Expanded repertoire of RASGRP2 variants responsible for platelet dysfunction and severe bleeding

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KEY POINTS

- Eleven pedigrees were identified with biallelic pathogenic variants in \textit{RASGPR2}, which encodes platelet CalDAG-GEFI.

- CalDAG-GEFI deficiency is a severe, recessive, non-syndromic platelet function disorder with defective aggregation to multiple agonists.
ABSTRACT

Heritable platelet function disorders (PFDs) are genetically heterogeneous and poorly characterised. Pathogenic variants in RASGRP2, which encodes calcium and diacylglycerol-regulated guanine exchange factor I (CalDAG-GEFI), have been reported previously in three pedigrees with bleeding and reduced platelet aggregation responses. To better define the phenotype associated with pathogenic RASGRP2 variants, we compared high-throughput sequencing and phenotype data from 2,042 cases in pedigrees with unexplained bleeding or platelet disorders to data from 5,422 controls. Eleven cases harboured 11 different, previously unreported RASGRP2 variants that were biallelic and likely pathogenic. The variants included five high-impact variants predicted to prevent CalDAG-GEFI expression and six missense variants affecting the CalDAG-GEFI CDC25 domain, which mediates Rap1 activation during platelet inside-out αIIbβ3 signalling. Cases with biallelic RASGRP2 variants had abnormal mucocutaneous, surgical and dental bleeding from childhood, requiring at least one blood or platelet transfusion in 78% of cases. Platelets displayed reduced aggregation in response to ADP and epinephrine, but variable aggregation defects with other agonists. There were no other consistent clinical or laboratory features. These data enable definition of human CalDAG-GEFI deficiency as a non-syndromic, recessive PFD associated with a moderate or severe bleeding phenotype and complex defects in platelet aggregation.
INTRODUCTION

Heritable platelet function disorders (PFD) are genetically heterogeneous rare diseases characterised by mucocutaneous, surgical and traumatic bleeding\(^1,2\) and reduced platelet responses to activating agonists.\(^3,4\) Human PFD have been associated with causal variants in more than 30 genes, often associated with distinctive clinical or laboratory phenotypes that inform selection of candidate genes for diagnosis.\(^5\) Several new PFD with less distinctive phenotypes have recently been identified using high-throughput sequencing.\(^6-8\) However, to date these have only been reported in small numbers of pedigrees and are incompletely characterised.

One such example is the PFD associated with loss-of-function variants in \textit{RASGRP2}, which encodes calcium and diacylglycerol-regulated guanine exchange factor I (CalDAG-GEFI). This PFD was first identified in a consanguineous pedigree with a homozygous p.G248W variant associated with bleeding and reduced platelet light transmission aggregation (LTA) responses to multiple agonists.\(^9\) CalDAG-GEFI deficiency has been reported subsequently in only three pedigrees worldwide.\(^10,11\)

In order to better define the phenotype and mutational spectrum of CalDAG-GEFI deficiency, we analysed data from a collection of 2,042 cases with unexplained bleeding or platelet disorders (BPD) to identify 11 new affected pedigrees.

METHODS

\textit{RASGRP2} was investigated in two study collections in which all participants were enrolled to the National Institute for Health Research BioResource - Rare Diseases between 2012 and 2016 after providing informed written consent (UK Research
Ethics Committee approval 13/EE/0325). The first collection comprised 1,472 cases or pedigree members with unexplained BPD and 5,422 cases with other rare inherited disorders who underwent whole exome or genome sequencing. The second collection comprised 570 other BPD cases analysed using the ThromboGenomics high-throughput platform, which captures variants in 81 tier one genes for BPD, designated by the ISTH Genomics Scientific and Standardisation Subcommittee.

Collection of clinical and laboratory phenotypes and coding with Human Phenotype Ontology (HPO) terms were performed as previously described. Variants from high-throughput sequencing were obtained using Isaac (Illumina, Inc.; San Diego, CA) or as described previously. Variants were not considered to be potentially pathogenic if the Variant Effect Predictor identified them as synonymous relative to canonical transcript ENST00000354024, or if the allele frequency was greater than 1/1000 in reference databases. Index cases in the NIHR BioResource were classified as having an indicator phenotype of CalDAG-GEFI deficiency if they had HPO terms indicating abnormal bleeding and reduced LTA responses to at least three agonists. Associations between rare and non-synonymous RASGRP2 variants in the NIHR BioResource and the presence or absence of the indicator phenotype were then assessed using the BeviMed method.

RESULTS AND DISCUSSION
The CalDAG-GEFI deficiency indicator phenotype of bleeding and reduced LTA responses to at least three agonists was present in 119 (9.7%) of 1,229 BPD index cases from the NIHR BioResource. Analysis of all the rare, non-synonymous
variants in the BPD and non-BPD index cases revealed a strong statistical association between the indicator phenotype and the presence of at least two RASGRP2 alleles, obtaining a posterior probability of association with recessive inheritance of 1 by inference using the BeviMed method (Figure 1A). The association was driven by eight different variants observed in eight BPD index cases as homozygous or compound heterozygous alleles (Figure 1B; pedigrees A-H in Figure 2A). There were a further 35 RASGRP2 variants which were not associated with the indicator phenotype or which were present as a single allele (Figure 1A). In the ThromboGenomics collection, there were four rare, non-synonymous RASGRP2 variants that were biallelic (Figure 1B) in 3 index cases with bleeding and reduced LTA responses to at least three agonists (pedigrees I-K in Figure 2A). Since this analysis, pedigree G has also been reported elsewhere.\textsuperscript{17}

Of the 11 likely pathogenic RASGRP2 variants, four resulted in frameshift or a stop codon when annotated against the RASGRP2 canonical transcript (Figure 1B, 1C). One further variant (11:64507638 G>C; pedigree K) was at the -3 position in the intron 5 splice region, adjacent to the splice acceptor site. The Alamut\textsuperscript{®} Visual splicing module (Interactive Biosoftware, Rouen, France) predicted this to result in loss of the native splice acceptor site and co-option of a cryptic acceptor site in exon 6, leading to deletion of nine codons and a stop gain at codon 125 (Figure 1C). Thus, a total of five variants were predicted to prevent full length CalDAG-GEFI expression.

All six missense variants predicted substitutions of amino acids that were conserved in CalDAG-GEFI orthologs in nine distantly related species, except for human
residue A345 that is a T residue in the Xenopus ortholog (Figure 1B and Supplementary table S1). All had high CADD pathogenicity scores (>20; within the top 1% of deleterious variants genome-wide)\textsuperscript{18} and were either absent or at very low allele frequency in the ExAC population database (Figure 1B).\textsuperscript{15} All the predicted substitutions were in the CDC25 domain of CalDAG-GEFI that is essential for guanine nucleotide exchange activity, and directly interacts with Rap1, the major platelet target for CalDAG-GEFI in the inside-out αIIbβ3 signalling pathway.\textsuperscript{19} This suggests that the missense variants reduce platelet function by altering CalDAG-GEFI function, although we cannot exclude reduced protein expression, as observed previously for the RASGRP2 p.Ser381Phe variant.\textsuperscript{11} There were no consistent phenotype differences between the cases with missense and high-impact variants.

The 11 likely pathogenic RASGRP2 variants occurred in a total of 11 unrelated cases (7 males) as homozygous (7 cases) or compound heterozygous (4 cases) alleles, indicating autosomal recessive inheritance. One pedigree (J) contained a sibling with biallelic RASGRP2 variants (Figure 2A). All the index cases had a first diagnosis of a bleeding disorder during childhood (age 1-14 years; Supplementary table S2). Abnormal bleeding was predominantly mucocutaneous or followed surgery or dental extraction. Gastrointestinal bleeding occurred in two cases (Figure 2B), but there was no intracranial bleeding. Of the nine index cases with available data, seven had required red cell or platelet transfusion on at least one occasion suggesting severe bleeding. There were no other consistently reported phenotypes. Platelet number and size were normal (Supplementary table S2).
All 11 index cases had reduced platelet LTA responses with ADP and epinephrine, and in some cases also with collagen, arachidonic acid and TRAP-6 (Figure 2C, 2D). Detailed analysis of A-II3 and B-II1 showed additionally that the velocity of aggregation to all tested doses of ADP, epinephrine and TRAP-6 was reduced compared with controls (Figure 2D). The normal platelet ultrastructure, nucleotide content and release of alpha (P-selectin exposure) and dense (CD63 exposure) granules in response to high dose TRAP14 indicated no defect in platelet granule biosynthesis. However, dense granule release with ADP was diminished in most tested cases, mirroring the functional defects observed with LTA (Supplementary table S2), similar to previous reports.9,10,11

This report of 11 new pedigrees significantly expands the reported cases with human CalDAG-GEFI deficiency and enables description of this disorder as an autosomal recessive, non-syndromic PFD that is associated with moderate or severe bleeding, similar to other disorders of αIIbβ3 integrin signalling20,21 and some types of Glanzmann thrombasthenia in which there is reduced expression of functional αIIbβ3 integrin.22 We show further that there is a consistent laboratory phenotype of reduced aggregation responses to ADP and epinephrine in all reported cases, but no defect in dense granule secretion, distinguishing this disorder from δ-storage pool disease. This distinctive phenotype is likely to assist genetic diagnosis of further pedigrees.
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AUTHOR CONTRIBUTIONS

SKW and MC wrote the paper with assistance from M-CA, ET and ADM. MC, DG, ET and SKW analysed data. EB, KH, MPL, CMM, PN, SGO, SR-V and CvG provided samples and clinical data. KD managed the ThromboGenomics programme. SP co-ordinated the NIHR BioResource – Rare Diseases BPD project
including ethics and governance. ST and CW provided sample logistics, QC and WGS oversight. KF, MAL and WHO contributed to the study design.

**DISCLOSURE OF CONFLICTS OF INTEREST**

The authors report no relevant conflicts of interest.

**REFERENCES**


FIGURE LEGENDS

Figure 1. Identification and characteristics of pathogenic variants in RASGRP2

A. BeviMed inference analysis applied to rare, non-synonymous RASGRP2 variants observed in all index cases from the NIHR BioResource. Cases were designated as having the indicator phenotype of CalDAG-GEFI deficiency if they had HPO terms indicating bleeding and reduced light transmission aggregation responses to at least three activating agonists. The indicator phenotype was present in 119 index cases and was absent in 5,982 index cases. The posterior probability of the association model was 1 (prior was 0.1) and the posterior probability of recessive inheritance was 1 (prior was 0.5). The RASGRP2 exons are represented by grey blocks. The bar chart above shows the marginal posterior probabilities of pathogenicity for individual variants observed in all NIHR BioResource index cases, conditional on an association under a recessive mode of inheritance. The bar chart beneath indicates whether the variant was observed in an index case with (pink) or without (blue) the indicator phenotype and whether present as a heterozygous and homozygous allele. Variants observed in index cases as compound heterozygous alleles are linked. B. The characteristics of the 11 likely pathogenic RASGRP2 variants across the NIHR BioResource (NBR) and ThromboGenomics (TG) collections. Variants were annotated against the canonical transcript ENST00000354024 using the Variant Effect Predictor (VEP). Population allelic frequencies are derived from the Exome Aggregation Consortium (ExAC). The likely pathogenicity of the variants is expressed as the Combined Annotation Dependent Depletion (CADD) score and the percentage conservation as the proportion of CalDAG-GEFI orthologs in nine species that have the same amino acid as human CalDAG-GEFI. C. Localization
and predicted consequence of the likely pathogenic *RASGRP2* variants identified in NIHR BioResource and ThromboGenomics collections (below protein diagram) and the previously reported variants 9-11 (above protein diagram). The exploded view shows the predicted consequence of the 11:64507638 G>C variant identified as a homozygous allele in case in ThromboGenomics case K II.1. Bioinformatic analysis of the variant sequence predicts preferential use of an alternative exonic splice acceptor, resulting in codon deletion, frameshift and a stop gain at codon 125.

**Figure 2: Characteristics of the 11 index cases with likely pathogenic biallelic *RASGRP2* variants.**

**A.** Pedigrees of the index cases (*) with likely pathogenic *RASGRP2* variants indicating the genotype of the index cases. The black symbols indicate cases with abnormal bleeding and reduced platelet aggregation responses. The white symbols indicate pedigree members without bleeding symptoms and the grey symbols, pedigree members unavailable for evaluation. +/V: heterozygous, V/V: compound heterozygous or homozygous for the variant allele.  

**B.** Annotation of 11 index cases with HPO terms for bleeding symptoms.  

**C.** Summary light transmission aggregation for all 11 index cases. Data are expressed as the proportion of index cases with any reported defect in aggregation responses to ADP, epinephrine (Epi), collagen (Coll), arachidonic acid (AA), TRAP-6 and ristocetin (Risto).  

**D.** Detailed light transmission aggregation response in cases All-3 and BII-1 in response to the stated agonist concentrations. Data are presented as the maximum aggregation and initial velocity of the aggregation responses.
Figure 1

A

B

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C

Genomic sequence

mRNA sequence next potential splicing consensus

Control: CAG.GGT.CAG/G.CCTACCT/AAAGGCGG помощь
K-I: CACAGGGGAGAGCCTACCT/AAAGGCGG помощь

5'→3' start...G.TGA.
Figure 2

A

1. p.N67Lfs*24
3. p.N330K
4. p.R494Afs*54
5. p.R494Afs*54/p.F181S
6. p.F497Sfs*22
7. p.F497Sfs*22
10. p.G305D
11. p.P125*

B

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- HP:0000421 Epistaxis
- HP:0001933 Subcutaneous hemorrhage
- HP:0000132 Menorrhagia
- HP:0006298 Prolonged bleeding after dental extraction
- HP:0004846 Prolonged bleeding after surgery
- HP:0002239 Gastrointestinal hemorrhage

C

% of cases with abnormal maximal aggregation

D

Maximal aggregation

- ADP 2.5µM
- ADP 5µM
- ADP 10µM
- Epi 300µM
- TRAP-6 5µM
- TRAP-6 10µM

- Controls
- p.N67Lfs*24 HOM