Identification of two novel mutations in RASGRP2 affecting platelet CalDAG-GEFI expression and function in patients with bleeding diathesis

Teresa Sevivas¹, José María Bastida², David S. Paul³, Eva Caparros⁴, Verónica Palma-Barqueros⁵, Margarida Coucelo⁶, Dalila Marques⁶, Francisca Ferrer-Marin⁶, José Ramón González-Porras⁷, Vicente Vicente⁴, Jesús María Hernández-Rivas⁸, Steve P. Watson⁹, Maria Luisa Lozano⁵, Wolfgang Bergmeier⁶, & José Rivera⁴,⁷

¹Servicio de Hematología Clínica del Centro Hospitalar e Universitario de Coimbra, EPE, S. Martinho do Bispo, Portugal, ²Servicio de Hematología, IBISAL-Hospital Universitario de Salamanca, Salamanca, Spain, ³Department of Biochemistry and Biophysics, and McAllister Heart Institute, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA, ⁴Servicio de Hematología y Oncología Médica, Hospital Universitario Morales Meseguer, Centro Regional de Hemodonación, Universidad de Murcia, Murcia, Spain, ⁵Grado de Medicina, Universidad Católica de Murcia (UCAM), Murcia, Spain, ⁶Institute of Cardiovascular Sciences, College of Medical and Dental Sciences, University of Birmingham, Birmingham, UK, and ⁷On behalf of “Inherited Platelet Disorders Project”, Hemorrhagic Diathesis Working Group, SETH, Spain

Abstract

The RASGRP2 gene encodes the Ca²⁺ and DAG-regulated guanine nucleotide exchange factor I (CalDAG-GEFI), which plays a key role in integrin activation in platelets and neutrophils. We here report two new RASGRP2 variants associated with platelet dysfunction and bleeding in patients. The homozygous patients had normal platelet and neutrophil counts and morphology. Platelet phenotyping showed: prolonged PFA-100 closure times; normal expression of major glycoprotein receptors; severely reduced platelet aggregation response to ADP and collagen (both patients); aggregation response to PAR1 and arachidonic acid markedly impaired in one patient; PMA-induced aggregation unaffected; platelet secretion, clot retraction, and spreading minimally affected. Genetic analysis identified two new homozygous variants in RASGRP2: c.706C>T (p.Q236X) and c.887G>A (p.C296Y). In both patients, CalDAG-GEFI protein was not detectable in platelet lysates, and platelet αIIbβ3 activation, as assessed by fibrinogen binding, was greatly impaired in response to all agonists except PMA. Patient neutrophils showed normal integrin expression, but impaired Mn²⁺-induced fibrinogen binding. In summary, we have identified two new RASGRP2 mutations that can be added to this rapidly growing form of inherited platelet function disorder.

Introduction

The guanine nucleotide exchange factor, CalDAG-GEFI, is critical for integrin signaling in platelets and neutrophils [1]. CalDAG-GEFI is activated in response to elevated cytoplasmic calcium concentrations, downstream of engagement of agonist receptors coupled to phospholipase C. Its main target is the small GTPase Rap1, an important regulator of integrin-mediated adhesion in different cell types [2]. Mice deficient in CalDAG-GEFI bleed after challenge due to a defect in platelet integrin signaling. More subtle defects in integrin function in neutrophils were also described [3]. Recent studies have identified five distinct variants in the gene encoding for CalDAG-GEFI, RASGRP2, all of them associated with markedly impaired platelet function and bleeding in the affected individuals [⁴−⁶]. Defects in neutrophil integrin activation were observed only in some patients [⁵]. We here describe two more RASGRP2 variants that affect integrin-mediated adhesion in patient platelets and neutrophils.

Methods

See supplemental information available online at publisher’s website.

Results and discussion

Here, we have characterized at a functional and molecular level two unrelated Portuguese children with lifelong bleeding complications (Figure 1A). No consanguinity could be assessed in either pedigree. One index case is a 4-year-old girl suffering from severe epistaxis, oral cavity bleeding, and spontaneous bruising since she was 8 months old (BAT-ISTH score [BS]: 9). The second proband (PI-family 2) is an 8-year-old boy also suffering from clinically relevant mucocutaneous bleeding (BS: 7), since the age of 1 year. He also presented with motor development delay. Bleeding complications in both patients have required occasionally hospitalization and medical intervention, including nasal packing, antifibrinolytic and desmopressin (only PI-family 2) treatments, iron therapy, and transfusion of platelets or red blood cells.
Both patients displayed, in at least two separate occasions, normal platelet and neutrophil counts and morphology and mild anemia (Figure 1A), normal blood coagulation parameters, and no overt signs of immunodeficiency or predisposition to bacterial infections. An inherited platelet disorder was first suspected in both children following observation of severely extended closure time in PFA-100 testing (>300s with both collagen/ADP and collagen/epinephrine cartridges). Both patients showed normal expression of major platelet surface glycoproteins (Figure S1). In contrast, they displayed impairment in their platelet aggregation in response to common agonists, which was more generalized and pronounced in P1-family1 (Figure 1B). Noteworthy, platelets
from both patients aggregated normally in response to PMA stimulation, a direct activator of protein kinase C (Figure 1B).

In agreement with their aggregation defect, platelets from both patients showed a marked defect in fibrinogen binding to activated αIIbβ3, when stimulated with ADP and low doses of agonists to PAR1, PAR4, or GPVI. However, fibrinogen binding was not affected when cells were activated with PMA (Figure 1C). Agonist-induced release of alpha and dense granules

---

Figure 2. Novel variants p.C296Y and p.Q236X lead to impaired CalDAG-GEFI expression in index cases. (A) Schematic representation for CalDAG-GEFI showing the different domains: Ras exchanger motif (REM), catalytic domain (CDC25), calcium-binding EF hands (EF) and C1-like domain (unknown function). The positions of the recently reported R113X, G248W, K309X, L360del, and S381F mutations[4–6] and the novel mutations C296Y and Q236X within the CDC25 domain are shown. (B) DNA from index cases was analyzed by high-throughput sequencing and novel mutations in RASGRP2 were identified. Figure shows the localization of the novel c.887G>A (p.C296Y) in P1-family 1 and c.706C>T (p.Q236X) in P1-family 2, within the RASGRP2 sequence. (C) Immunoblot analysis for CalDAG-GEFI (CDGI, polyclonal antibody raised against the N-terminus of the protein), Rasa3, Rap1, and β-actin in platelet lysates from the homozygous index cases (P) and healthy and unrelated controls (C). Both variants, C296Y and Q236X, severely impaired platelet expression of CDGI. Similar results were obtained with different antibodies directed against the N-terminus or the C-terminus of CDGI (not shown).
was also partially impaired (Figure S2), while clot retraction (Figure S3) and platelet spreading (Figure S4) were only minimally affected. We also observed normal expression (Table S1) but reduced activation of \( \beta_2 \) integrins in neutrophils from both patients (Figure 1D).

The various platelet function defects observed by aggregometry, flow cytometry, and PFA-100 are consistent with the altered platelet function previously described in patients with mutations in \textit{RASGRP2} [4–6]. High throughput sequencing, and thereafter Sanger sequencing, identified two novel mutations in \textit{RASGRP2}: c.887G>A in P1-family1 and c.706C>T in P1-family2. Both parents in family 1 were heterozygous for the c.887G>A mutation. In family 2, the mother was heterozygous for the c.706C>T mutation; DNA from the father was not available. While c.706C>T leads to a premature stop at amino acid position 236 (p.Q236X), c.887G>A leads to a single amino acid change (p.C296Y) in CalDAG-GEFI (Figures 2A, B). Interestingly, CalDAG-GEFI protein was not detectable in platelets from both homozygous patients (Figure 2C), suggesting that the p.C296Y variant is not tolerated; this conclusion is supported by bioinformatics analyses using the SIFT and Panther algorithms. Thus, six out of seven \textit{RASGRP2} variants identified so far in humans, two carried in patients described in this report and four previously reported [4–6], lead to CalDAG-GEFI deficiency in platelets. Noteworthy, in a companion manuscript in this issue of \textit{Platelets}, Bermejo et al. report another variant in \textit{RASGRP2} that leads to reduced but not absent expression of CalDAG-GEFI.

In summary, we here report two new variants in \textit{RASGRP2} that lead to altered integrin function in platelets and neutrophils. Consistent with previous studies, deficiency in CalDAG-GEFI leads to a moderate-to-severe bleeding diathesis but not to immune dysregulation in the affected patients. The stronger platelet aggregation defect observed in P1 of family 1 may suggest an additional signaling defect in this family.

Acknowledgments

This study was conducted according to the aims of the Project “Functional and Molecular Characterization of Patients with Inherited Platelet Disorders” (approved by the Hemorrhagic Diathesis Working Group of the Spanish Society of Thrombosis and Haemostasis). We thank the families for providing samples. We also thank Constantino Martínez and José Padilla for their help in some platelet studies and Sanger sequencing.

Declaration of interest

The authors declare no competing financial interests.

Funding

JMB group is supported by Gerencia Regional de Salud (GRS 1370/A/16). Research by the group of J.R. is supported by grants from Instituto de Salud Carlos III and Feder (PI14/01956 and CB15/00055). Research by the group of S.P.W is supported by the British Heart Foundation (BG/PG/13/36/30275; RG/09/007). W.B. is supported by grants from the National Institutes of Health (R01 HL130404 and R01 HL121650) and the American Heart Association (14EIA18910004).

Supplemental material

Supplemental data for this article can be accessed on the publisher’s website.

ORCID

Verónica Palma-Barqueros http://orcid.org/0000-0002-5699-0053
Margarida Coucelo http://orcid.org/0000-0002-3426-6363
María Luisa Lozano http://orcid.org/0000-0003-3148-7037
José Rivera http://orcid.org/0000-0003-4225-6840

References

1. Stefanini L, Bergmeier W. CalDAG-GEFI and platelet activation. Platelets 2010;21:239–243.
2. Stefanini L, Bergmeier W. RAP1-GTPase signaling and platelet function. J Mol Med (Berl) 2016;94:13–19.
3. Crittenden JR, Bergmeier W, Zhang Y, Piffath CL, Liang Y, Wagner DD, Housman DE, Graybiel AM. CalDAG-GEFI integrates signaling for platelet aggregation and thrombus formation. Nat Med 2004;10:982–986.
4. Canault M, Ghalloussi D, Grosdidier C, Guinier M, Perret C, Chelghoum N, Germain M, Raslova H, Peiretti F, Morange PE, Saut N, Pillois X, Nurden AT, Cambien F, Pierres A, Van Den Berg TK, Kuijpers TW, Alessi MC, Tregouet DA. Human CalDAG-GEFI gene (RASGRP2) mutation affects platelet function and causes severe bleeding. J Exp Med 2014;211:1349–1362.
5. Lozano ML, Cook A, Bastida JM, Paul DS, Irinu G, Cid AR, Adan-Pedroso R, Ramón González-Porras J, Hernández-Rivas JM, Fletcher SJ, Johnson B, Morgan N, Ferrer-Marin F, Vicente V, Sondek J, Watson SP, Bergmeier W, Rivera J. Novel mutations in RASGRP2, which encodes CalDAG-GEFI, abrogate Rap1 activation, causing platelet dysfunction. Blood 2016;128:1289–1292.
6. Kato H, Nakazawa Y, Kurokawa Y, Kashiwagi H, Morita D, Banno F, Honda S, Kanakura Y, Tomiyama Y. Human CalDAG-GEFI deficiency increases bleeding and delays αIbβ3 activation. Blood 2016;128:2729–2733.