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PLATELETS AND THROMBOPOIESIS

Enrichment of FLI1 and RUNX1 mutations in families with excessive bleeding and platelet dense granule secretion defects

Jacqueline Stockley, Neil V. Morgan, Danai Bem, Gillian C. Lowe, Marie Lordkipanidzé, Ban Dawood, Michael A. Simpson, Kirsty Macfarlane, Kevin Horner, Vincenzo C. Leo, Katherine Talks, Jayashree Motwani, Jonathan T. Wilde, Peter W. Collins, Michael Makris, Steve P. Watson, and Martina E. Daly, on behalf of the UK Genotyping and Phenotyping of Platelets Study Group

Introduction

Inherited platelet function disorders (PFDs), which cause excessive bleeding, are heterogeneous and can seldom be linked to a causative gene using clinical and laboratory phenotype alone. The advent of techniques allowing selective capture and enrichment of target gene sequences, coupled with next-generation sequencing (NGS) technology, has facilitated the rapid identification of disease-associated variants by enabling the simultaneous analysis of large numbers of genes. We have used NGS to index cases from 13 unrelated families recruited to the UK Genotyping and Phenotyping of Platelets (UK GAPP) study with significantly reduced platelet dense granule secretion using lumi-aggregometry.

Study design

Recruitment of patients and platelet phenotyping

Index cases and affected relatives from a subgroup of 13 families, recruited to the UK GAPP study on the basis of excessive clinical bleeding and a suspicion of an inherited platelet disorder, were investigated. These individuals were diagnosed with a platelet dense granule secretion defect following platelet phenotyping but had no other features of Hermansky-Pudlak syndrome. The inclusion and exclusion criteria for recruitment to the study, and the criteria for diagnosis of a dense granule secretion defect, have been described. Platelet aggregation and adenosine triphosphate (ATP) secretion were assessed in platelet-rich plasma using a rationalized panel of platelet agonists and a dual-channel Chronolog lumi-aggregometer. This study was approved by the National Research Ethics Service Committee West Midlands–Edgbaston (REC reference: 06/MRE07/36) and conducted in accordance with the Declaration of Helsinki.

Analysis of candidate genes

Genomic DNA was isolated from peripheral blood, and NGS of candidate PFD genes was undertaken on an ABI-SOLiD3+ or an Illumina Genome Analyzer IIx platform. Novel sequence variations identified by NGS were confirmed by conventional Sanger sequencing. Where possible, patients were selected for NGS analysis on the basis of having an affected relative who was also available for study.

Key Points

- Novel FLI1 and RUNX1 alterations were identified in 6 of 13 patients with excessive bleeding and platelet granule secretion defects.
- Two FLI1 alterations predicting amino acid substitutions in the DNA-binding domain of FLI1 abolished transcriptional activity of FLI1.

We analyzed candidate platelet function disorder genes in 13 index cases with a history of excessive bleeding in association with a significant reduction in dense granule secretion and impaired aggregation to a panel of platelet agonists. Five of the index cases also had mild thrombocytopenia. Heterozygous alterations in FLI1 and RUNX1, encoding Friend leukemia integration 1 and RUNT-related transcription factor 1, respectively, which have a fundamental role in megakaryocytopoiesis, were identified in 6 patients, 4 of whom had mild thrombocytopenia. Two FLI1 alterations predicting p.Arg337Trp and p.Tyr343Cys substitutions in the FLI1 DNA-binding domain abolished transcriptional activity of FLI1. A 4-bp deletion in FLI1, and 2 splicing alterations and a nonsense variation in RUNX1, which were predicted to cause haploinsufficiency of either FLI1 or RUNX1, were also identified. Our findings suggest that alterations in FLI1 and RUNX1 may be common in patients with platelet dense granule secretion defects and mild thrombocytopenia. (Blood. 2013;122(25):4090-4093)
Results and discussion

To date, 366 patients, from 292 unrelated families, have been recruited to the UK GAPP study, of whom 56 (15%) have been classified as having a secretion defect. NGS analysis of candidate PFD genes selected, where possible, on the basis of having an affected relative who was also available for study. Novel heterozygous alterations in either FLI1 or RUNX1 were identified in 6 of the families (F1 to F6) (Table 1). Two nonsynonymous FLI1 alterations, c.1009C>T and c.1028A>G, predicting p.Arg337Trp and p.Arg337Trp, respectively, and a nonsense mutation in codon 106 (c.317G>A; p.Trp106Stop) were also associated with alopecia, eczema or psoriasis, and recurrent viral infections in affected individuals in F1, and with mild thrombocytopenia, alopecia, and eczema in the affected individuals in F2 (Figure 1A-B).

Examination of platelet secretion and aggregation in all subjects carrying FLI1 or RUNX1 defects consistently revealed the predominant platelet abnormality to be a significant reduction in platelet ATP secretion in response to all agonists tested (Figure 1C, Table 1, supplemental Figure 2, and data not shown). As reported previously for patients with dense granule secretion defects, reductions in platelet aggregation in response to collagen and PAR-1 were observed in some, but not all, subjects and were usually more apparent at low or intermediate agonist concentrations (data not shown).3

FLI1 is a member of the ETS (E-twenty six) family of transcription factors, which is expressed primarily in hematopoietic cells and regulates genes expressed both early and late in megakaryocytogenesis, including GP6,6,7,8-10 Patients with Paris-Trousseau syndrome (PTS), who are hemizygous for FLI1 as a result of a deletion of chromosome 11q23, have an increased tendency to bleed, which is associated with thrombocytopenia, and enlarged platelets displaying giant α-granules.10,12 It is thought that transient monoallelic expression of FLI1 at an early stage during megakaryocytogenesis, coupled with hemizygous loss of FLI1 in patients with PTS, which results in a complete lack of FLI1 expression, accounts for the generation of 2 distinct subpopulations of normal and immature megakaryocytes in patients with PTS.10 The identification of alterations predicting substitutions in the DNA-binding domain of FLI1 and their association with symptoms in 2 families suggests a role for FLI1 in the pathogenesis of bleeding in these families.

We investigated whether the p.R337W and p.Y343C substitutions could alter the DNA-binding capacity of FLI1 by examining the ability of the recombinant FLI1 variants to bind to an ETS site in the GP6 promoter using the dual-luciferase reporter assay to measure

### Table 1. Genotypic and phenotypic characteristics of subjects with platelet dense granule secretion defects

| Family (F)/Patient identification* | FLI1 or RUNX1 alteration† | Effect | Platelet count (x 10^9/L)‡ | Mean platelet volume (fL) | ATP secretion (nmol/L x 10^9 platelets)§ | Mean platelet ATP secretion (nmol/L x 10^9 platelets) ||
|-----------------------------------|---------------------------|--------|---------------------------|--------------------------|------------------------------------------|--------------------------------------------|
| F1; I.1                           | FLI1; c.1009 C>T          | p.Arg337Trp | 244 | 10.3 | 0.37 | |
| F1; III.1                         | FLI1; c.1009 C>T          | p.Arg337Trp | 180 | 10.5 | 0.07 | |
| F2; II.5                          | FLI1; c.1028 A>G          | p.Tyr343Cys | 92 | 8.8 | NDII | |
| F2; II.1                          | FLI1; c.1028 A>G          | p.Tyr343Cys | 117 | 8.6 | NDII | |
| F3.1                              | FLI1; c.992-995del        | p.Asn331Thrfs*4 | 142 | 11.4 | 0.38 | |
| F3.2                              | FLI1; c.992-995del        | p.Asn331Thrfs*4 | 157 | 11.8 | 0.57 | |
| F4.1                              | RUNX1; c.508+1 G>T        | Splicing | 302 | 7.3 | 0.32 | |
| F4.2                              | RUNX1; c.508+1 G>T        | Splicing | 70 | 7.5 | ND | |
| F4.3                              | RUNX1; c.508+1 G>T        | Splicing | 130 | 7.1 | 0.62 | |
| F5                                | RUNX1; c.351+1G>T         | Splicing | 139 | 8.0 | 0.35 | |
| F6.1                              | RUNX1; c.317 G>A          | p.Trp106Stop | 100 | 8.0 | 0.25 | |
| F7                                | –                         | –       | 233 | 8.5 | 0.57 | |
| F8                                | –                         | –       | 190 | 8.5 | ND | |
| F9                                | –                         | –       | 205 | 8.4 | 0.48 | |
| F10                               | –                         | –       | 370 | 8.6 | 0.31 | |
| F11                               | –                         | –       | 225 | NA | 0.63 | |
| F12                               | –                         | –       | 245 | NA | 0.52 | |
| F13                               | –                         | –       | 114 | 10.9 | 0.12 | |

Heterozygous nucleotide changes present in FLI1 and RUNX1 and their predicted effects on the resulting RNA or protein are shown.

NA, not available; ND, not detectable.

†Alterations are numbered according to positions in the NM_002017.3 and NM_001754 transcripts for FLI1 and RUNX1, respectively.

‡Mean platelet counts are shown, the normal reference range is 150 x 10^9 to 400 x 10^9 platelets per L, and thrombocytopenia is defined as platelet count <150 x 10^9 platelets per L.

§ATP secreted in response to 100 μM of PAR-1 receptor–specific peptide SFLLRN; fifth percentile in healthy volunteers is 0.82 nmol/L x 10^9 platelets.

∥ATP secretion was measured in response to 1 U/mL of thrombin in the center where these subjects were recruited, against a normal reference range of 0.73 to 1.80 nmol/L x 10^9 platelets.
GP6 promoter activity. There was a complete loss in the ability of the R337W and Y343C FLI1 variants to induce GP6 promoter activity compared with that of WT-FLI1 ($P < .001$; Figure 1E). Furthermore, coexpression of either the R337W or the Y343C variant with WT-FLI1, to mimic heterozygosity, resulted in significant reductions in the transcriptional activity to $60\%$ of that of WT-FLI1 alone ($P < .01$), indicating that these mutations disrupt DNA binding of FLI1 and will cause a reduction in activation of megakaryocyte-specific genes (Figure 1E). Of interest is a previous study, which showed that R337 is critical for the function of nuclear localization signal 2 of FLI1 and that an R337A substitution caused a reduction in nuclear accumulation of FLI1. However, as with the R337W variant, the R337A substitution was shown to disrupt the DNA-binding ability of FLI1 and, consequently, to downregulate expression of FLI1-inducible megakaryocyte-specific genes.

As a member of the RUNT family of transcription factors, the role of RUNX1 in regulating megakaryopoiesis and platelet formation is well established. RUNX1 has been shown to cooperate with FLI1 in the late stages of megakaryopoiesis. Inherited RUNX1 mutations are recognized to lead to autosomal dominant thrombocytopenia and impaired platelet function and are associated with a propensity to myelodysplastic syndrome and acute myeloid leukemia. RUNX1-mediated silencing of the MYH10 gene is required for the switch from mitosis to endomitosis during megakaryocyte maturation. The persistence of MYH10 in platelets was recently proposed as a biomarker for RUNX1 alterations in patients with familial thrombocytopenia and for FLI1 deletions in PTS. The detection of MYH10 in platelets from patients carrying the p.Tyr343Cys and the p.Asn331Thrfs*4 FLI1 variations confirms the use of MYH10 detection as a biomarker for FLI1 alterations (Figure 1F). Indeed, immunoblot detection of MYH10 expression in platelets would be a useful initial screening test for inherited platelet disorders caused by abnormalities in RUNX1 or FLI1. Dosage of RUNX1 is thought to contribute to the risk of AML because missense mutations that result
in variants that can heterodimerize with normal RUNX1 and act in a dominant-negative manner to compete with the normal protein have been reported to be associated with a higher risk of hematologic malignancy than mutations resulting in haploinsufficiency. The absence of hematologic malignancies to date in the 3 families with RUNX1 mutations identified in this study, which were all predicted to result in haploinsufficiency, would support this observation.

The identification of RUNX1 or FLI1 alterations in 6 of 13 unrelated index cases with dense granule secretion disorder, 4 of whom had thrombocytopenia, suggests that defects in these genes will account for a significant proportion of patients with defects in platelet granule secretion and excessive bleeding.

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