Clinical and genetic aspects of Bernard-Soulier syndrome: searching for genotype/phenotype correlations

Anna Savoia,[1,2] Annalisa Pastore,[3] Daniela De Rocco,[2] Elisa Civaschi,[4] Mariateresa Di Stazio,[1] Roberta Bottega,[2] Federica Melazzini,[4] Valeria Bozzi,[4] Alessandro Pecci,[4] Silvana Magrin,[5] Carlo L. Balduini,[4] and Patrizia Noris[4]

[1]Department of Reproductive and Developmental Sciences and Public Medicine Sciences, University of Trieste, Trieste, Italy; [2]Laboratory of Genetics, Institute for Maternal and Child Health - IRCCS "Burlo Garofolo" - Trieste, Italy; [3]MRC National Institute for Medical Research, London, UK; [4]Department of Internal Medicine, University of Pavia and IRCCS Policlinico San Matteo Foundation, Pavia, Italy; and [5]Division of Hematology and BMT Unit, "V. Cervello" Hospital, Palermo, Italy

Funding: this work was supported by research grants from the Italian Ministry of Education, University and Research (PRIN 2008), IRCCS Burlo Garofolo (Grant: Ricerca Corrente 34/09), and Italian ISS (Istituto Superiore di Sanità; Grant: Italian/USA Rare Diseases).

Acknowledgment: AP was funded by MRC grant U117584256.

Manuscript received on September 21, 2010. Revised version arrived on November 17, 2010. Manuscript accepted on December 10, 2010.

Correspondence: Anna Savoia, Laboratory of Genetics, Institute for Maternal and Child Health - IRCCS "Burlo Garofolo", University of Trieste, via dell'Istria 65/1, 34137 Trieste, Italy. E-mail: savoia@burlo.trieste.it

The online version of this article has a Supplementary Appendix.

ABSTRACT

Background

Bernard-Soulier syndrome is a severe bleeding disease due to a defect of GPIb/IX/V, a platelet complex that binds the von Willebrand factor. Due to the rarity of the disease, there are reports only on a few cases compromising any attempt to establish correlations between genotype and phenotype. In order to identify any associations, we describe the largest case series ever reported, which was evaluated systematically at the same center.

Design and Methods

Thirteen patients with the disease and seven obligate carriers were enrolled. We collected clinical aspects and determined platelet features, including number and size, expression of membrane glycoproteins, and ristocetin induced platelet aggregation. Mutations were identified by direct sequencing of the *GP1BA*, *GP1BB*, and *GP9* genes and their effect was shown by molecular modeling analyses.

Results

Patients all had a moderate thrombocytopenia with giant platelets and a bleeding tendency whose severity varied among individuals. Consistent with expression levels of GPIbα always lower than 10% of control values, platelet aggregation was absent or severely reduced. Homozygous mutations were identified in the *GP1BA*, *GP1BB* and *GP9* genes; six were novel alterations expected to destabilize the conformation of the respective protein. Except for obligate carriers of a *GP9* mutation with a reduced GPIb/IX/V expression and defective aggregation, all the other carriers had no obvious anomalies.

Conclusions

Regardless of mutations identified, the patients' bleeding diathesis did not correlate with thrombocytopenia, which was always moderate, and platelet GPIbα expression, which was always severely impaired. Obligate carriers had features similar to controls though their GPIb/IX/V expression showed discrepancies. Aware of the limitations of our cohort, we cannot define any correlations. However, further investigations should be encouraged to better understand the causes of this rare and underestimated disease.

Key words: Bernard-Soulier syndrome, macrothrombocytopenia, GP1BA, GP1BB and GP9 mutations.

Citation: Savoia A, Pastore A, De Rocco D, Civaschi E, Di Stazio M, Bottega R, Melazzini F, Bozzi V, Pecci A, Magrin S, Balduini CL, and Noris P. Clinical and genetic aspects of Bernard-Soulier syndrome: searching for genotype/phenotype correlations. Haematologica 2011;96(3):417-423. doi:10.3324/haematol.2010.032631

Introduction

Although first described more than 60 years ago,[1] Bernard-Soulier syndrome (BSS) is an extremely rare inherited bleeding disorder the understanding of which is still evolving. It is characterized by the absence of a major carbohydrate-containing protein complex on the platelet surface[2] which results in a severe deficiency of four glycoproteins (GP): GPIbα, GPIbβ, GPIX, and GPV.[3,4] These polypeptides associate in the ratio 2:2:2:1 on the platelet membrane to form the GPIb/IX/V complex which is assembled in the endoplasmic reticulum and the Golgi apparatus before localizing on the surface.[5,6] The presence of GPIb/IX/V is essential for hemostasis, as the extracellular domain of GPIbα binds to the subendothelial von Willebrand factor that is exposed at vascular injury sites.

Since the first mutation was detected,[7] over 50 different alterations, missense, nonsense or frame shift mutations have been identified in *GP1BA*, *GP1BB*, and *GP9*, the genes encoding for the GPIbα, GPIbβ, and GPIX subunits, respectively.[8-13] Most of the mutations prevent the coordinated association of the complex, resulting in very low expression of GPIb/IX/V itself on the platelet membrane.[6] In other cases, the complex is normal or slightly decreased but unable to bind the von Willebrand factor.

Bernard-Soulier syndrome is usually transmitted as a recessive trait with giant platelets and severe bleeding tendency. However, there are families with dominant forms in which the affected individuals have only moderate thrombocytopenia and bleeding tendency, as well as an increased mean platelet volume.[11,14,15] In these patients, specific mutations of *GP1BA*, such as p.Leu73Phe, p.Ala156Val and p.Asn41His, allow GPIb/IX/V to correctly assemble and localize on the platelet membrane but prevent GPIbα from binding the von Willebrand factor.

Therefore, Bernard-Soulier syndrome is a complex disease that may be induced by monoallelic or biallelic mutations affecting different components of the GPIb/IX/V complex and resulting in quantitative defects and/or in a complete or partial misfunctioning of the von Willebrand factor receptor. Consistent with genetic heterogeneity, its phenotype is highly variable and has not been clarified likely because the rarity of the disease prevents the collection of cohorts of homogeneously investigated patients. Since a correct definition of the clinical and laboratory spectrum, together with accurate genotype/phenotype correlation studies, remains essential for understanding the molecular basis of the disease and managing patients appropriately, we describe clinical, biological and genetic features of the largest case series ever reported consisting of 13 patients observed during the last 15 years at our clinical institution.

Design and Methods

Patients

From 1995 to 2010 we diagnosed Bernard-Soulier syndrome in 13 patients with a lifelong bleeding disorder from ten unrelated families. Of the ten families, eight were from Italy and the oth-

Table 1. Clinical findings of 13 BSS patients from ten unrelated families.

Family	Patient (gender)	Age at diagnosis (years)	WHO bleeding scale[a]	Bleeding manifestation other than petechiae, ecchymoses, epistaxis, and gum bleeding	Therapy
F1	P1 (F)	50	4	Menorrhagia	Estroprogestinics, nasal packing, iron therapy, red cell and platelet transfusions
F2	P2 (F)	15	4	Menorrhagia	Estroprogestinics, nasal packing, iron therapy, platelet transfusions
F3	P3 (F)	30	4	Unrestrainable menorrhagia	Estroprogestinics, uterine tamponade, nasal packing, iron therapy, red cell and platelet transfusions
F4	P4 (M)	5	2	None	None
	P5 (F)	10	2	None	None
F5	P6 (M)	34	2	None	None
F6	P7 (M)	33	2	None	Steroids
	P8 (M)	35	2	None	Steroids
F7	P9 (F)[b]	8	4	Brain hemorrhage	Platelet transfusion with development of GPIb isoantibodies and refractoriness, hematopoietic stem-cell transplantation
	P10 (F)	7	3	None	Hematopoietic stem-cell transplantation
F8	P11 (F)	18	4	Menorrhagia, hemoperitoneum	Estroprogestinics, iron therapy, red cell and platelet transfusions
F9	P12 (F)	45	2	Menorrhagia	Steroids, intravenous immunoglobulins, splenectomy and removal of accessory spleen
F10	P13 (M)	51	4	Repeated gastrointestinal bleedings, retinal hemorrhage	Steroids, recombinant factor VIIa, splenectomy argon plasma coagulation of intestinal angiodysplasia, platelet tranfusions and refractoriness

[a]*WHO (World Health Organization) grade 0, no bleeding; grade 1, petechiae; grade 2, mild blood loss; grade 3, gross blood loss; grade 4, debilitating blood loss.* [b]*Another brother not included in this study developed isoantibodies after platelet transfusion and died of brain hemorrhage.*

ers (F4 and F5) from India (Table 1). We also examined 7 parents from five independent families. The investigated subjects gave informed consent to the studies, which were carried out in accordance with the Principles of the Declaration of Helsinki. The study was approved by the Ethical Committee of IRCCS San Matteo of Pavia, Italy.

Platelet analyses

Manual platelet counting

Whole blood platelet counts were performed on ethylene-diamine-tetra-acetic acid anticoagulated blood. After blood dilution in ammonium oxalate solution, the counting procedure was performed by optical microscopy in a Neubauer chamber as indicated by the International Committee for Standardization in Hematology.[16]

Platelet count and mean platelet volume by cell counter

Ethylene-diamine-tetra-acetic acid anticoagulated blood samples were analyzed for platelet count and mean platelet volume within two hours from sampling by the Sysmex XE-2100® (Sysmex Corporation, Kobe, Japan) which analyzes platelets by the impedance method. Instrument settings were those routinely used for blood cell counts according to the manufacturer's instructions.

Platelet diameters

Platelet diameters were measured by optical microscopy on May-Grünwald-Giemsa stained peripheral blood films and software-assisted image analysis (Axio-vision 4.5®, Carl Zeiss, Göttingen, Germany) as previously reported.[17]

Platelet aggregation

In vitro platelet aggregation was studied in citrated platelet rich plasma by the method of Born as previously reported.[15] To minimize the loss of denser platelets, the platelet rich plasma was obtained by sedimentation of blood for 20-30 min. Platelet agonists were: collagen 4 μg/mL (Mascia Brunelli, Milan, Italy), adenosine diphosphate 5 and 20 μM, and ristocetin 1.5 and 3 mg/mL (both from Sigma Chemical Co, St. Louis, MO, USA).

Flow cytometry

The expression of platelet membrane glycoproteins was investigated in platelet rich plasma by flow cytometry with an Epics XL flow cytometer (Coulter) as previously reported.[15] The following monoclonal antibodies were used: AP2 (Immunotech SA, Marseille, France), which recognizes GPIIb/IIIa (CD41/61); MB45 (CLB, Amsterdam, The Netherlands) and SZ2 (Immunotech) against GPIbα (CD42b); SZ1 (Immunotech) which recognizes GPIX (CD42a), and SW16 (CLB) against GPV (CD42d). MO2 (Coulter, Miami, FL, USA) was used as the negative control. Fluorescein isothiocyanate-conjugated goat anti-mouse antibodies (GAM-FITC) were also purchased from Coulter. Ten thousand platelet events were collected and the value of mean fluorescence, expressed in arbitrary units, was recorded. A sample from a healthy donor (the same since the beginning of this study) was run with the patients' samples as a control.

Mutation screening

The *GP1BA*, *GP1BB* and *GP9* genes were screened for mutations using genomic DNA samples from probands. Genomic fragments were analyzed by polymerase chain reaction using primers designed specifically to amplify the coding regions (the primer sequences are available upon request). Amplification reactions were carried out in a final volume of 35 μL containing 100-200 ng of genomic DNA, 2 units of TaqGold DNA polymerase (Applied Biosystem) under the following conditions: 95°C for 12 min followed by 30 cycles of 95°C for 40 sec, 58-64°C for 45 sec and 72°C for 45 sec, then final extensions of 72°C for 7 min. Polymerase chain reaction fragments were sequenced using the BigDye Terminator cycle Sequencing V3.1 (Applied Biosystems) and analyzed on an ABI PRISM 3130xl (Applied Biosystems). The same primer pairs were used for amplification and sequencing.

Bioinformatic analysis

Sequence analysis was supported by the FASTA (*http://www.ebi.ac.uk/Tools/fasta/index.html*), PFAM (*http://pfam.janelia.org/*) and SMART (*http://smart.embl-heidelberg.de/*) webservers. Structural analysis was based on the crystal structure of *GP1BA* available from the Protein Data Bank

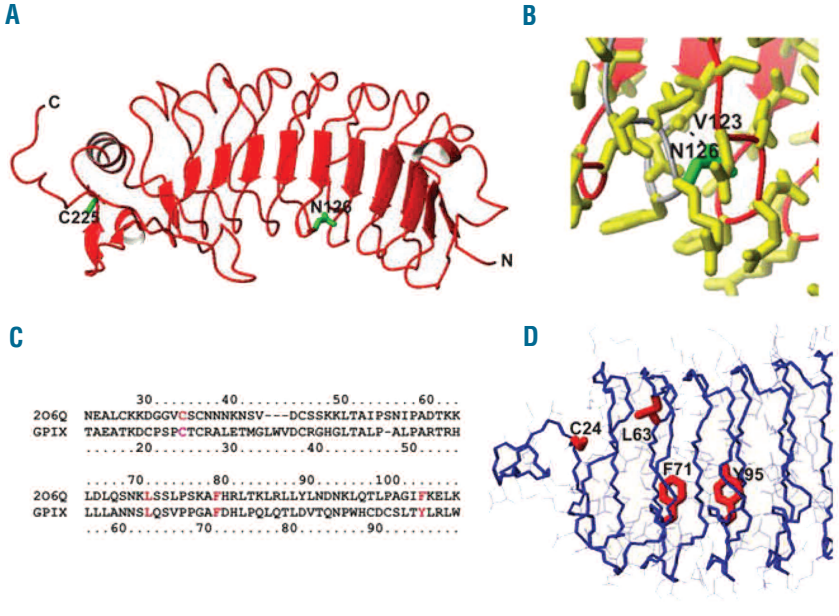

Figure 1. Effect of missense mutations on protein folding. (A, B) Ribbon representation of GPIbα (entry name 1GWB). (A) The side chains of Asn126 and Cys225 are indicated, as well as the positions of the N- and C-termini. (B) Close-up of the region around Asn126 to show the environment and its involvement in hydrogen bonds. A linear fog effect is applied to give a 3D impression. (C, D) Modeling of GPIX based on the coordinates of 2O6Q. (C) Alignment of the relevant region of the template with the sequence of GPIX. The positions that carry the observed mutations are marked in red. (D) Mapping the mutations into the structure using a thicker bond radius for the positions mutated.

(accession name 1GWB). Modeling of GPIX was achieved using the structure 2O6Q as a template.

Results

Variable expressivity of clinical features in BSS patients

We studied 13 patients diagnosed as having Bernard-Soulier syndrome from ten unrelated families (Table 1). Mean age at diagnosis was 26 years (range 5-51 years). Study subjects were 5 males and 8 females. Having been misdiagnosed with autoimmune thrombocytopenia, 4 patients (P7, P8, P12, and P13) had been previously treated with intravenous immunoglobulins and/or steroids, and/or splenectomized without receiving any benefit. Bleeding diathesis measured by the World Health Organization bleeding scale (grade 0, no bleeding; grade 1, petechiae; grade 2, mild blood loss; grade 3, gross blood loss; grade 4, debilitating blood loss) ranged from 2 to 4 (Table 1). All subjects had petechiae and suffered from recurrent ecchymoses, epistaxis, and gum bleeding. All menstruated women had a menorrhagia that required estroprogestinic treatment in 4 cases (P1, P2, P3, and P11) whereas menorrhagia was well tolerated in one (P12). In 3 patients, there were life-threatening, acute bleeding episodes, such as unstoppable menorrhagia requiring uterine tamponade (P3), brain hemorrhage (P9), and hemoperitoneum (P11). Five patients, all with the most severe bleeding score, received platelet transfusions to arrest bleeding episodes or as pre-surgical procedure, whereas 2 brothers (P9 and P10) underwent hematopoietic stem-cell transplantation from HLA-identical related donors, which cured their illness.[18] In addition to Bernard-Soulier syndrome cases, 7 obligate carriers from families 2, 3, 4, 5 and 7 were also included in the study (Table 2). They were asymptomatic without bleeding tendency.

Platelet count, size, and aggregation

All patients had platelet counts ranging from 34 to 82×10^9/L as determined by microscope technique (Table 2). Thrombocytopenia was always more severe when platelets were measured by cell counter. This observation confirms that the impedance counter is not reliable in macrothrombocytopenia because it does not recognize

Table 2. Platelet, GPIb/IX/V complex, and mutational features of 13 BSS patients and 7 obligate carriers.

Family	Individuals studied[a]	Platelet count mean (x10^9/L) (range)		Platelet diameter mean[b] (μm)	RIPA mean (% of control) (range)	Fluorescence intensity mean (% of control) (range)[c]			Mutational screening (range)		
		Microscope	Cell counter			GPIbα	GPIX	GPV	cDNA[d]	Protein[e]	Gene
F1	P1	58	23	3.8	7	7	18	7	c.376A>G[f]	p.Asn126Asp (p.Asn110Asp)	*GP1BA*
F2	P2	82	67	4.0	10	4	23	26	c.673T>A	p.Cys225Ser (p.Cys209Ser)	
	C1	268	287	3.1	97	102	78	91			
F3	P3	40	34	3.7	0	7	3	5	c.491dupA[f]	p.His164GlnfsX145	*GP1BB*
	C2	286	256	3.1	100	NA	NA	NA			
F4, F5	P4, P5, P6	57 (48-72)	47 (23-86)	4.1 (3.7-4.5)	8 (0-11)	5 (1-7)	6 (1-16)	18 (1-28)	c.70T>C	p.Cys24Arg (p.Cys8Arg)	
	C3, C4, C5	273 (208-388)	262 (202-380)	3.1 (2.8-3.4)	81 (71-94)	97 (70-108)	93 (68-108)	103 (71-123)			
F6	P7, P8	45 (38-52)	12 (6-18)	4.1 (4.0-4.2)	9 (7-11)	5 (3-7)	5 (4-7)	33 (30-36)	c.72T>G[f]	p.Cys24Trp (p.Cys8Trp)	*GP9*
F7	P9, P10	NA	37 (6-68)	4.1 (4.10-4.13)	2 (0-4)	2 (2-2)	6 (5-7)	8 (7-10)	c.188T>C[f]	p.Leu63Pro (p.Leu47Pro)	
	C6, C7	NA	182 (151-214)	2.8 (2.6-3.0)	29 (14-45)	47 (37-57)	53 (36-68)	53 (42-64)			
F8	P11	34	34	4.6	n.a.	9	5	9	c.212T>C	p.Phe71Ser (p.Phe55Ser)	
F9	P12	42	12	5.7	0	5	9	9	c.284A>G[f]	p.Tyr95Cys (p.Tyr79Cys)	
F10	P13	NA	47	4.6	0	10	0	NA	c.442dupG[f]	p.Val148GlyfsX67	

[a]P, BSS patients; C, obligate carriers. [b]Values obtained in 40 healthy subjects: mean 2.4 μm (range 1.9-3.4 μm). [c]RIPA: ristocetin induced platelet aggregation. Reported data describe aggregation induced by 3 mg/mL ristocetin in patients and by 1.5 mg/mL in family members. [d]Nucleotide A of the ATG translation initiation start site of the GP1BA *(GeneBank NM_000407.4),* GP1BB *(GeneBank NM_000407.4) and* GP9 *(GenBank NM_000174.3) cDNAs is indicated as nucleotide +1. eAmino acid positions of precursor glycoproteins. In brackets, amino acid position of mature forms without signal peptides, which are the first 16 residues for GPIbα and GPIX and the first 25 residues for GPIbβ. fNovel mutations.*

and enumerate very large platelets. Normal platelet counts ranging from 151 to 380×10⁹/L were instead observed in all 7 carriers.

Regarding platelet size, in none of the patients did the impedance instrument report the mean volume of platelets because of their abnormality. As determined in peripheral blood films, platelet mean diameter was larger than in controls varying from 3.7 to 5.7 μm (Table 2). In the obligate carriers, diameters were in the upper part of the normal range (Table 2).

Unaltered response of platelets was observed after adenosine diphosphate and collagen stimulation in both patients and carriers (*data not shown*). Ristocetin induced platelet aggregation using a dose of 3 mg/ml was absent or very low in all patients (Table 2). Aggregation induced by 1.5 mg/ml of ristocetin was reduced to 14-45 % in the parents of family 7, whereas it was normal in the remaining obligate carriers.

Identification of homozygous mutations in GP1BA, GPIBB, *and* GP9 *genes*

In order to determine the causative mutations, the *GP1BA*, *GP1BB*, and *GP9* genes were analyzed, allowing us to identify homozygous mutations in all families (Table 2). Segregation analysis confirmed the presence of heterozygous mutations in all available carriers. Of the eight alterations, six were novel mutations, including two frameshift duplications c.491dupA and c.442dupG in *GP1BB* and *GP9*, respectively and four amino acid substitutions, p.Asn126Asp in *GP1BA* and p.Cys24Trp, p.Leu63Pro, and p.Tyr95Cys in *GP9*. Of note, mutations at Cys24 corresponded to different substitutions: in families F4 and F5, as previously reported,[19] we observed a c.70T>C substitution which corresponds to a p.Cys24Arg substitution whereas in family F6 we identified the 70T>C mutation, which leads to p.Cys24Trp. All four amino acid substitutions affect residues that are well conserved during evolution (*Online Supplementary Figures S1 and S2*).

Platelet surface expression of GPIbα is always severely affected

As evaluated by flow cytometry, the GPIbα, GPIX and GPV expression on platelet membrane was reduced in all Bernard-Soulier syndrome patients. In particular, GPIbα was always less than 10% of the control value. GPIX was always hardly detectable in patients with mutations in the *GP9* or *GP1BB* genes. It was instead reduced to 18% or 23% when *GP1BA* was mutated. The expression of GPV was more variable, ranging from 5 to 33%. The GPIb/IX/V complex was normal or only slightly reduced in the 5 parents from families F2, F3, F4 and F5 (Table 2). It was clearly reduced in the 2 parents of family F7 carrying the p.Leu63Pro mutation, who also had impaired platelet aggregation after low-dose ristocetin.

Missense mutations are predicted to destabilize protein fold

To define the structural impact of the missense mutations identified in the patients, the role of the amino acid substitutions in GPIbα and GPIX was analyzed respectively using the crystal structure available in the Protein Data Bank (accession name 1GWB) and a model built by homology.

GPIbα is the subunit disulfide linked to GP1bβ. Its N-terminal 282 residues form a domain and contain binding sites for the von Willebrand factor and other factors involved in hemostasis.[5,20] This region consists of an N-terminal β-hairpin corresponding to residues 18-34 (we are adopting hereafter the 1GWB numbering; it is necessary to substract 16 to adhere to the amino acid numbering used by Uff *et al.*[20]) containing the Cys20–Cys33 disulfide, eight leucine rich repeats each consisting of ca. 24-residues (35–220), a di-sulphide knot structure (221–280) with the Cys225–Cys264 and Cys227–Cys280 disulfides, and an anionic sequence (285–298) with three sulfated tyrosines (292/294/295) (Figure 1A).[20] Of the mutations found in this subunit, the p.Asn126Asp substitution affects a position that corresponds to one well conserved residue of the leucine rich repeat consensus sequence (LxxLxLxxNxL). This asparagine is present in all leucine rich repeats of GPIbα but one, where it is substituted by a cysteine (Cys65), and in the leucine rich repeats of the other GPIb/IX/V subunits, suggesting its important role in protein structure. Indeed, in the crystal structure (1GWB), Asn110 is completely buried (13 Å exposed surface area) with the side chain pointing towards the hydrophobic core despite its hydrophilic character (Figure 1B). It could form a bifurcated hydrogen bond with the backbone carbonyls of Val123 (amide nitrogen to the oxygen distance 2.72 Å) and Ser124 (2.99 Å). Substitution of the residue into a charged group would require an even higher energetic cost to bury side chain, thus destabilizing the fold of the glycoprotein. The p.Cys225Ser mutation identified in family F2 and previously reported in another 2 cases[21,22] affects the Cys225–Cys264 disulfide, leading to a strong destabilizing effect on the fold.

Although the crystal structure of glycoprotein GPIX is not available, different structures in the PDB database have a significant sequence homology as detected by the FASTA server. A SMART search identifies leucine rich repeat motifs at the N- and C-termini between residues 19-55 and 85-136, respectively. GPIX also contains a signal peptide between residues 1 and 16 and a transmembrane sequence in the region 147-169. We used the structure 2O6Q as the template for modeling GPIX and rationalized the effects of the mutations. The two sequences align well with only a three residue deletion and one residue insertion in the region relevant for the mutations (Figure 1C). These affect residues Cys24, Leu63, Phe71 and Tyr95 of GPIX that correspond to residues Cys34, Leu71, Phe79 and Phe103 of the template. All four residues are buried in the hydrophobic core (Figure 1D) with exposed surface areas around between 0-2 Å.

Cys24 is in the leucine rich repeat motifs at the N-terminus and is the second cysteine of the CPXPCXC motif. Substitution with the bulky arginine or tryptophan can only strongly destabilize the fold. It also directly faces another Cys (38 in GPIX and 45 in 2O6Q) with which it could form a disulfide bridge. Leu63 is in the first leucine rich repeat motif and is surrounded by other hydrophobic residues with which it packs. It is in the loop between the strand and the so-called variable region and has the important function of allowing inversion of the chain direction. Substitution of this residue into a proline will restrict the conformational space and force a conformation that might be incompatible with loop formation, strongly interfering with protein fold. This mutation should have particularly severe effects on the protein stability. Phe71 and Tyr95 are in corresponding positions of the leucine rich repeat. Although not entirely conserved, these positions are often

occupied by aromatics and should pack against the rest of the protein. Their mutations into serine and cysteine, respectively, would introduce a hole in the structure and again have a destabilizing structural role.

Discussion

In this study we evaluated the largest cohort ever reported of 13 Bernard-Soulier syndrome patients, all studied at the same clinical unit, where their analysis is consistent and homogeneous. In all families we identified homozygous mutations of the *GP1BA*, *GP1BB* or *GP9* genes: frameshift duplications and seven missense mutations, only one being common to two families. Six of these are novel mutations, which impair the folding of the respective glycoproteins, as suggested by the molecular modeling studies.

The vast majority of the cases reported so far are homozygous for the same mutated allele inherited from parents, who are likely to have common ancestors. In addition to the 4 Italian cases previously reported,[23-26] those described in the present study are the only families identified in our country. This is in agreement with the view that Bernard-Soulier syndrome is an extremely rare disease although there is no doubt that its prevalence has largely been underestimated due to underreporting and/or misdiagnosis. Erroneous diagnoses of immune thrombocytopenic purpura were made, for instance, in 4 of our patients, including 2 brothers, which led to futile administration of corticosteroids and/or intravenus gamma globulin infusion and/or splenectomy. Because of these and other unfortunate misdiagnoses, Bernard-Soulier syndrome should always be considered in patients with macrothrombocytopenia whenever it cannot be excluded that they had low platelet count since birth.

Regardless of which gene is mutated, our patients all shared similar platelet features. Thrombocytopenia was never extreme, with platelet counts always higher than 34×10^9/L. Platelet diameters were much larger than normal and platelet aggregation induced by ristocetin was greatly impaired. Of note, bleeding diathesis was more severe than expected from platelet counts, and its severity varied considerably among patients without any apparent association with their platelet count. In particular, half of the patients had a bleeding tendency that exposed them to the risk of death and/or severely (grade 3 or 4) affected their quality of life, requiring platelet transfusions or hemopoietic stem-cell transplantation. The 3 patients with alterations of the *GP1BA* or *GP1BB* genes, as well as those with the p.Leu63Pro, p.Phe71Ser and p.Val148GlyfsX67 mutations of *GP9*, belong to this group. Consistent with our data, the cases previously reported to have the same mutations as in this group (p.Cys225Ser of *GP1BA* and p.Phe71Ser of *GP9*) were associated with a severe phenotype.[21,25,27] The other half of patients had a mild bleeding tendency (grade 2) without any additional manifestations to petechiae, ecchymoses, epistaxis and gum bleeding, which did not necessitate any particular interventions. These patients had mutations of the *GP9* gene affecting residue 24 (p.Cys24Arg and p.Cys24Trp) in 5 cases or residue 95 (p.Tyr95Cys) in one case. Of note, the only known mutation among this group was p.Cys24Arg, which was identified in a patient with mild clinical manifestations.[19]

Having excluded a potential correlation with platelet count, the bleeding severity does not even associate with the expression of the GPIb/IX/V receptor. When genes *GP1BA*, *GP1BB* or *GP9* are mutated, in the majority of cases the complex does not assemble in the endoplasmic reticulum and does not localize in the membrane.[5,6] Indeed, the expression level of GPIbα was less than 10% of the controls or hardly detectable in any of our patients regardless of their genetic profile. Aware that flow cytometry is not sensitive enough to determine low quantities of antigen, we cannot regard these conclusions as definitive and further studies using Western blot analysis are required before excluding any correlation between phenotype and glycoprotein expression on platelet membrane.

Concerning obligate carriers, all of them had similar clinical findings, with no bleeding diathesis, normal platelet counts and platelet diameters in the upper part of the normal range. Their GPIb/IX/V expression level was reduced to nearly 50% of the control value only in subjects with the p.Leu63Pro mutation (GPIX). Contrary to expectation, it was at the normal levels or partially reduced in carriers with p.Cys225Ser and p.Cys24Arg of the GPIbα and GPIX subunits, respectively. Such a discrepancy had already been observed previously, being the expression in obligate carriers with p.Cys225Ser (GPIbα) or p.Cys24Arg (GPIX) normal (or slightly reduced)[19,21] and close to 50% of controls in other cases.[7,28,29] We cannot explain these observations and any hypothesis would, at this time, be very speculative. As a matter of fact, we do not know the molecular mechanisms regulating the expression levels of the GPIb/IX/V complex in healthy subjects or how the mutations affect stability and function of its subunits. This requires further investigation to improve our knowledge of this aspect.

In the obligate carriers, the expression levels of GPIbα correlate with platelet response to ristocetin, being normal in subjects with normal amounts of GPIb/IX/V complex and reduced in those with a clear reduction of the complex. These observations are consistent with data from velocardiofacial syndrome, where microdeletions of chromosome 22q11 removing *GP1BB* associate with a 50% reduction of the GPIb/V/IX expression and impaired ristocetin-induced platelet aggregation.[30]

In conclusion, since we know that bleeding tendency is mediated by a multitude of factors, both genetic and environmental, we believe that our study sets a reference for further understanding the molecular bases of a rare but severe disease such as Bernard-Soulier syndrome. More work will be needed to further assess a correlation between mutations and phenotype, which would represent an important tool in patient management.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

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