M. M. Bleeker, C. E. Van der Schoot, A. E. G. Kr.Von dem Borne (1994b). Association of a variable number of tandem repeats (VNTR) in glycoprotein Ib α and HPA-2 alloantigens. *Thrombosis and Haemostasis* **72** (5): 757-761.

Smith, A. (1991). DNA sequence analysis by primed synthesis. *Methods in Enzymology* **65**: 560-581.

Smith T. F., M. S. Waterman (1983). Comparison of bio-sequences. *Advances in applied mathematics* **2**: 482-489.

Snyder F. (1989). Biochemistry of platelet activating factor: a unique class of biologically active phospholipids. *Proc Soc Exp Biol Med* **190**: 125

Sorrentino, R. ,C. Iannicola ,S. Costanzi, A. Chersi, and R. Tosi (1991). Detection of complex alleles by direct analysis of DNA heteroduplexes. *Immunogenetics* **33**: 118-123.

Tsuji T., S. Tsunehisa, Y. Watanabe (1983). The carbohydrate moiety of human platelet glycocalcin. The structure of the major SER/ THR-linked chain. *J Biol Chem* **285**: 6335- 6339

Vicente V., R. A. Houghten, Z. M. Ruggeri (1990). Identification of a site in the A chain of platelet glycoprotein Ib that participate in von Willebrand factor binding. *J Biol Chem* **265**: 274-280

Ware J., S. R. Russell, P. Marchese, M. Murata, M. Mazzucato, L. D. Marco (1993a). Point mutation in a leucine-rich repeat of platelet glycoprotein Ib- α resulting in the Bernard-Soulier syndrome. *J.Clin.Invest.* **92**: 1213-1220

Ware, J., SR. Russell, V. Vincente, RE.Scharf, A. Tomer, R. McMillan, Z. M. Rugerri (1990). Nonsense mutation in the glycoprotein Ib α coding sequence associated with Bernard-Soulier Syndrome. *Proc. Natl. Acad. Sci (USA)* **87**:2026-2030

Ware, J., S. Russel, P. Marchese, M. Mazzucato, L. De Marco (1993b). Point Mutation in a Leucine-rich Repeat of Platelet GP Ib α resulting in the Bernard Soulier Syndrome. *J. Clin. Invest.* **92**: 1213-1218.

weiss H. J., V. Turrito (1979). Prostacyclin (prostaglandin I₂, PG I₂) inhibits platelet adhesion and thrombus formation on subendothelium. *Blood* **53**: 244- 250

Weiss H. J., V. T. Turrito, H. R. Baumgartner (1978). Effect of shear rate on platelet interaction with subendothelium in citrated and native blood. I. shear rate-dependent decrease of adhesion in von Willebrand's disease and the Bernard-Soulier syndrome. *J Lab Clin Med* **92**: 750- 764

Weiss H., V. Turitto, H. Baumgartner (1986). Platelet adhesion and thrombus formation on subendothelium in platelets deficient in glycoproteins Iib-IIIa, Ib and storage granules. *Blood* **67**: 322-327

Wencel-Drake J. D., B. Dahlback, M. H. Ginsberg (1986). Ultrastructural localization of coagulation factor V in human platelets. *Blood* **68**: 244

Wenger R., A. Wicki, N. Kieffer (1989) the 5' flanking region and chromosomal localization of the gene encoding human platelet membrane glycoprotein Ib alpha gene. *85*: 517-524

Wenger, R. , N. Kieffer, A. Wicki, and K. Clemetson (1988). Structure of human blood platelet membrane glycoprotein Ib α gene. *Biochemical and Biophysical Research Communications.* **156** (1):,389-395.

White J., S. Burris, D. Hasegawa, M. Johnson (1984). Micropipette aspiration of human blood platelets, a defect in Bernard-Soulier Syndrome. *Blood* **63**: 1249

Wicki, A. , and K. Clemetson.(1985) Structure and function of platelet membrane glycoprotein Ib and V effects of leukocyte elastase and other proteins on platelets response to von Willebrand factor and thrombin. *Eur. J. Biochm.* **153**: 1-11

Wright, S. K. Michaelides, D. J. D. Johnson, N. C. West, and E. G. D. Tuddenham. (1993) Double heterozygosity for mutations in the platelet glycoprotein IX gene in three siblings with Bernard-Soulier Syndrome. *Blood* **81**: (9):2339-2347