Deletion of human GP1BB and SEPT5 is associated with Bernard-Soulier syndrome, platelet secretion defect, polymicrogyria, and developmental delay

Ingrid Bartsch1; Kirstin Sandrock1; Francois Lanza2; Paquita Nurden3; Anna Pavlova4; Andreas Greinacher5; Uta Tacke1; Michael Barth1; Anja Busse1; Johannes Oldenburg4; Martin Bommer4; Brigitte Strahm1; Andrea Superti-Furga1; Barbara Zieger1

1Department of Pediatrics and Adolescent Medicine, University Medical Center Freiburg, Freiburg, Germany; 2Inserm UMR_S 949, Etablissement Français du Sang – Alsace (EFS-Alsace), Université de Strasbourg, Strasbourg, France; 3Centre de Référence des Pathologies Plaquettaires, Hôpital Cardiologique, Pessac, France; 4Institute of Experimental Haematology and Transfusion Medicine, University Clinic Bonn, Bonn, Germany; 5Institute for Immunology and Transfusion Medicine, Ernst-Moritz-Arndt-University, Greifswald, Germany; 6Department of Internal Medicine III, University Hospital of Ulm, Ulm, Germany

Summary
The bleeding disorder Bernard-Soulier syndrome (BSS) is caused by mutations in the genes coding for the platelet glycoprotein GPIb/IX receptor. The septin SEPT5 is important for active membrane movement such as vesicle trafficking and exocytosis in non-dividing cells (i.e. platelets, neurons). We report on a four-year-old boy with a homozygous deletion comprising not only glycoprotein Ibβ (GP1BB) but also the SEPT5 gene, located 5′ to GP1BB. He presented with BSS, cortical dysplasia (polymicrogyria), developmental delay, and platelet secretion defect. The homozygous deletion of GP1BB and SEPT5, which had been identified by PCR analyses, was confirmed by Southern analyses and denaturing HPLC (DHPLC). The parents were heterozygous for this deletion. Absence of GPIbβ and SEPT5 proteins in the patient’s platelets was illustrated using transmission electron microscopy. Besides decreased GPIb/IX expression, flow cytometry analyses revealed impaired platelet granule secretion. Because the bleeding disorder was extremely severe, the boy received bone marrow transplantation (BMT) from a HLA-identical unrelated donor. After successful engraftment of BMT, he had no more bleeding episodes. Interestingly, also his mental development improved strikingly after BMT. This report describes for the first time a patient with SEPT5 deficiency presenting with cortical dysplasia (polymicrogyria), developmental delay, and platelet secretion defect.

Keywords
Inherited / acquired platelet disorders, paediatric haemostasis, platelet glycoproteins, platelet pathology / inherited, acquired

Introduction
Patients with Bernard-Soulier syndrome (BSS) suffer from bleeding symptoms such as epistaxis, haematomas and prolonged bleeding after mucocutaneous injury. Typically, patients with BSS show thrombocytopenia, giant platelets, and prolonged bleeding time (1). The glycoproteins (GP) GPIbα, GPIbβ, GPV and GPIX associate on the platelet surface to form the GPIb/IX receptor. In patients with BSS mutations in GP1BA, GP1BB, and GPIX (not in GPV) leading to the absence of the GPIb/IX receptor on the platelet surface have been described.

Originally, the septin gene SEPT5 has been identified immediately 5′ to the GP1BB gene (2). Meanwhile, 13 septin genes have been described in humans (SEPT1-SEPT12 and SEPT14) (3). Septins constitute a family of GTP-binding proteins implicated in a variety of cellular processes from cell polarity to cytokinesis (4). In addition, septins seem to regulate exocytosis in post-mitotic cells such as neurons and platelets (5, 6). Interestingly, the septins SEPT4, SEPT5 and SEPT8 have been shown to localise surrounding platelet granules and may contribute in such a way to platelet biology (7–9). Platelets from a SEPT5 knockout mouse show altered serotonin secretion and platelet aggregation suggesting that SEPT5 is involved in platelet secretion (7). In addition, SEPT5 deficiency seems to exert pleiotropic effects on a selected set of affective behaviours and cognitive processes as shown for SEPT5 knockout mice which demonstrated delayed acquisition of rewarded goal approach (10).

Both genes, SEPT5 and GP1BB, are localised on chromosome 22q11.2, a region important for DiGeorge/velo-cardio-facial syndrome (VCFS) (11). VCFS patients demonstrate heterozygous deletions either comprising 3 Mb (90%) or 1.5 Mb (10%) of chromosome 22q11.2. Depending on the exact location of the deletion...
patients with VCFS show parathyroid hypoplasia, thymic hypoplasia, heart defects, learning disabilities, and recurrent infections. Thrombocytopenia and/or -pathy can cause bleeding symptoms (12, 13).

The boy reported here demonstrates a homozygous deletion of only two contiguous genes: SEPT5 and GP1BB (~16.4 kb). Besides GP1BB and SEPT5 the deletion does not include adjacent genes. This genotype has never been reported before. The clinical phenotype of this patient combines that of BSS (bleeding disorder) with neurological abnormalities. The additional loss of SEPT5 in this patient seems to be responsible for the developmental retardation, the cortical dysplasia (polymicrogyria), and the platelet secretion defect.

Materials and methods

Platelet aggregation analyses

Citrate-anticoagulated blood samples were obtained after informed consent from the controls, patient and the parents, respectively. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared as described (14). Platelet aggregation was analysed on a platelet aggregometer APACT 4 (Labor Fibrintimer, Ahrensburg, Germany) using collagen (2.0 μg/ml; Nycomed, Vienna, Austria) and ristocetin (1.2 mg/ml; American Biochemical and Pharmaceutical Ltd, London, UK).

Transmission electron microscopy and immunogold staining

Samples were prepared as described previously (15) and incubated with anti-SEPT5 (8, 16) and polyclonal anti-GPIb/IX antibody (gift of Dr. B Steiner, Hoffmann-La Roche, Basel, Switzerland) (17). Bound antibodies were detected using goat anti-rabbit IgG conjugated to 10 nm gold particles (Amersham, Orsay, France). Samples were analysed with a Jeol JEM-1010 transmission electron microscope (Jeol, Croissy-sur-seine, France) as described (15). Controls included the absence of primary antibody or its substitution with an IgG antibody.

Platelet adhesion immunofluorescence test (PAIFT)

EDTA-anticoagulated blood and serum were used for determination of platelet-bound and free antibodies by PAIFT (18). Platelet GP complexes IIB/IX, IIb/IIa, Ia/IIa were determined semi-quantitatively using monoclonal antibodies: FMC25 (CD42a; Millipore, Schwalbach, Germany), G5 and G14 (CD41a and CD49b, respectively; gifts of Dr. Santos, University Giessen, Germany), and FITC-labelled goat anti-mouse IgG antibody (Dianova, Hamburg, Germany).

Flow cytometry

PRP was incubated separately with monoclonal antibodies as previously described for the PAIFT, washed, incubated with the FITC-labelled antibody and assessed by FACScan (Becton Dickensen, Heidelberg, Germany). Platelets were stained using monoclonal anti-CD41 FITC IgG1 (clone: P2, Immunotech, Marseille, France), anti-CD42b (clone AN51, DAKO, Copenhagen, Danmarks), anti-CD62P FITC IgG1 (clone: CLB-Thromb/6, Immunotech, Marseille, France), anti-thrombospondin-1 (TSP-1) PE IgG1 (clone: P10, Immunotech), and anti-CD63 FITC IgG1 (clone: CLB-gran12, Immunotech) antibodies, respectively. Isotypic mouse IgG1 FITC (clone: 679.1Mc7) and IgG1 PE (clone: 679.1Mc7) control antibodies were also obtained from Immunotech. Target antigen analysis was performed for CD41-positive particles only. The percentage of specific fluorescence-positive platelets was obtained after subtraction of unspecific mouse IgG binding.

Southern analyses

Genomic DNA from the patient and a healthy control was digested with BamHI analysing SEPT5. Digested DNA was separated on a 0.7 % agarose gel and transferred to a Hybond N+ nylon membrane (GE Healthcare, Freiburg, Germany). After prehybridisation, the membrane was hybridised with a [α-32P] dCTP-labelled probe overnight. After washing the membrane was analysed by autoradiography (Kodak™ BioMax® MRI Film; PerkinElmer, Rodgau, Germany).

Semi-quantitative Multiplex PCR and HPLC analyses

For detection of SEPT5 and GP1BB large deletions a semi-quantitative multiplex PCR was performed and analysed in a semi-automated high throughput HPLC system (WAVE, Transgenomic Ltd, Omaha, NE, USA) as described previously (19). The chromatograms of patient's samples were normalised according to the internal HGH (human growth hormone) peak and superposed to those of a normal control. The peak height of each exon of the investigated sample and the normal control were compared and a ratio for each peak was calculated (patient/control). Ratios between 0.2 and 0.6 were accepted to represent a large deletion of the corresponding fragment.

PCR analyses and sequencing

Genomic DNA was isolated from EDTA-anticoagulated blood (Qiagen, Hilden, Germany). The genes encoding SEPT5 and GPIbβ were amplified by PCR as described previously (20). Puriﬁed DNA fragments were directly sequenced.
Results

Patient

We report on a four-year-old boy who had been admitted to the hospital several times during the first year of his life (1994/1995) because of severe epistaxis, mucosal bleeding episodes, haematomas and petechiae. The blood smear showed giant platelets (Fig. 1A) and a severely prolonged Mielle bleeding time (>15 minutes) was measured. His platelet counts were between 30–70 G/l and mean platelet volume (Coulter Counter) was 11.0 fL. The platelet GPIb/IX expression was reduced. The bone marrow showed many megakaryocytes and normal white and red blood cell differentiation.

The boy was diagnosed with left side hemiparesis. Magnetic resonance imaging (MRI) of the head showed cortical dysplasia with polymicrogyria of the right hemisphere (parietal lobes) (Fig. 1B). A significant perisylvian substantial disruption which almost reached the basal ganglia was detected. There was no sign of intracranial bleeding. The electroencephalogram at the age of seven months showed focal activation on the right hemisphere. He never had seizures. From infancy onwards he presented combined developmental problems in motor coordination, language and speech development with severe attentional and perceptional deficits and he needed special education. At the age of seven years he spoke and understood short, incomplete sentences. Socio-emotional deficits were present at home, in kindergarten/school as well as on the paediatric ward, with severe aggressiveness at school and at home. Because of his behavioural deficits the standardised developmental testing was not possible. Observational assessment revealed moderate to severe mental retardation.

In the years 1998–2000 he suffered from recurrent petechiae and multiple haematomas and presented with several life-threatening mucosal bleeding episodes in the course of upper respiratory infections (4x/year). After tooth extraction he was bleeding for several days. He received multiple platelet and red blood cell transfusions in the first years of his life. In 2001 the bleeding symptoms were so severe (epistaxis for several weeks) that bone marrow transplantation (BMT) from a HLA-identical unrelated donor was performed at the age of seven years. Afterwards the platelet counts normalised and the boy did not show bleeding symptoms any longer.

Interestingly, the boy’s neuropsychiatric symptoms were changed after BMT. Hyperactivity and attention deficits improved as did speech development, social competence at home, at school, and at our hospital. Oppositional conflicts decreased at home. His mother described him as significantly changed.

The family belongs to a gypsy tribe. According to the parents’ information they are not consanguineous. The parents showed no increased bleeding tendency.

Platelet aggregation analyses

The boy’s platelets did not agglutinate after stimulation with ristocetin. Parents’ platelet function was normal.

Transmission electron microscopy and immunogold staining

Immune transmission electron microscopy of the platelets showed clearly reduced levels of SEPT5 and GPIb/IX on the patient’s platelets compared to the control (Fig. 2A, B).
Southern analyses

Southern analyses of patient’s genomic DNA revealed absence of \textit{SEPT5} in contrast to the parents’ or the control’s DNA. The BamHI digest of the control’s and the parents’ DNA revealed a 2.0 kb fragment using a probe binding to exon 13 of \textit{SEPT5} (Fig. 4).

Denaturing high performance liquid chromatography (DHPLC) analyses

DHPLC analysis of \textit{SEPT5} in the patient demonstrated no peak in the chromatogram for the multiplex amplified fragments representing exon 4, 6, 9, 11 and 13, indicating a homozygous deletion of the \textit{SEPT5} gene (Fig. 5A). Investigation of the remaining exons indicated a deletion spanning the whole gene (data not shown). In the parents peak heights for all exons were reduced to half of the control ones hinting to a heterozygous large deletion of the whole gene.

DHPLC analysis of patient’s \textit{GP1BB} revealed a homozygous deletion of exon 1 (lack of signal for exon 1) compared to the healthy control (Fig. 5B). However, exon 2 of \textit{GP1BB} was detectable most likely because of the 3’ untranslated region of \textit{GP1BB} which is still present in the patient. In both parents the peak height for exon 1 was reduced more than half of the control peak indicating a heterozygous large deletion of the fragment. Exon 2 was normally presented.

PCR analyses and sequencing

Interestingly, the entire \textit{SEPT5} gene which is located 5’ of \textit{GP1BB} was deleted. Up to 5,000 bp 5’ of \textit{SEPT5} were not detectable. PCR reactions from several exons of the \textit{SEPT5} gene were performed before and after BMT: exons 10 to 13 were absent before BMT and clearly detectable after BMT (Fig. 6A). Another gene, Claudin5, which is located about 200 kb 5’ of \textit{SEPT5}, was normally detectable (data not shown).

PCR analyses revealed that there was no signal for the patient’s \textit{GP1BB} gene, whereas the \textit{GP1BA} and \textit{GPIX} genes were detectable (data not shown). Interestingly, exon 1, intron 1 and partly exon 2 of \textit{GP1BB} were deleted on both alleles. In exon 2 the deletion includes the coding region of \textit{GP1BB}, whereas the 3’ untranslated region was detectable (Fig. 6B). The same PCR reactions were performed after BMT; as for the healthy control, all fragments of the \textit{GP1BB} gene were reproducible.

Taken together, only two genes, \textit{GP1BB} and \textit{SEPT5}, including 5,000 bp of the \textit{SEPT5} promoter region are homozygously deleted (Fig. 6C).

Flow cytometry and platelet adhesion immunofluorescence test (PAIFT)

Flow cytometry analyses of patient’s platelets revealed severely decreased GPIbα expression (28%) (Fig. 3A) and absence of GPIX expression (data not shown). Expression of CD62P (11%, normal range 90–100%) and of thrombospondin-1 (15%, normal range 90–100%) was severely reduced, expression of CD63 was 73% (normal range 90–100%) and expression of GPIIb/IIIa was 74% (Fig. 3B-E). Expression of GPIA/IIa (90%) (Fig. 3F) and GPIV (88%) were normal.

On the patient’s platelets the absence of GPIX expression was also determined using PAIFT (data not shown). In the parents’ platelets GPIX expression was slightly reduced.
Figure 3: Flow cytometry analyses. Expression levels of GPIbα (A), CD62P (B), thrombospondin-1 (TSP-1) (C), CD63 (D), GPIIb/IIIa (E), and GPIa/IIa (F) on patient’s platelets were detected by flow cytometry analyses.

Figure 4: Southern analysis of SEPT5. Shown is the autoradiograph of the patient’s and the control genomic DNA hybridised with a specific SEPT5 probe. The BamHI digest reveals a DNA fragment in the control DNA but not in the patient’s DNA (left panel). The SEPT5 fragment is detectable in the mother’s and father’s genomic DNA (right panel).
Discussion

We report on a four-year-old patient with Bernard-Soulier syndrome (BSS) who presented with severe bleeding symptoms and mental retardation. The patient demonstrated typical BSS features such as giant platelets, absent platelet agglutination after ristocetin stimulation, and reduced expression of platelet GPIb/IX receptor. Surprisingly, our flow cytometry data showed a striking discrepancy between α- and δ-granule-secretion suggesting a severe α-granule secretion defect. The δ-granule-dependent epitope CD63 was almost normal whereas all α-granule-dependent epitopes (CD62P, receptor bound TSP-1) remained low. Interestingly, this patient with BSS (reduced levels of GPIb/IX expression) additionally presented with a platelet granule secretion defect which may have been caused by the SEPT5 deletion. In accordance, the patient’s bleeding symptoms were extremely severe compared to other BSS patients. Bleeding episodes during infancy were several times life-threatening and required many platelet transfusions.
The severe platelet secretion defect seems to have aggravated the bleeding symptoms. Interestingly, the boy also presented with cortical dysplasia (polymicrogyria) on the right hemisphere and showed left side hemiparesis and developmental retardation.

Molecular genetic analyses revealed that the GP1BB gene is homozygously deleted causing the clinical entity of BSS. So far, no BSS patient suffering from developmental retardation has been reported. Accordingly, a GPIbβ-deficient mouse model of BSS displays macrothrombocytopenia and a severe bleeding phenotype, but no neurological impairments (21, 22).

GP1BB and SEPT5 are both located within the chromosomal region 22q11.2 which is deleted in patients with DiGeorge/velocardio-facial syndrome (VCFS) (11). Some of the patients with VCFS show cognitive, behavioural, and psychiatric impairments (23). In addition, patients with combined clinical phenotypes of BSS and VCFS have been reported (12, 13). The patient described
in this study does not show the typical VCFS phenotype (i.e. heart defects, parathyroid defect, thymus defect). Clinically, the boy presented with a bleeding disorder and mental retardation. Interestingly, he carries a homozygous deletion of not only GP1BB but also at the adjacent SEPT5 gene. Therefore, these data possibly indicate a novel contiguous gene syndrome. SEPT5 has been shown to play an important role in regulated secretion in platelets and neurons (5, 7, 9, 24). Therefore, we hypothesise that the deletion of SEPT5 contributed to the boy’s platelet secretion disorder, the cortical dysplasia (polymicrogyria), and neuropsychiatric symptoms. Several case reports of VCSF also noted severe cortical malformations manifesting with polymicrogyria (25). The neuro-mechanical model of Van Essen claims that the pattern of decreased folding with large gyri could be due to weak local interconnections in frontal and parietal lobes (26). This would go along with the findings that the septin SEPT5/7/11 complex is critical for dendrite branching and dendritic-spine morphology (27). The SEPT5 deficiency seems to cause a platelet secretion defect, cortical dysplasia (polymicrogyria) and developmental delay. The bleeding disorder was cured by BMT.

Conflict of interest
None declared.

References


