Somatic mutations associated with leukemic progression of familial platelet disorder with predisposition to acute myeloid leukemia

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The familial platelet disorder with predisposition to acute myeloid leukemia (FPD/AML) is an autosomal dominant disorder characterized by germline *RUNX1* alterations. Missense, nonsense and frameshift mutations as well as intragenic duplications and large deletions of *RUNX1* (21q22) have been reported in less than 45 pedigrees. Transformation to acute leukemia (AL) occurs with an incomplete penetrance and at a variable age in FPD/AML patients, suggesting that germline *RUNX1* mutations alone are insufficient to induce AL. Thus this disorder represents a unique model to study leukemic progression. *RUNX1* mutation could be considered like an inherited 'first hit', leading to a preleukemic state, but additional genetic alterations are required for the formation of fully transformed leukemic cells. To date, the most frequent additional event implicates an acquired mutation in the second allele of *RUNX1*.[1] Despite this, recurrent mutations coinciding with transformation to AL are not yet well defined. Recently, somatic mutations in the cell cycle regulator *CDC25C* were identified in 53% of FPD/AML patients. *CDC25C* mutations appeared to occur in the preleukemic clone, and subsequent mutations in other genes, such as *GATA2*, coincided with leukemia progression.[2] Moreover, a number of somatic mutations have been recently described to significantly co-occur with somatic *RUNX1* aberrations in AML, including mutations in the Polycomb-associated genes *ASXL1/2* in t(8;21) AML, as well as in *RUNX1*-mutated AML.[3] Intriguingly, an acquired *ASXL1* mutation has been also described in a FPD/AML patient who developed T-ALL.[4] Given these recent discoveries and the lack of recurrent mutations known to coincide with leukemic progression of FPD/AML patients, we explored the status of 44 AML-associated genes in 25 individuals from 15 FPD/AML pedigrees. Interestingly, we identified a second alteration of *RUNX1* in all patients who developed AML, in contrast to patients who developed T-ALL.

The FPD/AML patients were identified from 2005 to 2014 (Supplementary Material, Supplementary Figure S1A), and biological samples were collected after informed consent, in accordance with the Declaration of Helsinki. Genomic DNA was extracted from peripheral blood or bone marrow mononuclear cells, using standard procedures. Next-generation sequencing (NGS) was performed at thrombocytopenia stage if the patients

Table 1. Characteristics of FPD/AML patients

Patient	*Pedigree*	*Sex*	*Germline RUNX1 alteration*	*Age at AL*	*AL*	*Acquired RUNX1 anomalies*	*Other acquired anomalies identified*	*Reference*
1	1/brother	M	p.R177Q	NA	No	No	No	[1]
2	1/brother	M	p.R177Q	NA	No	No	DNMT3A[a]	[1]
3	1/proband	M	p.R177Q	60	AML	p.A160T	FLT3-ITD, PHF6, KIT	[1]
4	1/son	M	p.R177Q	28	T-ALL	No	PHF6, WT1, NOTCH1	[1]
5	2/proband	F	p.Q308RfsX259	55	AML	p.G138PfsX12	ND	[1]
6	2/daughter	F	p.Q308RfsX259	NA	No	No	No	[1]
7	2/daughter	F	p.Q308RfsX259	24	T-ALL t-AML 5 years later	ND No	ND KRAS, t(1;3)(p36;q26)	[1] [1]
8	3	F	Complete deletion of *RUNX1*	12	AML	Duplication of RUNX1-deleted chromosome	No	[1]
9	4	F	p.R139X	48	AML	p.R139X (CN-LOH)	KRAS[a], RAD21	[7]
10	5/proband	M	p.P218S	NA	No	No	No	
11	5/identical twin	M	p.P218S	NA	No	No	No	
12	6	M	p.G108V	14	T-ALL	No	WT1, FLT3-ITD, FLT3-TKD	
13	7	F	p.D305TfsX262	37	AML	Duplication of *RUNX1*-mutated chromosome	SRSF2, WT1, TET2	
14	8	F	p.H377PfsX191	12	AML	p.S114P	SF3B1	
15	9	F	p.G108V	NA	No	No	No	
16	10	M	p.G143RfsX43	36	AML	p.K83Q	BCORL1, FLT3-ITD	
17	11/proband	M	p.T169R	NA	No	No	No	
18	11/daughter	F	p.T169R	NA	No	No	No	
19	11/daughter	F	p.T169R	NA	No	No	No	
20	11/son	M	p.T169R	NA	No	No	No	
21	12	F	Complete deletion of *RUNX1*	NA	No	No	No	[6]
22	13	F	p.T219RfsX8	43	AML	p.T219RfsX8 (CN-LOH)	CBL, MPL, TP53, WT1, del(11)(q21)	[8]
23	14/proband	F	p.T121HfsX9	NA	No	No	No	
24	14/son	M	p.T121HfsX9	6	AML	ND	ND	
25	15	F	p.A129E	42	AML	Duplication of *RUNX1*-mutated chromosome	SRSF2	[1]

Abbreviations: AML, acute myeloid leukemia; AL, acute leukemia; CN-LOH, copy-neutral loss of heterozygosity; F, female; *FLT3*-ITD, *FLT3* internal tandem duplication; *FLT3*-TKD, *FLT3* tyrosine kinase domain mutation ; M, male; NA, not applicable; ND, not determined; T-ALL, T-acute lymphoblastic leukemia; t-AML: therapy-related AML. [a]Acquired mutation at thrombocytopenic stage.

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were thrombocytopenic or at leukemia stage if they developed AL. For one patient, samples for both stages have been collected (patient 9). For patients who progressed to AL, samples were also collected at the time of complete remission to confirm acquired status of mutations. NGS was performed using a custom-designed 44 gene panel (MiSeq, Illumina, San Diego, CA, USA), including the entire coding region of *ASXL1*, *ASXL2*, *CDC25C*, *BCOR*, *BCORL1*, *BRAF*, *CSF3R*, *CALR*, *CBL*, *CEBPA*, *DNMT3A*, *ETV6*, *EZH2*, *FBXW7*, *FLT3*, *GATA1*, *GATA2*, *IDH1*, *IDH2*, *JAK2*, *JAK3*, *KIT*, *KRAS*, *MPL*, *NIPBL*, *NPM1*, *NOTCH1*, *NRAS*, *PHF6*, *PTEN*, *PTPN11*, *RAD21*, *RUNX1*, *SETBP1*, *SF3B1*, *SMC1A*, *SMC3*, *SRSF2*, *STAG2*, *TET2*, *TP53*, *U2AF1*, *WT1* and *ZRSR2*, with a median depth of 2692×. For *CDC25C*, the entire coding region was covered with a mean depth of 2633× (range: 1837–3656). Hotspot locations at codons 233–234 described by Yoshimi *et al.*[2] were visually checked without any filters. Depth at these codons was >3000 for all patients. *FLT3* internal tandem duplication (*FLT3*-ITD) was detected as previously described.[5] *RUNX1* loss of heterozygosity was deducted from variant allele frequencies (VAF) found by NGS and standard cytogenetic analysis. For the patient 25, quality of DNA did not allow a good quantification of the VAF. For the patients 5, 7 and 24, NGS was not performed at AL stage owing to a lack of DNA. We report here the mutations already described for these patients.[1] Cytogenetic G-banding analysis was performed according to standard methods in each center. Comparative genomic hybridization array analysis was performed for patients 8 and 21 as previously described.[1,6]

Characteristics of the 25 FPD/AML patients studied here are reported in Table 1.[1,6–8] Of the 15 pedigrees, 8 were described for the first time. Germinal *RUNX1* mutations were heterogeneous

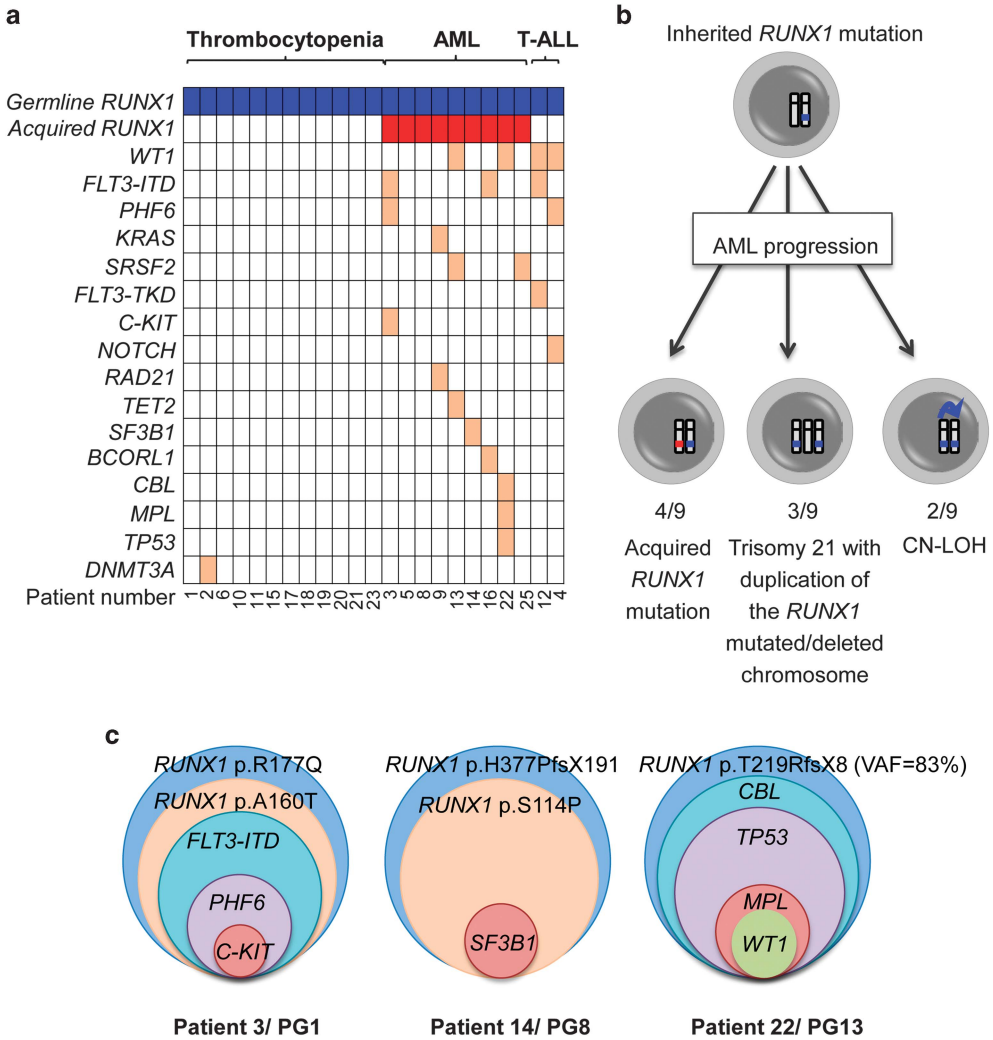

Figure 1. Associated mutations in FPD/AML patients. (**a**) Aberrations of *RUNX1* and associated mutations in FPD/AML patients at all stages. Germline *RUNX1* aberrations (mutations and deletions) are represented in blue and acquired *RUNX1* aberrations (second mutation, mutation by copy-neutral loss of heterozygosity (CN-LOH) and duplication of *RUNX1*-mutated or -deleted chromosome) in red. Other acquired mutations are represented in orange. Twenty-three patients are represented; the patients 7 and 24 have been excluded because of a lack of material at T-ALL and AML diagnosis, respectively. The patient 9 is represented at AML stage. (**b**) Acquired *RUNX1* secondary events in FPD/AML patients who developed AML. Of the 10 patients who developed AML, DNA was available for 9. Of these 9, all had a second somatic event affecting the other allele of *RUNX1*: 4 had a novel acquired *RUNX1* mutation, 3 had trisomy 21 with duplication of the *RUNX1*-mutated or -deleted chromosome and 2 harbor a second mutation by CN-LOH. *RUNX1* germline mutation is represented in blue, *RUNX1* acquired mutation in red. (**c**) Schematic representation of mutations found in FPD/AML patients who developed AML. The circles are proportional to the variant allelic frequency found for each mutation (values in Supplementary Table S1). Representative examples of three patients are shown (patient 3 from pedigree 1, patient 14 from pedigree 8, patient 22 from pedigree 13) and highlight that the second aberration of *RUNX1* is an early event in leukemia transformation. PG, pedigree; VAF, variant allelic frequency.

with six missense mutations, one non-sense mutation, six frame-shift mutations and two large deletions of *RUNX1*. Most mutations (8/13) were located in the Runt homology domain (DNA-binding domain), where the majority of *RUNX1* mutations in FPD/AML are reported (Supplementary Figure S1B).[9] Of the 25 patients, 13 patients developed AL, 10 AML and 3 T-ALL, attesting to the importance of *RUNX1* mutations to the myeloid as well as T-lymphoid lineage. The age of AML onset was heterogeneous (6–60 years), whereas the patients who developed T-ALL were younger (14–28 years).

Mutational analysis identified a second leukemogenic event in all patients who developed AL. Consistent with our previous report,[1] we identified a second aberration of *RUNX1* in all AML patients, for whom material was available (9/10). Of the nine patients, six have a mutation in the second allele, including two by copy-neutral loss of heterozygosity, and three have a duplication of the *RUNX1*-mutated or -deleted chromosome (Figures 1a and b). These data highlight the importance of RUNX1 dosage in AML development. Analysis of the variant allelic frequency (Supplementary Table S1) was critical in identifying copy-neutral loss of heterozygosity, as well as duplication of the *RUNX1*-mutated allele. In contrast to AML transformation of FPD/AML, most *RUNX1* mutations in sporadic AML are monoallelic.[10] These results suggest that FPD/AML should be suspected in the diagnosis of AML in any patient with a *RUNX1* biallelic mutation or with a single *RUNX1* mutation with a VAF > 50%, which could indicate trisomy 21 with a duplication of the mutated chromosome or copy-neutral loss of heterozygosity. Routine analysis of *RUNX1* mutant allele ratios may greatly facilitate identification of FPD/AML patients in the clinical setting. These findings in FPD/AML are analogous to what has been observed in familial MDS/AML owing to *CEBPA* mutations or in recently described familial MDS/AML with mutations in the RNA helicase *DDX41*, where biallelic mutations have been reported at AML transformation.[11,12] Subsequent germline *CEBPA* mutations have been found in 10% of a cohort of AML patients harboring biallelic *CEBPA* mutations. Interestingly, no additional *RUNX1* mutations were found in patients who developed T-ALL.

Additional mutations identified at the AL stage were heterogeneous and affected genes recurrently implicated in leukemogenesis. This included mutations affecting signaling intermediates (*FLT3*, *KRAS*, *KIT*, *MPL*, *CBL*, *NOTCH1*), tumor suppressors (*TP53*, *WT1*, *PHF6*, *BCORL1*), cohesins (*RAD21*), splicing proteins (*SRSF2*, *SF3B1*) or DNA methylation (*TET2*, *DNMT3A*) (Table 1, Figure 1a).[13] A possibility for this heterogeneity is that, as low levels of wild-type RUNX1 cause genetic instability in FPD/AML,[14] the cells are more prone to acquire mutations. Analysis of the allelic frequency for each somatic mutation reveals that, in most of the cases, the second *RUNX1* alteration is one of the initial events for leukemia progression (Supplementary Table S1, Figure 1c). None of these additional mutations were found in samples at complete remission (available for 10/13 patients), attesting to their role in leukemogenesis.

In contrast to the AL stage, no additional mutations were found in patients at the thrombocytopenic stage, except for one patient with a *DNMT3A* mutation and another with a *KRAS* mutation. Interestingly, this last patient (patient 9) developed AML and analysis of samples from both stages revealed that the minor clone with the *KRAS* mutation (4%) expanded at the leukemic stage with a VAF at 45% (Supplementary Table S1).

Surprisingly, no association between germline *RUNX1* mutation and *ASXL1/2* mutations were found. We also did not identify *CDC25C* or *GATA2* mutations in these pedigrees. This is intriguing given that *CDC25C* mutations were recently identified in 7/13 FPD/AML patients, including 4 at AML stage and 3 at thrombocytopenia stage.[2] One possible explanation could be the geographic origin of the patients, as the patients described with *CDC25C* and *GATA2* mutations were from Japan and the majority of the patients described here are from France. Additional studies will be needed to corroborate this hypothesis and evaluate more precisely the frequency of *CDC25C* and *GATA2* mutations in FPD/AML.

In this study, we analyzed a large cohort of FPD/AML patients for a panel of genes recurrently mutated in AML, including many genes never studied previously in the context of FPD/AML, and identified genetic events co-existing with germline *RUNX1* mutations in all patients at AL stage. These events implicate signaling, RNA splicing or epigenetic regulation genes, reflecting the heterogeneity of additional mutations with AL progression in FPD/AML. Moreover, a second acquired aberration of *RUNX1* was associated with AML progression in all patients, suggesting that biallelic *RUNX1* alterations are crucial for AML development.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

N Duployez, MB, SG, AR and CP performed genetic analysis and analyzed mutational data. IA-D, N Duployez, NB and CP conceptualized the idea, designed the research and analyzed data. N Dhédin, DR, BN, CB, TL, M-JM, RF, P-GH, HR, VL-C and CP provided samples and data. IA-D, N Duployez, J-BM, OA-W and CP wrote the manuscript, which was approved by all the authors.

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