FIVE NEW PEDIGREES WITH INHERITED RUNX1 MUTATIONS CAUSING FAMILIAL PLATELET DISORDER WITH PROPENSITY TO MYELOID MALIGNANCY (FPD/AML)

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ABSTRACT

Familial platelet disorder with propensity to myeloid malignancy (FPD/AML) is an autosomal dominant syndrome characterised by platelet abnormalities and a predisposition to myelodysplasia (MDS) and/or acute myeloid leukemia (AML). The disorder, caused by inherited mutations in RUNX1, is uncommon with only 14 pedigrees reported. We screened 10 families with a history of more than one first degree relative with MDS/AML for inherited mutations in RUNX1. Germline RUNX1 mutations were identified in 5 pedigrees with a 3:2 predominance of N-terminal mutations. Several affected members had normal platelet counts or platelet function, features not previously reported in FPD/AML. The median incidence of MDS/AML among carriers of RUNX1 mutation was 35%. Individual treatments varied but included haematopoietic stem cell transplantation (HSCT) from siblings before recognition of the inherited leukemogenic mutation. Transplantation was associated with a high incidence of complications including early relapse, failure of engraftment and post-transplantation lymphoproliferative disorder. Given the small size of modern families and the clinical heterogeneity of this syndrome, the diagnosis of FPD/AML could be easily overlooked and may be more prevalent than previously recognized. Therefore, it would appear prudent to screen young patients with MDS/AML for RUNX1 mutation, prior to consideration of sibling HSCT.
INTRODUCTION

Familial platelet disorder with propensity to myeloid malignancy (FPD/AML) is an autosomal dominant disorder characterised by mild-moderate thrombocytopenia, abnormalities of platelet function and a propensity to develop myelodysplasia (MDS) and/or acute myeloid leukemia (AML). Since the discovery of germline mutations in \textit{RUNX1} (previously \textit{AML1} or \textit{CBFA2}) in FPD/AML in 1999\cite{1}, 14 pedigrees have been described with confirmed germline \textit{RUNX1} mutations.\cite{2-7} Considerable phenotypic and genotypic variability exists. Most mutations are unique to the individual pedigree with only a single incidence of the same mutation in 2 distinct pedigrees.\cite{1,5} The median incidence of myeloid malignancy in FPD/AML is 35\% such that these individuals with germline \textit{RUNX1} mutations are at significant risk of leukemia development.

\textit{RUNX1} is a key regulator of definitive hematopoiesis and of myeloid differentiation.\cite{8} The protein belongs to a small family of transcription factors which share homology for a region designated ‘runt homology domain’ (RHD). The RHD directs binding of RUNX1 to DNA sequences of target genes and also mediates interaction between RUNX1 and the \(\beta\)-subunit, core-binding factor \(\beta\) (CBF\(\beta\)). The gene is commonly dysregulated in sporadic cases of MDS and AML, mostly by translocations in AML\cite{9} and by point mutations in MDS.\cite{10} An exception occurs in the most undifferentiated subtype of AML, FAB class M0, in which point mutations occur in \(\sim\)20\% of cases.\cite{11-13} A similar frequency (\(\sim\)25\%) is observed in certain subtypes of \textit{de novo} MDS/AML (including refractory anemia with excess blasts [RAEB], AML with multilineage dysplasia and AML following MDS)\cite{10} with an even higher frequency in
therapy-related MDS/AML and radiation-associated MDS/AML (tested in survivors of the atomic bomb in Hiroshima).14

Familial RUNX1 mutations generally cluster to the N-terminal region of the gene within the RHD in exons 3-5, often disrupting DNA binding but allowing ongoing dimerization with CBFβ. Familial mutations have been reported less frequently in the C-terminal region and these typically maintain DNA binding and dimerization with loss of the trans-activation region. The nature of RUNX1 mutations in de novo MDS/AML appears similar to that seen in FPD/AML with a predominance of point mutations in the RHD.1,2,10,14

Regardless of the cause of MDS/AML, effective treatment options are limited and the only curative therapy for MDS is hematopoietic stem cell transplantation (HSCT) with its relatively high treatment-related mortality and morbidity. Inherited RUNX1 mutations can have implications for the selection of donors for HSCT, as one would prefer to exclude a sibling donor with the same leukemogenic mutation. Herein, we describe 5 previously unreported pedigrees with FPD/AML in which some affected individuals demonstrate clinical features not previously reported in the disorder and in which several individuals underwent HSCT from affected siblings prior to detection of the inherited RUNX1 mutation, often with poor outcomes. These cases exemplify the risks of sibling donor HSCT in FPD/AML and highlight the need for vigilance for the familial presentation of RUNX1 mutation.

**METHODOLOGY**
Patients

Individuals from families with > 1 first-degree relative with MDS or AML were selected for investigation. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki and independent ethics approval was obtained from the BC Cancer Agency Research Ethics Board (Canada) and the Riverside Ethics Committee for Barts and the London NHS Trust (UK).

Mutation analysis of RUNX1

Peripheral blood, bone marrow aspirate and/or saliva samples were obtained from study subjects. Genomic DNA was extracted from peripheral blood or bone marrow aspirate mononuclear cells using phenol and chloroform, following standard procedures. Saliva was collected in Oragene vials (DNA Genotek, Ottawa, Canada) and DNA was purified and extracted following manufacturer’s instructions.

Exons 1, 3-6, 7b and 8 of RUNX1 were separately amplified by polymerase chain reaction (PCR) using intronic primers as previously described. PCR products were purified using gel columns (Montage, Millipore Corporation, Bedford, USA), sequenced using the respective forward and reverse primer and analysed on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, USA). Mutations were confirmed either by repeat sequencing or by cloning sequencing using the plasmid vector pCR 2.1 TOPO (Invitrogen, Carlsbad, USA).

RESULTS
Individuals from 10 families were investigated. Five families had a history of at least one individual with platelet abnormalities and all of these demonstrated novel RUNXI mutations. The remaining 5 pedigrees were heterogeneous, have no detectable mutations in RUNXI or CEBPA and continue to be investigated. The 5 FPD/AML pedigrees labelled A, B, C, D and E and their corresponding mutations (3 N-terminal, 2 C-terminal) are presented in Figure 1. Clinical characteristics are presented in Table 1. Supplementary Figure 1 demonstrates a schematic representation of the positions and consequences of the 5 mutations in relation to the normal RUNXI protein.

**Pedigree A:** The proband (I-2) was followed for many years with moderate thrombocytopenia and a presumed diagnosis of chronic immune thrombocytopenic purpura (ITP). Her daughter (II-2) developed MDS at age 37 without previous detection of thrombocytopenia. II-2 underwent 2 HSCTs from her HLA-matched sibling (II-1) who had normal blood counts at the time. She relapsed 8 months following the first HSCT but remains well and in complete remission (CR) 6 years following the second HSCT. I-2 subsequently developed MDS at age 63 and died of AML at age 64. The history of MDS in mother and daughter prompted investigations of the other siblings. II-3 was noted to have moderate thrombocytopenia and a history of easy bruising only in association with trauma. His bone marrow aspirate and trephine biopsy demonstrated mild dysplastic features, which were consistent with but not diagnostic of MDS. II-1 was also noted to have mild, asymptomatic thrombocytopenia, which was not present when he was selected as the HSCT donor for his sister. The final sibling II-4 had a history of childhood ALL at age 12, treated
with intensive chemotherapy. He has been in a CR for nearly 30 years with normal blood counts.

Genomic DNA was extracted from saliva, peripheral blood and bone marrow from II-1, II-3 and from saliva from II-4. DNA from the diagnostic MDS bone marrow was available for II-2. A 7 base pair (bp) deletion in exon 8 (1007_1013del) was detected in all available samples from Pedigree A causing a frameshift mutation with a new termination at amino acid 563 (G336fsX563).

_Pedigree B:_

The proband III-1 presented for investigation of familial thrombocytopenia. She had been treated with splenectomy for a presumptive diagnosis of chronic ITP in her 20s and had maintained a platelet count between 50-100 x 10⁹/L with no further treatment. Platelet function studies had revealed an abnormal aggregation response to collagen and epinephrine. She reported a family history of many individuals with thrombocytopenia and mild bleeding tendencies and of a history of “leukemia” in her father (II-2) and grandfather (I-1), who were both deceased and for whom further clinical information could not be obtained. Her brother (III-4) had a lifelong history of epistaxis and was also noted to have thrombocytopenia at age 20. He developed AML with normal karyotype at age 28 and was treated with an HSCT from his sister (III-2), who had a normal platelet count. The donor cells failed to engraft after the first HSCT and he was re-transplanted 18 months later from the same donor, but subsequently died of graft versus host disease. III-1’s paternal aunt (II-5) had a long history of excessive bleeding with a release-type platelet aggregation defect in
response to ADP, collagen, epinephrine and arachidonic acid, but with a normal platelet count. She had similarly undergone a sibling HSCT (for AML with monosomy 5) from a brother (II-6). II-6 had mild thrombocytopenia and mild dyserythropoiesis with a normal karyotype on bone marrow analysis, prior to being selected as the transplant donor. II-5 developed post-transplant lymphoproliferative disorder and died 3 months after the HSCT. Several of II-5's other siblings (II-1, II-3, II-7) are also followed for moderate thrombocytopenia as are III-1's children (IV-1, IV-2) whose platelet function screening reveal abnormal closure times on PFA-100 testing.

Genomic DNA was extracted from saliva from III-1. DNA from the diagnostic bone marrow aspirates for II-5 and III-4 were also available. A single bp insertion was detected in all available samples from Pedigree B in exon 3 (83insG) causing a frameshift mutation and an early truncated protein at amino acid 109 (A28fsX109).

**Pedigree C**: The proband (II-2) and her sister (II-1) were diagnosed with moderate thrombocytopenia in childhood after their father (I-1) died of AML at age 29. II-2 was later diagnosed with MDS (refractory anemia with multilineage dysplasia, with trisomy 8) at age 45, underwent an unrelated donor HSCT and is currently well. II-2’s children, III-2 and III-3 have asymptomatic, mild thrombocytopenia. II-1 has a lifetime history of thrombocytopenia and easy bruising, previously diagnosed as chronic ITP. A bone marrow aspirate and biopsy of II-1 at age 49 revealed MDS (refractory anemia with ringed sideroblasts) with normal karyotype, which has not yet required treatment.
Genomic DNA was extracted from saliva from I-2, II-1, II-2, III-2 and III-3 and from peripheral blood from II-1. A missense mutation was detected in exon 4 (286G→C) in all samples collected from affected members (II-1, II-2, III-2, III-3) from Pedigree C (D96H). The unaffected family member, I-2 lacked the mutation.

**Pedigree D:** The proband (II-1) presented at age 21 with mild thrombocytopenia and easy bleeding. Her mother (I-2) had previously been followed for thrombocytopenia, diagnosed at a similar age. Both women were also noted to have platelet function abnormalities with absent aggregation to epinephrine and reduced aggregation to collagen and ADP. I-2 developed MDS (chronic myelomonocytic leukaemia with 11q23 deletion) in her late 40s and died 8 years later. I-2’s brother (I-3) died of AML at age 64 after a history of easy bruising but a normal platelet count and normal platelet function tests. His daughter (II-3) was noted to have thrombocytopenia at age 12, developed MDS with normal karyotype at age 23 and quickly progressed to AML. She died from complications of pancytopenia during treatment.

Genomic DNA was available from the peripheral blood of II-1. A single nucleotide substitution of G→A was detected at the splice donor site between exon 3 and intron 3, in II-1 predicted to cause altered splicing. Two previously described pedigrees have been reported with splice site mutations at the exon3/intron3 boundary but these were splice acceptor mutations, resulting in a K90fs predicted protein product.

**Pedigree E:** The proband (I-2) had a history of chronic thrombocytopenia since childhood. She developed MDS with normal karyotype at age 52 and received an HSCT from her brother (I-3) with normal engraftment and no early transplant
complications. Her son (II-2) was treated for biphenotypic AML at age 8 and received an HSCT from his sister (II-1) at that time. He had no history of thrombocytopenia or bleeding tendency prior to his diagnosis of AML. He remains well 26 years following the HSCT. Neither II-1 nor II-2 currently have thrombocytopenia.

Genomic DNA was extracted from peripheral blood and saliva from I-2 and II-2 and from peripheral blood of the donor sibling, II-1. A nonsense mutation was detected in exon 7b (877C→T) in blood and saliva of I-2, resulting in an early termination at amino acid 292 (R292X). II-1 demonstrated a wildtype RUNXI configuration. Of note, although her son, II-2, had childhood AML, R292X was not detected in his saliva or peripheral blood DNA. Since it is unlikely that the occurrence of AML in this individual represents a sporadic event, it seems probable that the tissues tested for mutation are of donor (II-1) origin.15

DISCUSSION

Investigation of families with MDS/AML is often limited by the fact that modern pedigrees are small, living-affected family members are few and DNA is rarely available from deceased patients. Despite these difficulties, previous investigations detected germline mutations in RUNXI and CEBPA, genes also frequently mutated in de novo MDS/AML. This study reveals 5 new cases of inherited RUNXI mutations and provides new clinical insight into the resultant syndrome of FPD/AML. Though familial presentation of MDS and/or AML is rare, the detection of these new FPD/AML families, comprising 50% of the pedigrees investigated, suggests that inherited RUNXI mutations may occur more frequently than previously appreciated.
The increasing availability of molecular testing, along with a heightened awareness of this syndrome may also lead to the identification of additional families in the near future.

Fourteen families have been previously reported with inherited \textit{RUNXI} mutations causing FPD/AML\textsuperscript{1-7}. In these cases, there was a strong predominance of N-terminal mutations with just 3 families having germline C-terminal mutations. In our series, 3 N-terminal and 2 C-terminal germline \textit{RUNXI} mutations were detected. These new cases suggest that effective screening of the \textit{RUNXI} gene should not be restricted to the RHD and that C-terminal mutations may be more frequent than previously recognized.

Although the molecular basis of FPD/AML as caused by germline \textit{RUNXI} mutations is constant, the clinical phenotype of affected individuals is heterogeneous. The most consistent clinical feature is that of a mild to moderate bleeding tendency due to platelet functional and/or quantitative abnormalities. This feature is absent in many affected individuals in our 5 pedigrees. Several affected family members lacked a bleeding history and thus did not have platelet function studies performed. Platelet counts were also frequently normal. Individuals within Pedigree A were noted to develop mild thrombocytopenia only in their 40s with no pre-existing history of bleeding tendency. Similarly, II-5 from Pedigree B had a normal platelet count 5 years prior to her diagnosis and death from AML, but with a mild bleeding tendency and release-type platelet function abnormalities. Our results suggest that neither complete blood counts nor platelet function studies are a sensitive test for inherited \textit{RUNXI} mutations. Of note, in Pedigree A, a carrier also developed sporadic
childhood ALL, suggesting that the phenotype of FPD/AML may not be restricted to myeloid malignancies. This may not be unreasonable given the importance of \textit{RUNXI} in ALL (with \~25\% of pediatric ALLs involving the \textit{TEL-AML1} [\textit{ETV6-\textit{RUNXI}}] translocation).\textsuperscript{16}

Overall, fewer than 50\% of individuals who inherit a \textit{RUNXI} mutation develop MDS/AML and there is a large range in the prevalence of malignancy between families (20-60\%).\textsuperscript{17} Our series confirms the median incidence of MDS/AML of 35\% among carriers of germline \textit{RUNXI} mutations. The age at presentation of MDS/AML (6-75 years) is also variable but not extended by our series.\textsuperscript{17} This large age range is an important feature in that it may obscure the diagnosis of FPD/AML if offspring develop MDS/AML before their parents/grand-parents. The accumulation of clinical data from additional families will hopefully help to identify the factors that are important in the development of overt malignancy in individuals with inherited \textit{RUNXI} mutations; however, no information is currently available to predict which patients will develop malignancy or when. The latency period required before onset of overt leukemia suggests that secondary mutations are necessary to trigger MDS/AML and that a heterozygous \textit{RUNXI} mutation is insufficient alone.

Investigations of \textit{de novo} cases of MDS/AML with \textit{RUNXI} mutations have revealed several secondary mechanisms involved in the development of overt malignancy. Half of AML M0 cases demonstrate biallelic \textit{RUNXI} mutations while MDS cases frequently harbour additional karyotypic abnormalities.\textsuperscript{12,18} However, similar recurrent secondary aberrations have not been observed in FPD/AML; in particular, acquired mutations in the wild-type \textit{RUNXI} allele are not observed. This is in contrast...
to familial AML involving germline CEBPA mutations in which somatic C-terminal mutations are acquired, in addition to the germline N-terminal mutations, promoting overt leukemia. Secondary cytogenetic abnormalities occur frequently in FPD/AML but these are not consistent within or between pedigrees. These studies highlight the importance of serial monitoring and collection of material from affected individuals to allow for investigation of the secondary events that promote overt malignancy, which is currently lacking.

Previous studies suggest that the occurrence of distinct mutations between families contributes to the variable phenotypes of FPD/AML. Michaud et al demonstrated that some mutant proteins exert dominant-negative effects and that the pedigree with the highest incidence of leukemia had the highest predicted dominant-negative activity. Evidence of dominant-negative activity was recently confirmed by Matheny et al, when they examined the effects of different RHD mutations on RUNX1 function. The family with the highest incidence of leukemia among our pedigrees was Pedigree D (75%) in which the mutation occurred early in the RHD and would be predicted to act by a mechanism of haploinsufficiency.

Unfortunately, outcomes for MDS/AML are generally poor and current treatment options are unsatisfactory, with HSCT being the only potentially curative treatment for MDS. Long-term data on the outcomes of patients treated for FPD/AML-associated leukemias is limited, making a determination of prognosis in these patients difficult. Until recently, only a single family with FPD/AML had been reported in which HSCT was undertaken from a sibling donor who also possessed a germline RUNXI mutation. In that case, the recipient had slow and incomplete engraftment.
and within 2 years, developed AML of donor origin. The donor-derived AML was treated with an unrelated donor HSCT but the patient subsequently died of an EBV-associated post-transplantation lymphoproliferative disorder. A later report by Kirito et al described a family in which members were noted to have thrombocytopenia once investigated as potential HSCT donors for their MDS-affected sibling. The presence of thrombocytopenia in several family members prompted screening for germline \textit{RUNXI} mutations, confirming an inherited predisposition and preventing the siblings from being selected as HSCT donors.\textsuperscript{7} Our series includes 3 additional families in which siblings were used as HSCT donors, prior to documentation of an inherited \textit{RUNXI} mutation. Individual II-5 from Pedigree B received an HSCT from an affected sibling and subsequently died of post-transplantation lymphoproliferative disorder. It is notable that this is the second reported case of a fatal EBV-related lymphoproliferative disorder in FPD/AML.\textsuperscript{3} Another individual within this family (III-4) had a sibling HSCT with failure of engraftment. In Pedigree A, the donor (II-1) was noted to have normal peripheral blood counts and bone marrow morphology prior to HSCT but developed thrombocytopenia 5 years later. The recipient of the HSCT (II-2) relapsed quickly after a first transplant but was re-transplanted with the same sibling donor and is now well 6 years later. Pedigree E also involves sibling HSCT, in this case with good results. These cases, although few, suggest that results of HSCT from siblings with \textit{RUNXI} mutations have a high risk of failure.

The small size of modern families along with the variability in age at presentation of disease in affected individuals will result in inherited \textit{RUNXI} mutations being easily overlooked. A single case of MDS/AML within a small family may be a subtle presentation of FPD/AML. The current description of 5 new cases of familial \textit{RUNXI}
mutations illustrates that this syndrome is likely more prevalent than previously appreciated. This highlights the possibility that some presumed cases of de novo MDS/AML with RUNXI mutations could represent unrecognised cases of FPD/AML. This occurrence was recently observed in the setting of inherited CEBPA mutations where Corbacioglu et al found germline CEPBA mutations in 2 of 18 AML patients with known CEBPA mutations. Therefore, we recommend that young patients with MDS/AML, irrespective of family history, be investigated for RUNXI mutations to select those whose families may also be at risk.

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Figure 1 Pedigrees for the 5 FPD/AML families and their corresponding DNA sequencing traces for the mutated RUNX1 gene and the corresponding wild type sequence. Within the pedigrees, squares denote males and circles denote females. Black boxes denote confirmed RUNX1 mutations, dark grey boxes denote a history of MDS/AML, pale grey boxes represent a history of thrombocytopenia and unfilled boxes represent unaffected individuals. Sequence traces for A and B represent the
sequence analysis of the cloned mutated allele while C, D and E are direct from patient genomic DNA. A 7 bp deletion in exon 8, B 1 bp insertion in exon 3, C C→G missense mutation in exon 4, D splice donor site mutation in intron 3 and E C→T nonsense mutation. The nucleotide numbers are relative to the start codon ATG defined as +1 of the AML1b transcript (blast accession no: L34598) as previously reported.15
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<th>Age at diagnosis of MDS/AML (years)</th>
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<td>Collagen - ↓ response</td>
<td>Epinephrine no response</td>
<td>21</td>
<td>None</td>
</tr>
<tr>
<td>D II-3</td>
<td>Unknown</td>
<td>Yes</td>
<td>Unknown</td>
<td>12</td>
<td>AML evolved from MDS (RAEB) with normal karyotype</td>
<td>23</td>
<td>SNA</td>
</tr>
<tr>
<td>E I-2</td>
<td>Yes</td>
<td>Yes</td>
<td>Unknown</td>
<td>10</td>
<td>MDS</td>
<td>52</td>
<td>R292X (C terminal)</td>
</tr>
<tr>
<td>E II-2</td>
<td>No</td>
<td>Unknown</td>
<td>Unknown</td>
<td>NA</td>
<td>AML (biphenotypic)</td>
<td>8</td>
<td>No mutation detected (post-HSCT samples)</td>
</tr>
</tbody>
</table>

** ** borderline thrombocytopenia (140-150 x10^9/L)
* - Also demonstrated defective dense granule function measured by flow cytometry
† - IV-1 and IV-2 demonstrated closure times on PFA-100 to Collagen/Epinephrine of >300s (normal 84-176s) and Collagen/ADP of 136-142s (Normal 63-111s)
Five new pedigrees with inherited \textit{RUNX1} mutations causing familial platelet disorder with propensity to myeloid malignancy (FPD/AML)