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 Padua Medical School, Padova, Italy

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Methods online supplementary Appendix

Flow cytometry

For flow cytometry, 22.5 μL of PRP (native or 1:10 diluted with Tyrode's buffer if platelets were >50×10⁹/L) were incubated with mAbs in a 25 μL final volume reaction for 15 mins. at room temperature. Appropriate normal and isotopic controls were set at each experiment.

Stained platelets were resuspended in 500 μl of Tyrode's buffer pH7.4, tested on a BD FACScan (BD Becton Dickinson, Heidelberg, Germany) and analyzed with CellQuest rel. 3.1 software. For each sample, 10,000 events were acquired. Excitation was induced with an argon laser at a wavelength of 488 nm with a 530 nm band-pass filter. The platelet population was gated in a characteristic size (forward scatter) and granularity (side scatter) gate, and identified by antiCD41a staining. Fluorescence histograms were used to quantify fluorescence related to mAbs binding and mean channel fluorescence (MCH) values were recorded after setting appropriate marker regions including >98% of positive events.

The MCH values (arbitrary units) recorded for anti-GPIbα (SZ2; LJ-Ib1; LJ-Ib10; AK2; HIP1 clones) and anti-CD41a mAbs were normalized using MCH values of healthy controls as 100% expression rates.

The normalized binding rates of patients were compared with those of controls using Student's two-tailed t-test. p values <0.05 were considered statistically significant.

Western Blotting

After addition of 0,2 volumes of PBS/EDTA 2%, control and patients' ACD anticoagulated PRP was spun for 5 mins. at 180 g and 70 g respectively, in order to limit red cell contamination. The supernatant was transferred to new tubes and centrifuged at 800 g for 20 mins. Pellets were resuspended in appropriated volumes of Tyrode's buffer pH7.4 after addition of proteases inhibitors cocktail (Sigma).

Aliquots of 10^7 platelets were lysed with 2X Laemmli sample buffer [10% glycerol, 1% sodium dodecyl sulphate (SDS), 5% b-mercaptoethanol, 50 mmol/L Tris HCl pH 6.8, 0.05% bromophenol blue] and boiled for 5 mins. Lysates were conserved at –80°C if not used immediately. SDS electrophoresis was performed on 7.5% polyacrilamide gels running at 25 mA at RT for 1 hour and 30 mins. Proteins were transferred to nitrocellulose membranes (Biorad Laboratories, Hercules, CA, USA) in a buffer containing Tris (25 mmol/L), glycine (192 mmol/L) and 20% methanol at 350 mA at RT for 2 hrs and 30 mins. Residual binding sites were blocked overnight at 4°C with a solution of 10 mmol/L Tris, 150 mmol/L sodium chloride (pH7.6) and 1% Tween 20 (TBST) with the addition of 3% BSA. Thereafter the membrane was washed three times with TBST and incubated for one hour at room temperature with the mouse monoclonal antibody LJ-Ib10 at a final concentration of 6 μg/mL in TBST. The primary antibody was removed and the blot has been washed twice with TBST and once in TBS and incubated with HRP conjugate anti-mouse antibody (GE Healthcare, UK), diluted 1:8000 in TBST for 1 one hour. Membranes were washed and treated with enhanced chemiluminescence reagents (GE Healthcare, UK) before exposure to X-ray film (30 secs).

Genetic analysis

Genomic DNA was extracted from whole blood with the WIZARD Genomic DNA Extraction kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

The entire coding region and part of the exon flanking sequences were amplified using three different pairs of primers, as previously described by Savoia *et al.*:[1] 1F/2R, 3F/3R, 4F/5R. Three separate PCR reactions were obtained (1F/2R; 3F/3R; 4F/5R) in 25 μL mixtures containing 200ng of genomic DNA, 15 pmoles of each primer and the appropriate volume of 2X PCR Master mix (Promega, Madison, WI, USA). Amplification proceeded after 5 mins of denaturation at 94°C in a GeneAmp 2400 (Applied Biosystems) thermal cycler device for 32 cycles programmed as follows: denaturation for 30 secs. at 94°C; annealing for 45 secs. at 60°C; elongation for

1'30 secs. at 72°C. Amplicons were controlled on 1.5% agarose gel stained with ethidium bromide.

Amplified DNA fragments, purified after ExoSap (GE Healthcare, UK) digestion, were sequenced directly according to the Big Dye terminator (Applied Biosystems, Foster City, CA; USA) cycling method on an ABI PRISM 3100 Genetic DNA Sequence Analyzer (Applied Biosystems, Foster City, CA, USA). The primers used were: 1F; 2F; 2R; 3F; 4F; 5R; 7F, as described by Savoia *et al*.,[1] and 3'HPA2, as described by Unkelbach *et al*.[2]

Crystallographic analysis

The crystallographic data relating to the N-terminal domain of GPIbα (PDB code:1P9A)[3] at 1.7 Angstrom resolution was used in our modeling study. The mutant N41H was generated using the *Mutate* protocol implemented in the Molecular Operating Environment (MOE, rel. 2006.08) suite.[4] Before running molecular dynamics simulations, hydrogen atoms were added to both wild-type and N41H mutant using the MOE suite. To minimize contact between hydrogen atoms, both protein structures were submitted to Amber94[5] energy minimization until the /rms/ of the conjugate gradient was <0.15 kcal mol^-1 Å^-1 keeping the heavy atoms fixed in their original positions. The conformational behavior of both proteins was calculated using the MOE program and Amber94 force field. For the solvent, the Generalized Born implicit water model was used.[6] A dielectric permittivity, e=1, was assumed and the van der Waals interactions were cut off at 0.8 nm. The SHAKE algorithm,[7] with a tolerance of 0.0005 Å) was applied to all bonds, using a 2 fs time step and maintaining a temperature of 300 K by Berendsen temperature coupling. The duration of the molecular dynamics simulations was fixed at one nanosecond.

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