Novel point mutation in a leucine-rich repeat of the GPIbα chain of the platelet von Willebrand factor receptor, GPIb/IX/V, resulting in an inherited dominant form of Bernard-Soulier syndrome affecting two unrelated families: the N41H variant

Silvia Vettore,[1] Raffaella Scandellari,[1] Stefano Moro,[2] Anna Maria Lombardi,[1] Margherita Scapin,[1] Maria Luigia Randi,[1] and Fabrizio Fabris[1]

[1]Department of Medical and Surgical Sciences and [2]Molecular Modeling Section, Department of Pharmaceutical Sciences, University of Padova Medical School, Padova, Italy

ABSTRACT

In Italy, a significant proportion of patients with autosomal dominant inheritance of macrothrombocytopenia have been recognized as having heterozygous Bernard-Soulier syndrome carrying the Bolzano-type defect. This condition prompted a systematic review of our outpatients with chronic isolated macrothrombocytopenia. We recognized that the affected members of two unrelated families represented a new variant of heterozygous Bernard-Soulier Syndrome with autosomal dominant inheritance. Sequencing analysis of the GPIbα gene revealed a novel heterozygous mutation, A169C, resulting in an N41H substitution in the protein. This aminoacid belongs to the first leucine-rich repeat of the chain. The molecular modeling suggests that the replacement of the N41 with a histidine (N41H) drastically disturbs the structure of the first portion of GPIbα N-terminal, directly involved in von Willebrand factor binding. As a consequence, platelet aggregation to 1.2 mg/mL of ristocetin is slightly impaired and flow cytometry reveals a reduced binding of monoclonals directed against N-terminal epitopes of the GPIbα.

Key words: glycoprotein Ibα, Bernard-Soulier, congenital thrombocytopenia.

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Introduction

In recent years, we have enrolled several families with hereditary macrothrombocytopenia. The disease was characterized and diagnosed using a diagnostic algorithm proposed by the Italian Platelet Study Group (GPS),[1,2] enabling the different syndromes to be distinguished. Patients who did not fit the above classification have been assigned to the group of chronic isolated macrothrombocytopenia (CHMT) or Mediterranean thrombocytopenia.[3,4] This form included defects with a similar phenotype, i.e. mild or no hemorrhagic diathesis, autosomal dominant inheritance of the trait, mild thrombocytopenia, variable percentages of giant platelets and a variable aggregation response to 1.5 mg/mL ristocetin. Such patients are often wrongly diagnosed as having autoimmune idiopathic thrombocytopenia.[5] Although in most cases the molecular defect remains unknown, a significant percentage of these patients have recently been recognized in Italy as being affected by the Bolzano Bernard-Soulier syndrome (BSS) variant, which is characterized by Ala156Val substitution in the GPIbα protein[6] near the anionic sulfated region of the last leucine-rich repeat (LRR).[7,8] Most of the causative mutations in BSS affect the GPIbα subunit of the platelet membrane glycoprotein Ib/IX/V complex,[9,10] so we performed sequencing analyses on the *GPIbα* gene in the affected members of two families who had previously been classified as CHMT. The study revealed a new mutation in the first LRR motif in the N-terminal domain of GPIbα, where asparagine 41 (one of the best-conserved residues of the LRR consensus sequence) was substituted by a histidine (N41H mutation).

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Correspondence: Fabrizio Fabris, Chair of Internal Medicine, Department of Medical and Surgical Sciences, via Giustiniani 1, 35128 Padova, Italy.
E-mail: fabrizio.fabris@unipd.it
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Design and Methods

The study of the characterization of patients with CHMT[1,3,4] followed the guidelines approved by the local ethical committee (Prot n. 1192P) and patients gave their consensus according to the Helsinki Declaration. Eleven affected (family 1: III-2, III-3, III-12, III-13, IV-10; V-7; family 2: II-3, II-6, III-1, III-4, IV-2) and six unaffected members (Family 1: III-1, III-14, V-8; family 2: III-3, IV-1, IV-3) of two unrelated families from northern Italy were studied (Figure 1). The two propositi presented a life-long history of mild thrombocytopenia (70/100×10^9 plt/L) with 20/30% of giant platelets (diameter >8 μm). No abnormal bleeding was reported with the exception of occasional epistaxis and prolonged bleeding time after major surgery. The other affected members of the two families presented similar histories, with mild macrothrombocytopenia (range: 60-100×10^9 plt/L; 10-40% giant platelets).

Blood samples were collected from healthy controls, patients and relatives in 3.8% Na citrate as anticoagulant for aggregations and flow cytometry studies, and in acid citrate dextrose to wash platelets and obtain platelet lysates.

Ristocetin-induced platelet aggregation (RIPA) at 1.2 mg/mL and 1.5 mg/mL was carried out on platelet rich plasma (PRP) according to the Born method using a Chronolog (Haverton, PA, USA) lumi-aggregometer.[11] Platelet aggregation values were expressed as the maximal percentage difference between the light transmission of PRP (0%) and platelet-poor plasma (100%).

We used flow cytometry to test the following monoclonal antibodies (mAbs) directed against surface platelet glycoproteins: antiCD41a-FITC (clone PM6/248, Serotec, Oxford, UK) against GPIIb/IIIa; anti-CD42a-FITC (clone GR-P, Serotec) against GPIX. Different clones of antiCD42b were used to better characterize the N-terminal region of GPIbα:

- clone SZ2 FITC-conjugated (Immunotech, Marseille, France) recognizing a narrow epitope of GPIbα (Tyr276-Glu282) in the anionic sulfated sequence flanking the LRR region at its C-terminal end.[12] SZ2 monoclonal antibody is currently considered conformation-sensitive for the diagnosis of Bernard-Soulier type Bolzano.[6]
- clone LJ-Ib1, kindly provided by Dr. ZM Ruggeri, recognizes an epitope located between residues His1-Arg293 of the GPIbα chain; its platelet binding is typically absent in BSS Bolzano variant.[7,13]
- clone LJ-Ib10, kindly provided by Dr. ZM Ruggeri, recognizes an epitope located between residues Ala238 and Arg293 of GPIbα.[7,8]
- both AK2 (Dako, Carpinteria, CA, USA) and HIP1 (Pharmingen, San Diego, CA, USA) clones bind the His1-Thr81 fragment of the GPIbαchain,[14,15] but their epitopes seem to be localized exactly on a level with the first LRR, where Asn41 lies (AK2) and in the vicinity of the second LRR (HIP1). Their binding seems to be highly sensitive to Glu40 mutation.[15] All primary mAbs used were coupled with secondary anti-mouse IgG-FITC (Sigma-Aldrich, Saint Louis, MO, USA).

For flow cytometry methods, Western blot, genetics[6,16] and crystallographic analysis details see the *Online Supplementary Appendix*.

Results and Discussion

Using 1.5 mg, RIPA was normal in several members of Family 1 (Figure 1A: propositus III-13, IV-2, IV-10) and Family 2 (Figure 1B: propositus IV-2, III-4), while it was reduced in two members of Family 2 (Figure 1B: II-3, III-1). Using 1.2 mg of ristocetin, RIPA was slightly decreased in both propositi (58% and 60% *vs.* 72±9%; mean±standard deviation of normal controls). By flow-cytometry, mean GPIIb/IIIa (CD41a) and GPIX (CD42a) expression levels were increased in all affected subjects of both families, depending on the size of their larger platelets (Figure 2A). The SZ2 mAb binding rate, recognizing a narrow epitope between Tyr276 and Glu282 of GPIbα, was normal in patients compared with controls suggesting a normal glycoprotein expression.

Conversely, all the other anti- CD42b mAbs clones (LJIb1, LJIb10, AK2, HIP1) directed against the GPIbα N-term appeared to be variously reduced in all affected subjects. It was also confirmed by the ratio between the binding rate of different mAbs versus the expression of CD41.

Western blot analysis of platelet lysates with LJIb10 showed an increased amount of the denaturized GPIbα band in affected subjects (Figure 2B) that seems to correlate with the amount of protein extract load into the gel as indicated by comparison of CD41 intensity.

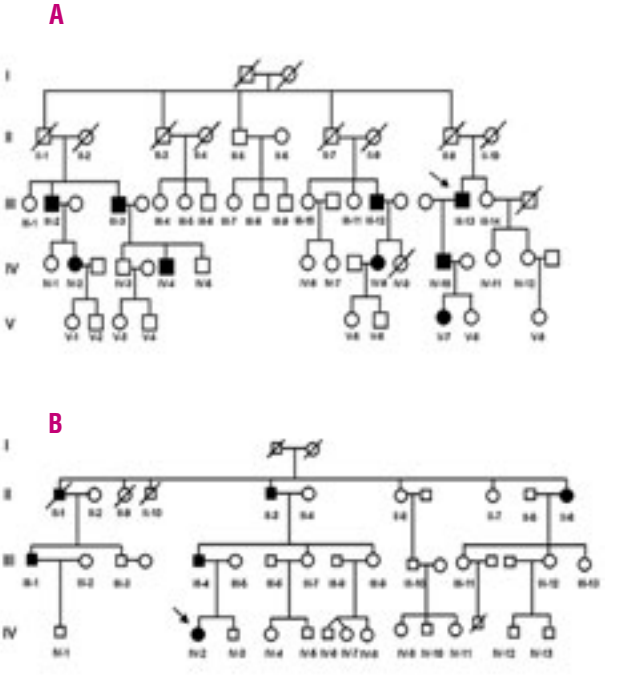
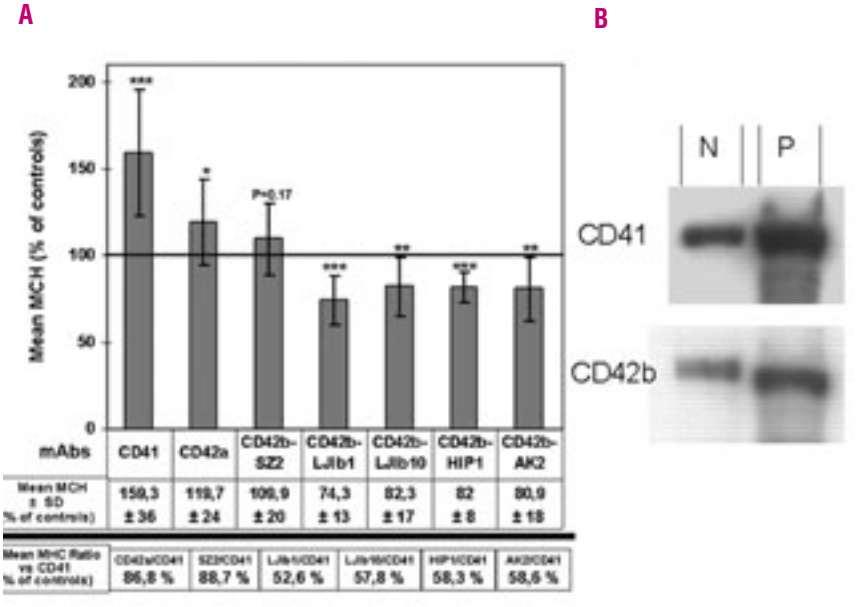


Figure 1. (A) Pedigree of Family 1. (B) Pedigree of Family 2. Affected (black box) and unaffected (white box) members. The arrows indicates the propositi, and the bars the deceased members.

The apparent discrepancy between an impaired binding of N-term specific anti-CD42b mAbs obtained on flow cytometry and the increased amount of GPIbα seen on Western Blot suggested a point mutation occurring in the patients' vWF receptor N-terminal domain. Genetic studies confirmed our hypothesis. In fact, DNA sequencing of the GPIbα gene revealed a heterozygous A>C transversion at the nucleotide +169, resulting in the Asn41His (N41H) substitution in the GPIbα protein sequence (Figure 3A). This mutation, that we named the Padova variant, was found in all affected subjects of both families, and was lacking in all the healthy relatives and the 100 controls sequenced. To our knowledge, this is the first description of such an alteration in



mAbs	CD41	CD42a	CD42b-SZ2	CD42b-LJIb1	CD42b-LJIb10	CD42b-HIP1	CD42b-AK2
Mean MCH ± SD (% of controls)	159,3 ± 36	119,7 ± 24	109,9 ± 20	74,3 ± 13	82,3 ± 17	82 ± 8	80,9 ± 18
Mean MHC Ratio vs CD41 (% of controls)	CD42a/CD41 86,8 %	SZ2/CD41 88,7 %	LJIb1/CD41 52,6 %	LJIb10/CD41 57,8 %	HIP1/CD41 58,3 %	AK2/CD41 58,6 %	

Figure 2A. Flow cytometry results. Binding of mAbs to platelet surface glycoproteins in affected patients of both families. The binding rate is expressed as a percentage of that of normal controls (Mean MCH ± SD). Mean values and standard deviations were obtained from 8 affected patients (III-12; III-13; IV-10; V-7 of Family 1; II-3; III-1; III-4; IV-2 of Family 2), 4 normal controls and 4 unaffected members from both families (Family 1: III-14; V-8. Family 2: III-3; IV-3). Upper data grid. The mean fluorescence intensity obtained for each patient was normalized to normal controls (100%). The black line in the graph highlights 100% binding. The mean of the patients' fluorescence values was compared with that of controls using Student's two-tailed t-test: *=$p< 0.05$; **=$p<0.0098$; ***=$p<0.0001$. Bottom data grid: the Mean MHC value found for each mAb binding rate is reported as ratio versus CD41 MHC mean value. The trend of these normalized percentages confirms the decreased binding of LJIb1, LJIb10, AK2 and Hip1 mAbs.

Figure 2B. Western Blot. Lane N: Normal control. Lane P: N41H Patient. Total lysates from 10^7 platelets were tested with SZ22 mAb for CD41 and LJIb10 for CD42b. The increased amount of CD42b seems to correlate with the amount of protein extract load into the gel as indicated by comparison of CD41 intensity between patients and controls.

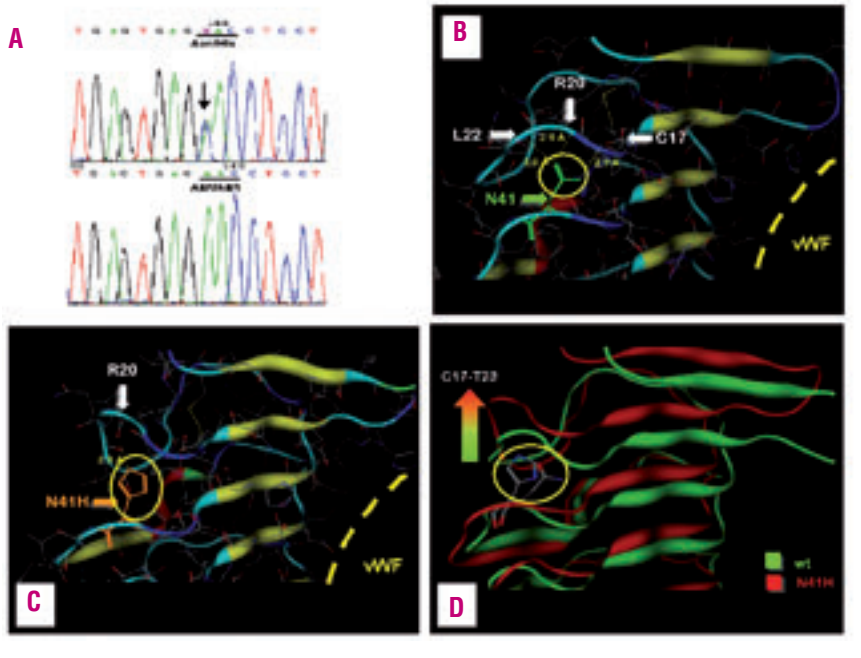


Figure 3. (A) Sequence analysis. Upper analysis. Upper panel: the heterozygous A>C transversion in the patient's DNA leads to the amino acid substitution Asn41His (41H) in the GPIba protein. Lower panel: normal DNA sequence. (B) N-terminal domain of wild-type GPIba. The cluster of stabilizing interactions between asparagine 41 (N41) and the protein backbond of cysteine 17 (C17) arginine 20 (R20), and leucine 22 (L22), observed in the crystallographic data relating to the N-terminal domain of human platelet receptor GPIbα (PDB code: 1P9A). Hydrogen atoms have been deliberately omitted. (C) N-terminal domain of mutated GPIbα. The conformational state of the mutant N41H after 1 nanosecond of molecular dynamics simulation at 300°K only one stabilizing interaction between H41 and R20 is detectable. Hydrogen atoms have been deliberately omitted. (D) Protein variation after N41H mutation. Superimposed conformational states of wild-type (in green) and N41H mutant (in red) after one nanosecond of molecular dynamics simulation at 300°K. The movement of the C17-T23 loop is emphasized. All atoms, except for asparagine 41 in the wild-type and histidine 41 in the N41H mutant (yellow circles), have been deliberately omitted.

BSS patients. The N41H substitution is located in the first LRR, in its sixth position.

The asparagine in the sixth position is one of the conserved residues in the LRR consensus sequence (LXL-SXNXLXXLPXG/LL/FXXLXXLXX). This aminoacid is conserved in all LRR of GPIbα except for the second, where asparagine is substituted by a cysteine, and in all the LRR of other components of the GPIb/V/IX complex.[10]

Moreover, the N in the sixth position is also conserved in other species, being present in the LRR consensus of canine and mouse GPIbα sequences,[17] suggesting that protein sequence integrity in this region is very important. In particular, N41 and E40 residues of GPIbα were subjects of *in vitro* mutation studies in order to determine the N-terminal structure and function of this chain.[14,15] The conserved asparagines are known to form intra-repeat hydrogen bonds, stabilizing the curved solenoid structure belonging to the vWF-binding site. In particular, a small surface of ~900 Å2, including the NH_2-terminal β finger and the first LRR, binds the $\alpha^1\beta^2$, $\beta^3\alpha^2$ and $\alpha^3\beta^4$ loops located on the bottom surface of the A1 vWF domain. Namely, in this context, N41 takes effect through three stabilizing interactions with the protein backbone of cysteine 17 (C17), arginine 20 (R20) and leucine 22 (L22), (Figure 3B). Indeed, molecular dynamics simulations clearly show that the wild-type N-terminal domain is conformationally stable during the trajectory of one nanosecond. Conversely, substitution of asparagine 41 with a histidine (N41H) drastically disturbs the conformational behavior of the first portion of the N-terminal region. The mutant N41H lost two of the three stabilizing interactions (only the interaction with R20 was maintained) during the molecular dynamics simulation for one nanosecond (Figure 3C). As a consequence, the loop defined by asparagine 17 (N17) and threonine 23 (T23) changes position, moving up from N41, and also its conformation (Figure 3D).

One of the GPIbα residues directly involved in binding the vWF A1 domain is H37, very close to the conserved N41 residue mutated in our patients.[18] We might surmise that the substitution could disrupt the environment needed for the proper alignment of H37 with its counterpart, vWF. This would explain the reduced response to ristocetin at 1.2 mg/mL and the diminished binding of LJIb1, LJIb10, AK2 and HIP1 mAbs, recognizing epitopes within the N-terminal of the GPIbα chain. SZ2 mAbs, whose epitope is located far from the mutated region, maintains normal behavior in flow cytometry analysis. We can conclude that N41H mutation could be responsible for the autosomal dominant variant of BSS found in these two unrelated families, and that the pathogenic mechanism is probably related to a modified binding capacity of the vWF receptor, as suggested by the RIPA test, mAbs binding variations and molecular modeling. Nevertheless, the pathological effects of this genetic variant of GPIbα on the functional interaction with the vWF and on megakaryocytopoiesis is beyond the scope of the present paper, and further studies are needed to confirm our hypothesis. However, there is evidence that GPIbα is responsible for platelet formation and morphology. GPIbα -null mice have a platelet phenotype typical of BS syndrome,[19] patients with type IIb-von Willebrand have abnormal proplatelet formation[20] and HIP1 mAb against N-terminal domain of GPIbα strongly inhibits proplatelet formation.[21]

It is worthy of note here that HIP1, like LJIb1, LJIb10, AK2 mAbs, can be considered conformation-sensitive for N41H mutation of CD42b. Their combination with the SZ2 clone, which is not so sensitive to N41H mutation, but is diagnostic for BSS Bolzano variant, may provide a useful tool for screening A169C (N41H) mutation carriers before genetic analysis, even in heterozygous patients.

Authorship and Disclosures

SV performed the research and wrote the paper; RS contributed to the clinical study of the patients; SM designed the molecular modeling; AML performed the sequencing analysis; MS performed the platelet function tests; MLR contributed to the analytical tools; FF designed the research and critically discussed the paper.

The authors reported no potential conflicts of interest.

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