Phenotype description and response to therapies in *DIAPH1*-related disorder

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Abstract

DIAPH1-related disorder (D-RD) is a recently discovered syndromic heritable thrombocytopenia associated with *DIAPH1* variants near the carboxyl terminus of the DIAPH1 protein. We report a detailed analysis of the haematological characteristics of 16 D-RD cases from five pedigrees with three different heterozygous *DIAPH1* variants. All the variants predicted truncation of the carboxyl terminus diaphanous autoregulatory domain and loss of a conserved regulatory RRKR motif that mediates DIAPH1 autoinhibition, thereby suggesting a common gain-of-function effect. All the D-RD cases had hearing loss associated with haematological abnormalities including thrombocytopenia (median platelet count 111 $x10^9$/l), enlarged platelets (median MPV 12.7 fl) and mild neutropenia (median neutrophil count 1.33 $x10^9$/l), but no demonstrable red cell or lymphoid abnormalities. The thrombopoietin (TPO) receptor agonist eltrombopag partially restored defective pro-platelet formation in cultured megakaryocytes from three unrelated D-RD cases. However, TPO receptor signalling was unaltered in D-RD platelets compared with controls. Eltrombopag administration to one D-RD case for 28 days before orthopaedic surgery improved platelet counts sufficiently for surgery to proceed without platelet transfusion.

Key points

- *DIAPH1*-related disorder (D-RD) has a bi-lineage haematological phenotype of macrothrombocytopenia and neutropenia associated with hearing loss
- Eltrombopag increased pro-platelet formation from cultured D-RD megakaryocytes and improved circulating platelet counts *in vivo*

The heritable thrombocytopenias (HT) are genetically heterogeneous rare disorders in which reduced circulating platelet mass may be accompanied by non-haematological features.[1,2] Amongst the recently discovered HT, *DIAPH1*-related disorder (D-RD; OMIM #124900) was initially reported in two pedigrees with macrothrombocytopenia associated with hearing loss. This phenotype segregated with a heterozygous p.R1213* variant in *DIAPH1,* predicted to truncate the encoded protein diaphanous homolog 1 (DIAPH1) in the carboxyl terminus diaphanous autoregulatory domain (DAD).[3] It was hypothesised that this conferred gain-of-function to DIAPH1, resulting in megakaryocyte (MK) cytoskeletal dysregulation and impaired proplatelet formation.[3] Thrombocytopenia and hearing loss were subsequently reported in further pedigrees with *DIAPH1* DAD variants [4-6] supporting the designation of D-RD as a distinct syndromic HT. However, other reports of pedigrees with similarly positioned variants report a hearing loss phenotype, but not haematological findings.[7] [8]

In order to clarify the characteristics associated with *DIAPH1* DAD variants, we report a detailed haematological analysis of three previously reported pedigrees[3,6] (A,D and E) and two new D-RD pedigrees (B and C), including the outcome of treatment with the thrombopoietin (TPO) -receptor agonist eltrombopag. Cases were identified through the National Institute for Health Research BioResource -Rare Diseases (Pedigrees A-C; UK REC 13/EE/0325) and Functional and Molecular Characterization of Patients with Inherited Platelet Disorders (Pedigrees D-E; Centro Regional de Hemodonación, Universidad de Murcia, IMIB-Arrixaca, CIBERER-U765, Murcia) programmes. Phenotype collection and high-throughput sequencing were as reported previously.[6,9,10]

The five pedigrees comprised 16 cases with heterozygous *DIAPH1* variants within the DAD (10 males, current ages 2-78 years; Figures 1A). These included the variants predicting p.R1213* (previously reported in pedigrees A and E[3,6] and here in new pedigree D) and p.A1210GfsTer31 (previously reported in an unrelated pedigree[4] and here in new pedigree B). Cases from pedigree C harboured a novel inversion with breakpoints in *DIAPH1* introns 26 and 27, predicting in-frame skipping of exon 27 (p.E1192_Q1220del). Consistent with this, platelet cDNA corresponding to *DIAPH1* exons 26-28 was smaller in pedigree C compared with controls and did not contain exon 27 sequence (supplemental Figure S1). All the *DIAPH1* variants predict truncation within the DIAPH1 DAD (Figure 1B) resulting in loss of the RRKR motif, and for p.E1192_Q1220del also the MDxLLExL motif. These conserved regulatory sequences within the DAD mediate DIAPH1 autoinhibition by competitive binding at the Rho GTPase activation site in the GBD/FH3 domain.[11]

Abnormal bleeding was reported in six D-RD cases and was predominantly mild and mucocutaneous (Figure 2A). Three D-RD cases had previously received platelet transfusions to prevent surgical or obstetric bleeding. The three D-RD cases from pedigree E had multiple hospital attendances with predominantly respiratory tract or cutaneous infections. Bilateral sensorineural hearing loss was detected at neonatal screening or in early childhood in all cases and progressed through childhood. Twelve cases required hearing aids and one case underwent successful cochlear implantation (supplemental Figure S2). There were no other consistently reported clinical features.

All of the 16 D-RD cases displayed mild thrombocytopenia on at least one occasion (median of all recorded platelet counts 111 x10^9/l; range 13-209) and enlarged platelets (median MPV 12.7 fl; range 9.3-19.8). Eleven cases also displayed neutropenia on at least one occasion (median of all recorded neutrophil counts 1.33 x10^9/l; range 0.50-4.30) (supplemental Table S1). These numerical abnormalities were confirmed by peripheral blood smears, but there were no other morphological abnormalities (Figure 2B). Bone marrow biopsies from cases A-2 and D-3 revealed normal architecture and localisation of cells. Case A-2 additionally demonstrated reduced granulopoiesis and MK that were normal in number but small with hypolobated nuclei (Figure 2C). Neutrophil adhesion, degranulation, reactive oxygen species generation and extracellular trap formation was the same in D-RD cases with all three of the *DIAPH1* variants as controls (supplemental Table S2). There were no consistent abnormalities in immunoglobulin concentrations, total or subset lymphocyte numbers or lymphocyte proliferation responses (supplemental Table S2). There was no consistent red cell phenotype.

In order to evaluate the TPO-receptor agonist eltrombopag (Novartis, UK) as a potential therapy to increase platelet count in D-RD, we first evaluated the effect of eltrombopag on TPO receptor signalling in D-RD platelets. In platelets from healthy controls, eltrombopag activates TPO receptor signalling pathways to a lesser extent than TPO and does not enhance agonist-mediated activation.[12] In keeping with this, immune thrombocytopenia patients receiving eltrombopag show no increase in platelet activation *in vivo.*[13] In platelets from D-RD cases with all three variants, clinically relevant concentrations of eltrombopag stimulated only very weak

phosphorylation of pJAK2$^{Y1007/1008}$ and pSTAT5α/β^{Y694} compared to TPO, similar to the effect in control platelets (Figure 2D).

It was reported previously that MK derived from blood CD34+ cells from D-RD case A-2 with the p.R1213* gain-of-function variant displayed abnormal clustering and reduced pro-platelet production as well as abundant and disorganised actin structures when cultured with TPO.[3] This finding was reproduced in MK from case B-4 (p.A1210GfsTer31) and case C-9 (p.E1192_Q1220del), supporting a common effect from the different DAD variants (Figure 2E). For all three cases, culture of MK with eltrombopag instead of TPO, or addition of eltrombopag to TPO reduced the number of abnormal MK clusters and partially reversed defective pro-platelet production (Figure 2F).

We monitored the clinical effect of eltrombopag in case C-9 (*DIAPH1* p.E1192_Q1220del) who in addition to D-RD also had severe osteoarthritis of the right hip requiring arthroplasty. However, because of previous platelet transfusions, case C-9 had acquired anti-HLA A1, A23, A24, A80, B51 and B76 IgG alloantibodies resulting in platelet refractoriness. Eltrombopag 50 mg was administered once daily from day-29 before surgery, increased to 75mg once daily from day-9 until day-1. The platelet count determined using the PLT-F detection endpoint (Sysmex XN series analyser, Kobe, Japan) increased from a baseline of 19 x10^9/l to 75 x10^9/l on day-1 before surgery (Figure 2G). The patient also received tranexamic acid for 72 hours from the start of surgery, but not platelet transfusion. Haemostasis was satisfactory (total estimated blood loss 500 ml vs 493 ml for controls undergoing similar surgery[14]) and there were no adverse events.

This largest ever reported series of 16 cases illustrates that D-RD is a dominant disorder characterised by macrothrombocytopenia and neutropenia associated with highly penetrant hearing loss. This analysis also shows that there are no neutrophil function, lymphoid lineage or red cell abnormalities. Although neutropenia most likely accounted for the recurrent infections observed in one D-RD pedigree (E), clinical immunodeficiency was absent in the other cases. No cases had renal disease, cataracts or neutrophil inclusions distinguishing D-RD from *MYH9*-related disorder in which there may also be macrothrombocytopenia and hearing loss.[15-17] The D-RD phenotype was associated with chain truncation variants close to the carboxyl terminus in the DAD and resulted in loss of a conserved sequence motif responsible for autoinhibitory interactions within the DIAPH1 protein[11]. This suggests a distinct molecular pathogenesis for D-RD, consistent with a gain-of-function effect.

This report also illustrates that eltrombopag partly rescues defective pro-platelet formation in D-RD MK cultured *in vitro*, but that TPO receptor signalling responses in D-RD platelets are unaltered. We provide proof of concept that similar to previous observations in *MYH9*-related disorder[18,19], short term eltrombopag may enable temporary correction of platelet counts in D-RD cases before surgery, enabling avoidance of allogenic platelet transfusion.

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AUTHOR CONTRIBUTIONS

SKW and ADM wrote the paper with assistance from KD, KF and JR. CB, MLL, SGO, TS and CHT provided samples and clinical data. WNE provided bone marrow and blood smear analysis. NM provided DNA sequencing and analysis for pedigree E. CK, SFM, CT and SKW performed laboratory experiments and analysed data. KD

and KG managed and chaired the ThromboGenomics programme respectively. SP co-ordinated the NIHR BioResource – Rare Diseases BPD project including ethics and governance. KF, ML, WHO and ADM contributed to the study design.

DISCLOSURE OF CONFLICTS OF INTEREST

The authors report no relevant conflicts of interest.

FIGURE LEGENDS

Figure 1. Variants in *DIAPH1* associated with DIAPH1-related disorder.

(**A**) Pedigree diagrams demonstrating co-segregation of the *DIAPH1* variants with sensorineural hearing impairment (black shading), and haematological abnormalities (red shading) in five pedigrees. The white shaded symbols indicate unaffected pedigree members. The + and V symbols indicate wild type and variant *DIAPH1* alleles respectively. The grey symbols indicate pedigree members with no data available. Index cases are indicated by the * symbol. (**B**) Schematic representation of *DIAPH1* and DIAPH1 protein divided into functional domains, including the diaphanous autoregulatory domain (DAD) near the carboxyl terminus. The expanded box shows the wild type DAD amino acid sequence with the positions of the regulatory RRKR and MDxLLExL sequence motifs indicated by the red shading. The predicted impact of the variants associated with D-RD is shown compared to the reference sequence. The abnormal carboxyl terminus amino acid sequence predicted from the p.A1210GfsTer31 variant is indicated in red font. The position of exon 27 residues that are absent with the p.E1192_Q1220del variant are indicated with the dashed line.

Figure 2. Detailed evaluation of DIAPH1-related disorder (D-RD) cases.

(**A**) Annotation of the 16 D-RD cases with human phenotype ontology terms for bleeding symptoms. Red shading indicates the presence of the bleeding symptom. Grey shading indicates a symptom was not applicable due to patient age or gender. (**B**) Representative peripheral blood smears from D-RD case A2 illustrating macrothrombocytopenia and morphologically normal neutrophils. Original magnification x40 (left) and x100 (right). (**C**) Haematoxylin and eosin stained bone

marrow biopsy from D-RD case A2. Granulopoiesis was reduced with few examples of mature neutrophils. Megakaryocytes were normal in number but generally small with hypolobated nuclei (arrowed). Original magnification x40 (left) and x100 (right). (**D**) Representative immunoblot using monoclonal antibodies recognising pSTAT5α/β^{Y694}, total STAT5α/β, pJAK2$^{1007/1008}$ and total JAK2 of lysates from case A-2 and control platelets stimulated with eltrombopag (0-30 μM) or thrombopoietin (TPO; 100 ng/mL). The histograms of the ratio of phosphorylated to total densitometry signal of three D-RD cases (A-2, B-4 and C-9) combined show that eltrombopag causes markedly reduced STAT5α/β and JAK2 phosphorylation compared to TPO and that the extent of phosphorylation in D-RD platelets is the same as controls. The data are representative of three independent experiments expressed as mean ± standard error of the mean. (**E**) Representative immunofluorescence confocal microscopy images of differentiated peripheral blood CD34+ derived MKs at day 12 of culture visualized using anti-integrin β3 (green; CD61) and phalloidin (red; F-actin) staining for case A-2 and controls. In the presence of thrombopoietin (TPO) the D-RD MKs show abnormal clustering, reduced pro-platelet formation and abnormal distribution of F-actin when compared with controls. Reduced pro-platelet formation and cluster formation is partially rescued in culture conditions containing eltrombopag (EP). Bars represent 20 μm. (**F**) Corresponding histograms of aggregate data from duplicate MK differentiation experiments from three unrelated healthy controls and cases A-2, B-4 and C-9 with each of the three D-RD variants cultured with TPO (5 uM), EP (3 uM) or TPO (2.5 uM) with EP (2 uM). Data are expressed as mean and standard error of the mean of the percentage of all cultured MK that associate in clusters and the percentage that are forming proplatelet extensions as specified in the supplement methods. A one-

way ANOVA was used for statistical analysis. *** $p<0.0001$, ** $p<0.001$, * $p<0.05$, ns not significant. (**G**) The time course of the haematological response to oral eltrombopag administered to case C-9 before elective hip arthroplasty at day 0. Platelet counts were determined using a Sysmex XN analyser using impedance (PLT-I), fluorescence (PLT-F) and optical (PLT-O) endpoints.

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