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1 **Somatic mutations associated with leukemic progression of FPD/AML**

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3 Iléana Antony-Debré,^{1,2,*} Nicolas Duployez,^{3,4,*} Maxime Bucci,³ Sandrine Geffroy,^{3,4} Jean-Baptiste
4 Micol,^{2,5,6} Aline Renneville,^{3,4} Nicolas Boissel,⁷ Nathalie Dhédin,⁷ Delphine Réa,⁷ Brigitte Nelken,⁸
5 Céline Berthon,^{4,9} Thierry Leblanc,¹⁰ Marie-Joëlle Mozziconacci,¹¹ Rémi Favier,^{2,12} Paula G. Heller,¹³
6 Omar Abdel-Wahab,⁵ Hana Raslova,² Véronique Latger-Cannard,^{14,*} and Claude Preudhomme^{3,4,*}

7
8 ¹Department of Cell Biology, Albert Einstein College of Medicine, New York, USA; ²INSERM
9 UMR1170, Institut Gustave Roussy, University Paris-Sud 11, Equipe labellisée Ligue Contre le
10 Cancer, Villejuif, France; ³Laboratory of Hematology, University Lille Nord de France, Lille, France;
11 ⁴INSERM UMR-S 1172, Lille, France; ⁵Human Oncology and Pathogenesis Program and Leukemia
12 Service, Memorial Sloan-Kettering Cancer Center and Weill Cornell Medical College, New York,
13 USA; ⁶Department of Hematology, Institut Gustave Roussy, Villejuif, France; ⁷Department of
14 Hematology, Hôpital Saint-Louis, University Paris Diderot, Paris, France; ⁸Department of Pediatric
15 Hematology, CHU of Lille, Lille, France; ⁹Department of Hematology, CHU of Lille, Lille, France;
16 ¹⁰Department of Pediatric Hematology, Hôpital Robert Debré, Paris, France; ¹¹Department of
17 Molecular Biology and Cytogenetics, Institut Paoli-Calmettes, Marseille, France; ¹²Laboratory of
18 Hematology, Hôpital Trousseau, Paris, France; ¹³Department of Hematology Research, Instituto de
19 Investigaciones Médicas Alfredo Lanari, Universidad de Buenos Aires, Consejo Nacional de
20 Investigaciones Científicas y Técnicas, Buenos Aires, Argentina; ¹⁴Laboratory of Hematology,
21 Nancy University Hospital, Nancy, France

22 ***These authors contributed equally to this work**

23
24 **Corresponding author:** Pr Claude Preudhomme, Laboratory of Hematology, INSERM UMR-S 1172,
25 Professor Leclercq Boulevard, 59037 Lille Cedex, France

26 Tel: 33 (0)3 20 44 47 82

27 claude.preudhomme@chru-lille.fr

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

54 The FPD/AML patients were identified from 2005 to 2014 (Supplementary Material,
55 Supplementary Figure 1A) and biologic samples were collected after informed consent, in accordance
56 with the Declaration of Helsinki. Genomic DNA was extracted from peripheral blood or bone marrow
57 mononuclear cells, using standard procedures. Next generation sequencing (NGS) was performed at
58 thrombocytopenia stage if the patients were thrombocytopenic or at leukemia stage if they developed
59 AL. For one patient, samples for both stages have been collected (patient 9). For patients who
60 progressed to AL, samples were also collected at the time of complete remission to confirm acquired

61 status of mutations. NGS was performed using a custom-designed 44 gene panel (MiSeq, Illumina[®]),
62 including the entire coding region of *ASXL1*, *ASXL2*, *CDC25C*, *BCOR*, *BCORL1*, *BRAF*, *CSF3R*,
63 *CALR*, *CBL*, *CEBPA*, *DNMT3A*, *ETV6*, *EZH2*, *FBXW7*, *FLT3*, *GATA1*, *GATA2*, *IDH1*, *IDH2*, *JAK2*,
64 *JAK3*, *KIT*, *KRAS*, *MPL*, *NIPBL*, *NPM1*, *NOTCH1*, *NRAS*, *PHF6*, *PTEN*, *PTPN11*, *RAD21*, *RUNX1*,
65 *SETBP1*, *SF3B1*, *SMC1A*, *SMC3*, *SRSF2*, *STAG2*, *TET2*, *TP53*, *U2AF1*, *WT1* and *ZRSR2*, with a
66 median depth of 2692X. For *CDC25C*, the entire coding region was covered with a mean depth of
67 2633X [range: 1837-3656]. Hotspot locations at codons 233-234 described by Yoshimi *et al.*² were
68 visually checked without any filters. Depth at these codons was > 3000 for all patients. *FLT3* internal
69 tandem duplication (*FLT3*-ITD) was detected as previously described.⁵ *RUNX1* loss of heterozygosity
70 was deducted from variant allele frequencies (VAF) found by NGS and standard cytogenetic analysis.
71 For the patient 25, quality of DNA didn't allow a good quantification of the VAF. For the patients 5, 7
72 and 24, NGS was not performed at AL stage due to a lack of DNA. We report here the mutations
73 already described for these patients.¹ Cytogenetic G-banding analysis was performed according to
74 standard methods in each center. Comparative genomic hybridization array analysis was performed for
75 patients 8 and 21 as previously described.^{1,6}

76 Characteristics of the 25 FPD/AML patients studied here are reported in **Table 1**.^{1,6-8} On the
77 15 pedigrees, 8 were described for the first time. Germinal *RUNX1* mutations were heterogeneous
78 with 6 missense mutations, 1 non-sense mutation, 6 frameshift mutations and 2 large deletion of
79 *RUNX1*. Most mutations (8/13) were located in the Runt homology domain (DNA binding domain),
80 where are reported the majority of *RUNX1* mutations in FPD/AML (**Supplementary Figure 1B**).⁹ Of
81 the 25 patients, 13 patients developed AL, 10 AML and 3 T-ALL, attesting to the importance of
82 *RUNX1* mutations to the myeloid as well as T-lymphoid lineage. The age of AML onset was
83 heterogeneous (6-60 years), whereas the patients who developed T-ALL were younger (14-28 years).

84 Mutational analysis identified a second leukemogenic event in all patients who developed AL.
85 Consistent with our previous report,¹ we identified a second aberration of *RUNX1* in all AML
86 patients, for whom material was available (9/10). Of the 9 patients, 6 have a mutation in the second
87 allele, including two by copy-neutral loss of heterozygosity (CN-LOH), and 3 have a duplication of
88 the *RUNX1*-mutated or -deleted chromosome (**Figure 1A, B**). These data highlight the importance of

89 RUNX1 dosage in AML development. Analysis of the variant allelic frequency (**Supplementary**
90 **Table 1**) was critical in identifying CN-LOH, as well as duplication of the *RUNX1* mutated allele. In
91 contrast to AML transformation of FPD/AML, most *RUNX1* mutations in sporadic AML are
92 monoallelic.¹⁰ These results suggest that FPD/AML should be suspected in the diagnosis of AML in
93 any patient with a *RUNX1* biallelic mutation, or with a single *RUNX1* mutation with a VAF >50%,
94 which could indicate trisomy 21 with a duplication of the mutated chromosome or CN-LOH. Routine
95 analysis of *RUNX1* mutant allele ratios may greatly facilitate identification of FPD/AML patients in
96 the clinical setting. These findings in FPD/AML are analogous to what has been observed in familial
97 MDS/AML due to *CEBPA* mutations, or in recently described familial MDS/AML with mutations in
98 the RNA helicase *DDX41*, where biallelic mutations have been reported at AML transformation.^{11,12}
99 Subsequent germline *CEBPA* mutations have been found in 10% of a cohort of AML patients
100 harboring biallelic *CEBPA* mutations. Interestingly, no additional *RUNX1* mutations were found in
101 patients who developed T-ALL.

102 Additional mutations identified at the AL stage were heterogeneous and affected genes
103 recurrently implicated in leukemogenesis. This included mutations affecting signaling intermediates
104 (*FLT3*, *KRAS*, *KIT*, *MPL*, *CBL*, *NOTCH1*), tumor suppressors (*TP53*, *WT1*, *PHF6*, *BCORL1*),
105 cohesins (*RAD21*), splicing proteins (*SRSF2*, *SF3B1*) or DNA methylation (*TET2*, *DNMT3A*) (**Table**
106 **1, Figure 1A**).¹³ A possibility for this heterogeneity is that, as low levels of wild type RUNX1 cause
107 genetic instability in FPD/AML,¹⁴ the cells are more prone to acquire mutations. Analysis of the
108 allelic frequency for each somatic mutation reveals that in most of the cases, the second *RUNX1*
109 alteration is one of the initial events for leukemia progression (**Supplementary Table 1, Figure 1C**).
110 None of these additional mutations were found in samples at complete remission (available for 10/13
111 patients), attesting to their role in leukemogenesis.

112 In contrast to the AL stage, no additional mutations were found in patients at the
113 thrombocytopenic stage, except for one patient with a *DNMT3A* mutation and another with a *KRAS*
114 mutation. Interestingly, this last patient (patient 9) developed AML and analysis of samples from both
115 stages revealed that the minor clone with the *KRAS* mutation (4%) expanded at the leukemic stage
116 with a VAF at 45% (**Supplementary Table 1**).

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

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

132

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141

142 **Authorship Contribution:**

143 N.Du, M.B, S.G, A.R and C.P performed genetic analysis and analyzed mutational data.

144 I.A-D, N.Du, N.B and C.P conceptualized the idea, designed the research and analyzed data.

145 N.Dh, D.R, B.N, C.B, T.L, M-J.M, RF, P-G.H, H.R, V.L-C, C.P provided samples and data.
146 I.A-D, N.Du, J-B.M, O.A-W and C.P wrote the manuscript, which was approved by all the authors.

147

148 **Conflict-of-interest disclosure:**

149 The authors declare no competing financial interests.

150 Supplementary information is available at Leukemia's website

151

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210 **Figure legends**

211

212 **Figure 1. Associated mutations in FPD/AML patients**

213 (A) Aberrations of *RUNXI* and associated mutations in FPD/AML patients at all stages. Germline
214 *RUNXI* aberrations (mutations and deletions) are represented in blue and acquired *RUNXI* aberrations
215 (second mutation, mutation by copy neutral loss of heterozygosity and duplication of *RUNXI* mutated
216 or deleted chromosome) in red. Other acquired mutations are represented in orange. 23 patients are
217 represented; the patients 7 and 24 have been excluded because of a lack of material at T-ALL and
218 AML diagnosis, respectively. The patient 9 is represented at AML stage.

219 (B) Acquired *RUNXI* secondary events in FPD/AML patients who developed AML. Of the 10 patients
220 who developed AML, DNA was available for 9. Of these 9, all had a second somatic event affecting
221 the other allele of *RUNXI*: 4 had a novel acquired *RUNXI* mutation, 3 had trisomy 21 with duplication
222 of the *RUNXI* mutated or deleted chromosome and 2 harbor a second mutation by copy neutral loss of
223 heterozygosity (CN-LOH). *RUNXI* germline mutation is represented in blue, *RUNXI* acquired
224 mutation in red.

225 (C) Schematic representation of mutations found in FPD/AML patients who developed AML. The
226 circles are proportional to the variant allelic frequency found for each mutation (values in
227 Supplementary Table 1). Representative examples of 3 patients are shown (patient 3 from pedigree 1,
228 patient 14 from pedigree 8, patient 22 from pedigree 13) and highlight that the second aberration of
229 *RUNXI* is an early event in leukemia transformation. Abbreviations: PG: pedigree, VAF: variant
230 allelic frequency.

231

232 **Supplementary Figure 1. Characteristic of FPD/AML pedigrees studied**

233 (A) Family trees of the newly described pedigrees (PGs). PGs 6, 7, 9, 10, 11, 14 are represented.

234 (B) Gene diagram depicting *RUNXI* anomalies in the FPD/AML pedigrees. *RUNXI* isoform b is
235 represented. Abbreviations: RHD: Runt homology domain, TAD: transactivation domain. In blue:
236 frameshift mutation, in orange: missense mutation, in green: nonsense mutation, red square: mutations
237 found to predispose to leukemia development in FPD/AML patients

238 **Supplementary information**

239

240 **Supplementary Material.**

241 The characteristics of each new pedigree and the significance of RUNX1 mutation are described in the

242 supplementary Material.

243

Figure 1

