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SHORT COMMUNICATION



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

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**Abstract**

The *RASGRP2* gene encodes the $Ca^{2+}$ 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 $Mn^{2+}$-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.

**Keywords**

Bleeding, dysfunction, platelets, RASGRP2, signaling

**History**

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## 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 (P1-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 P1-family 2) treatments, iron therapy, and transfusion of platelets or red blood cells.

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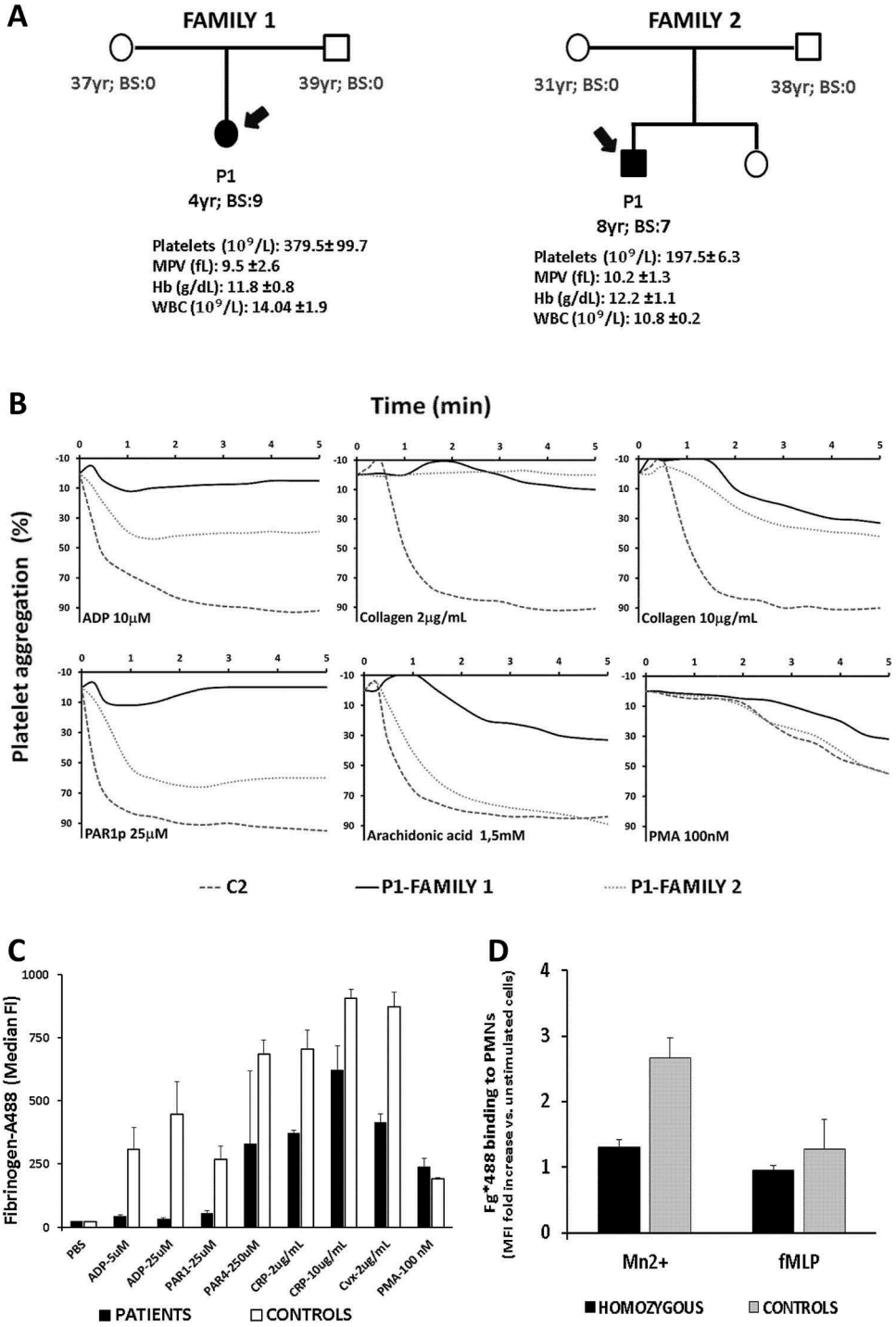


Figure 1. Platelet and neutrophil integrin function defects in two unrelated children suffering from lifelong bleeding diathesis. (A) Pedigrees of the two families studied. The index cases in each family are indicated with black arrows and their major blood parameters are shown. Bleeding in patients and family members was evaluated and scored (BS) using the Bleeding Assessment tool from ISTH. MPV: mean platelet volume; Hb: hemoglobin; WBC: white blood cells. (B) Platelet aggregation in response to the indicated platelet agonists was evaluated in unadjusted platelet-rich plasma from patients (P) and a healthy and unrelated control (C2). (C) αIIbβ3 integrin activation: Platelets from index cases and healthy and unrelated controls (controls) (combined data from two subjects) were stimulated under static conditions (30 min at RT) with the indicated agonist in the presence of fibrinogen-Alexa 488. The median fluorescence intensity [MFI] was determined by flow cytometry. (D) β2 integrin activation. Neutrophils from patients and healthy and unrelated controls (controls) (combined data from two subjects) were stimulated with the indicated agonists and the binding of Alexa Fluor 488-fibrinogen was evaluated by flow cytometry. Values are MFI ± SEM from data obtained in the two patients and two healthy controls.

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

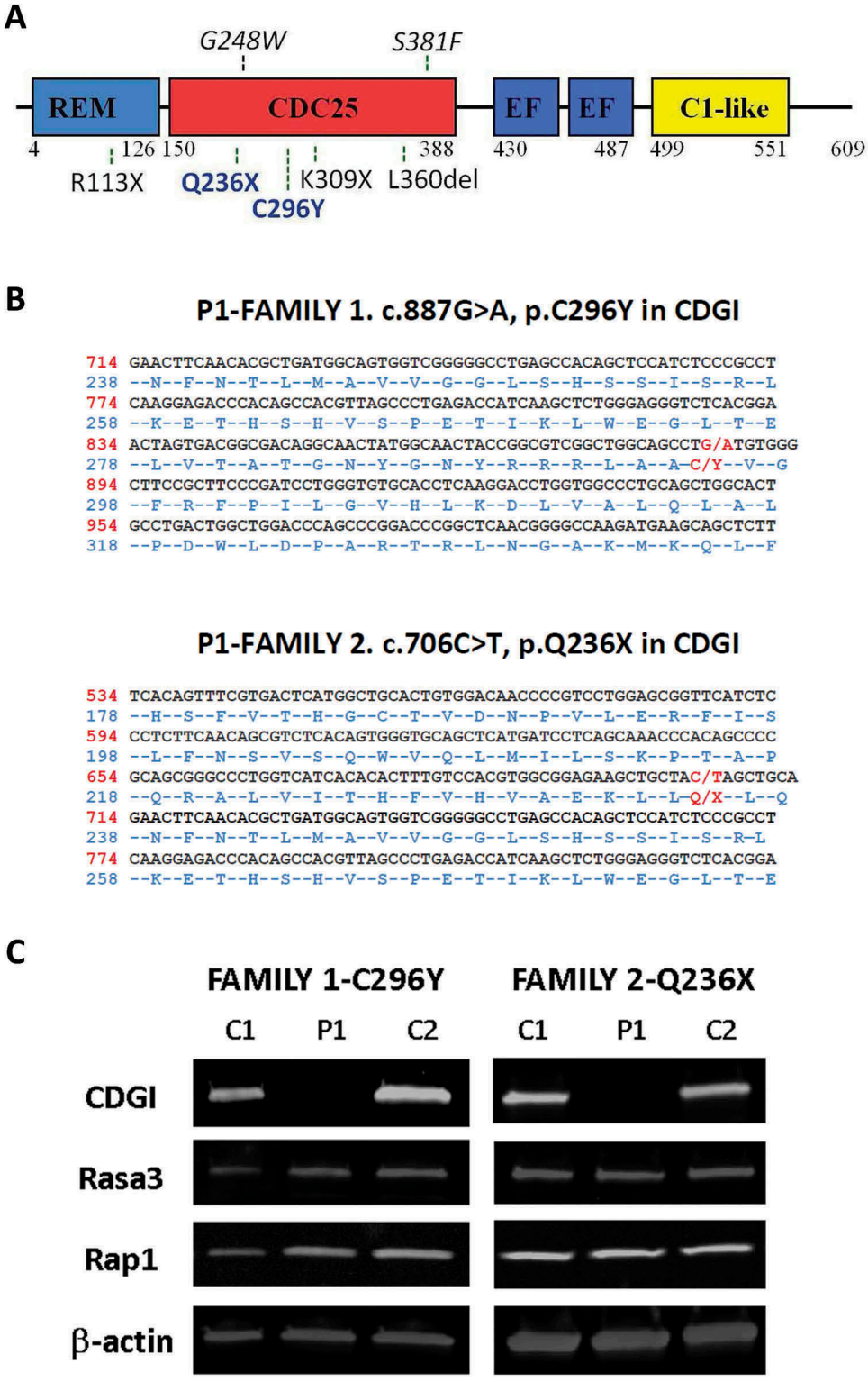


Figure 2. Novel variants p.C296Y and p.Q236X lead to impaired CalDAG-GEFI expression in index cases. (A) Schematic representation for CalDAG-GEFI showing the different domains: Ras exchanger motif (REM), catalytic domain (CDC25), calcium-binding EF hands (EF) and C1-like domain (unknown function). The 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-family1 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).

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

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 β2 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 *RASGRP2* [4–6]. High throughput sequencing, and thereafter Sanger sequencing, identified two novel mutations in *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.C296Yvariant is not tolerated; this conclusion is supported by bioinformatics analyses using the SIFT and Panther algorithms. Thus, six out of seven *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 *Platelets*, Bermejo et al. report another variant in *RASGRP2* that leads to reduced but not absent expression of CalDAG-GEFI.

In summary, we here report two new variants in 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.

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## Declaration of interest

The authors declare no competing financial interests.

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## Supplemental meterial

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

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