



Distinct clonal anomalies involving in acute myeloid leukemia at diagnosis and after bone marrow transplantation

Etienne Braekeleer, Nathalie Douet-Guilbert, Audrey Basinko, Marie-Josée Bris, Frédéric Morel, Christian Berthou, Claude Férec, Marc Braekeleer

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Corresponding Author: Prof Marc De Braekeleer,

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First Author: Etienne De Braekeleer

Order of Authors: Etienne De Braekeleer; Nathalie Douet-Guilbert; Audrey Basinko; Marie-Josée Le Bris;
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Response to Reviewers: ANSWERS TO THE REVIEWER

First major comment (association of 7q deletions and microdeletions of the RUNX1 gene): the discussion on this subject was limited to the association of 7q deletions and RUNX1 mutations (page 5).

Second major comment (other genes involved): we completed the paragraph by adding the 3 genes located in the interval between both BACs with a description of "our" candidate gene (page 5).

Third major comment (RUNX1 constitutively prone to breakage): indeed, as suggested by the reviewer, we think there was a pre-leukemic stem cell in which acquired RUNX1 alterations cooperated with pre-existing lesions to result in full blown acute leukemia. This was added in the last paragraph of the manuscript (page 6).

All three minor comments were taken care of.

DISTINCT CLONAL ANOMALIES INVOLVING *RUNX1* IN ACUTE MYELOID LEUKEMIA AT DIAGNOSIS AND AFTER BONE MARROW TRANSPLANTATION.

Etienne De Braekeleer^{1,2,3}, Nathalie Douet-Guilbert^{1,2,3}, Audrey Basinko^{1,2,3}, Marie-Josée Le Bris³, Frédéric Morel^{1,2,3}, Christian Berthou^{1,4}, Claude Férec^{1,2,5}, Marc De Braekeleer^{1,2,3}

¹ Université de Brest, Faculté de Médecine et des Sciences de la Santé, Brest, France.

² Institut National de la Santé et de la Recherche Médicale (INSERM), U613, Brest.

³ CHU Brest, Hôpital Morvan, Service de Cytogénétique, Cytologie et Biologie de la Reproduction, Brest.

⁴ CHU Brest, Hôpital Morvan, Service d'Hématologie clinique, Brest.

⁵ CHU Brest, Hôpital Morvan, Laboratoire de Génétique moléculaire et d'Histocompatibilité, Brest.

Running title: Distinct *RUNX1* anomalies in AML

Corresponding author: Pr. Marc De Braekeleer
Laboratoire de Cytogénétique
Faculté de Médecine et des Sciences de la Santé
Université de Bretagne Occidentale
22, avenue Camille Desmoulins
CS 93837
F-29238 Brest cedex 3
France

Phone: + 33 (0)2 98 01 64 76
Fax: + 33 (0)2 98 01 81 89
E-mail: marc.debraekeleer@univ-brest.fr

**DISTINCT CLONAL ANOMALIES INVOLVING *RUNX1* IN ACUTE MYELOID
LEUKEMIA AT DIAGNOSIS AND AFTER BONE MARROW
TRANSPLANTATION.**

Dear Editor,

The *RUNX* gene family includes three evolutionarily conserved genes (*RUNX1*, *RUNX2* and *RUNX3*) encoding transcription factors involved in cell lineage differentiation during development and various forms of cancer [1]. *RUNX1* gene (also known as *AML1 PEBP2 α B*, or *CBFA2*), located in chromosome 21q22.3, is crucial for the establishment of definite hematopoiesis and the generation of hematopoietic stem cells in the embryo. *RUNX1* acts as a key regulator of hematopoiesis through the regulation of various hematopoietic genes [1-3].

RUNX1 impairment resulting from mutation, deletion, translocation or amplification is reported to contribute to the pathogenesis of acute leukemias [4]. We report here distinct clonal anomalies involving the *RUNX1* gene occurring in the course of the disease in a patient with acute myeloblastic leukemia (AML).

This 51-year-old man was first seen in February 2006 because of persistent rhinitis and recurrent epistaxis. Physical examination showed no adenopathy or hepatosplenomegaly. The peripheral blood count revealed a leukocytosis of $8.6 \times 10^9/l$ with 37% blasts, 12% neutrophils, 25% monocytes and promonocytes, a hemoglobin level of 10.1g/l and a platelet count of $105 \times 10^9/l$. The bone marrow aspirate showed hypercellularity with 68% blasts positive for CD13, CD33, CD117, MPO and CD65. AML-M1 was diagnosed (FAB classification). The patient received induction chemotherapy according to the LAM2001 protocol (Idarubicin and Cytarabin) followed by re-induction because of chemoresistance at day 15. The patient also received central nervous system prophylaxis with intrathecal

1 injections. A complete remission was obtained. An allogenic bone marrow transplantation
2 (BMT) was performed in August 2006. The patient relapsed in December 2006. Bone marrow
3 aspirate revealed 54% blast cells whose morphology and immunophenotype (positive for
4 CD13, CD33, CD34, CD117) appeared identical to the blasts at diagnosis. The patient
5 received a second BMT in February 2007. A complete remission was obtained. An isolated
6 cutaneous granulocytic sarcoma localized at the penis, with normal bone marrow morphology,
7 was diagnosed in July 2008. Surgery followed by radiotherapy was applied. In November
8 2008, a gingival granulocytic sarcoma and a medullar relapse, with 45% blasts, were
9 observed. Induction chemotherapy failed to obtain a remission, the sole result being a
10 stabilization of the leukocytosis.
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26 R-banded karyotypes were established from sequential bone marrow aspirate samples that had
27 been cultured for 24 hours synchronized by FUdR. Fluorescence *in situ* hybridization (FISH)
28 analyses were performed using whole chromosome painting (WCP) of chromosomes 3, 7, 11,
29 21, 22 and arm specific probes (PCP) 3p, 3q (QBIOgene®, Illkirch, France), telomeric probes
30 of chromosome 3 (Abbott®, Rungis, France) and locus specific identifier (LSI) MLL,
31 BCR/ABL1, TEL/AML1 (Abbott®) as well as bacterial artificial chromosome (BAC) clones
32 RP11-13M6, RP11-198D7, RP11-434P20, RP11-91K9, RP11-245C23, RP11-12L14 located
33 on the long arm of chromosome 3 (3q26.3) and BAC RP11-77G18 covering *AML1*.
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51 At diagnosis, a sole abnormal clone was detected. Karyotype was interpreted as
52 46,XY,der(7)t(7;11)(q31;q14)[7]/46,XY[15].ish der(7)(WCP7+,MLL+), resulting in partial
53 monosomy 7q and partial trisomy 11q including trisomy *MLL* without rearrangement (Table
54 1) (Figs 1-A & 2-A).
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At 1st relapse following the 1st BMT (December 2006), conventional cytogenetics, completed by FISH studies, revealed a new abnormal clone showing *AML1* rearrangement: 46,XY,t(3;11;22;21;3)(p13;q14;q12;q22;q26)[15]/46,XY[5].ish t(3;11;22;21;3)(WCP3+, PCP3p+,PCP3q+,WCP11+,MLL+,WCP3+,PCP3p+,WCP22+,BCR+,WCP11+,MLL+; WCP21+,AML1+,WCP22+;WCP3+,PCP3p+,PCP3q+,AML1+) (Fig 1-B). No *MLL* amplification was detected by FISH. FISH with BAC clone (RP11-77G18) covering the *RUNX1* gene showed one signal on der(21) and one signal on der(3) (Fig 2-B). Breakpoints on 3q occurred between RP11-434P20 and RP11-245C23 with deletion of RP11-91K9. This translocation disappeared; interphase FISH on cells from the penis granulocytic sarcoma (July 2008) showed three *RUNX1* signals, presumably signing re-emergence of the translocation (Table 1).

FISH analyses using TEL/*AML1* probe were performed retrospectively at diagnosis and all along the disease. They revealed an abnormal clone with *RUNX1* deletion (undetected by banding techniques), distinct from the clone with *MLL* amplification, present at diagnosis (Table 1) (Fig 2-A). With chemotherapy and BMT, this clone disappeared until July 2008 before being again observed in bone marrow cells 4 months before the 2nd medullary relapse was evidenced (Table 1).

The *RUNX1* gene is known to be frequently deregulated in human leukemia through different mechanisms [4]. In the case reported here, at the time of AML diagnosis, an overlooked clone with *RUNX1* deletion was present. It coexisted with a partial monosomic 7q/trisomic 11q clone, consistently observed in AML. Although hemizygous loss of *RUNX1* function is suspected to play a key role in the development of myelodysplastic syndrome/acute

myeloblastic leukemia (MDS/AML) patients, it is not sufficient to develop overt leukemia and acquisition of additional genetic alterations is necessary [5, 6].

RUNX1 mutations were found to be significantly associated with monosomy 7 or partial deletion of the long arm of chromosome 7 in studies of MDS/AML patients [6, 7]. *RUNX1* mutations were suggested to be the major factor to develop MDS/AML in conjunction with additional gene alterations belonging to the RTK-RAS signal transduction pathway [6]. Interestingly, in our case, *RUNX1* deletion and 7q deletion did not occur in the same cells. However, an additional *RUNX1* mutation could not be excluded.

At first relapse, none of the clones present at diagnosis was detected but the *RUNX1* gene was rearranged in a complex 3;21 translocation involving five chromosomes. The t(3;21)(q26;q22) is a recurrent abnormality in therapy-related AML, occurring primarily after treatment with topoisomerase II inhibitors [8]. Three genes at 3q26, *EVII*, *MDS1* and *EAP* are known to be fused with *RUNX1* in the (3;21) translocation [9, 10]. In our case, the breakpoint at 3q26 is 8 megabases distal to the *EVII-MDS1-EAP* cluster, arguing for the involvement of another gene. Three genes have been mapped in the interval between RP11-434P20 and RP11-245C23. These are *KCNMB2*, a calcium channel, *WIG1*, a wildtype p53-induced gene, and *TBLIXR1*, a transducin-beta-like 1 receptor 1 (<http://www.ncbi.nlm.nih.gov/mapview/>). *WIG1* could be a good candidate to be fused with *RUNX1* as it is located in the nucleus and contains three zinc finger domains and a putative nuclear localization signal. Unfortunately, no cells taken at first relapse were available for further cytogenetic and molecular studies to characterize this partner gene.

This report raises the question of whether the *RUNX1* gene was constitutively prone to breakage in the patient. Whether there was a pre-leukemic stem cell in which acquired *RUNX1* alterations cooperated with pre-existing lesions to result in full blown acute leukemia remains hypothetical.

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but recurrent, cytogenetic pathway in blastic phase of chronic myeloid leukemia.
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Table 1. Conventional and molecular cytogenetic results obtained during the course of the disease.

Dates	Disease stage	Conventional cytogenetics	FISH
February 2006	Diagnosis	BM: 46,XY,der(7)t(7;11)(q31;q14)[7]/46,XY[15]	M: 3 MLL & 2 AML1 signals (32%) M: 2 MLL & 1 AML1 signal (8%) I: 2 MLL & 1 AML1 signal (14.5%)
March 2006	Re-induction	BM: 46,XY[22]	M: 3 MLL & 2 AML1 signals (2%) M: 2 MLL & 1 AML1 signal (12%) I: 3 MLL & 2 AML1 signals (1%) I: 2 MLL & 1 AML1 signal (10%)
July 2006	1 st complete remission	BM: ND	M: 2 MLL & 2 AML1 signals (100%) I: 2 MLL & 2 AML1 signals (100%)
October 2006	After 1 st BMT	BM: ND	M: 2 MLL & 2 AML1 signals (100%) I: 2 MLL & 2 AML1 signals (100%)
December 2006	1 st relapse	BM: 46,XY,t(3;11;22;21;3)(p13;q14;q12;q22;q26)[15]/46,XY[5]	M: 2 MLL & 3 AML1 signals (75%)
March 2007	After 2 nd BMT	BM: ND	M: 2 MLL & 2 AML1 signals (100%) I: 2 MLL & 2 AML1 signals (100%)
June 2007	2 nd complete remission	BM: 46,XY[21]	M: 2 MLL & 2 AML1 signals (100%) I: 2 MLL & 2 AML1 signals (100%)
October 2007	2 nd complete remission	BM: 46,XY[21]	M: 2 MLL & 2 AML1 signals (100%) I: 2 MLL & 2 AML1 signals (100%)
February 2008	2 nd complete remission	BM: 46,XY[21]	M: 2 MLL & 2 AML1 signals (100%) I: 2 MLL & 2 AML1 signals (100%)
July 2008	Granulocytic sarcoma	GS: no metaphases	I: 2 MLL & 3 AML1 signals (58%)
July 2008		BM: 46,XY[50]	M: 2 MLL & 2 AML1 signals (100%) I: 2 MLL & 1 AML1 signal (7%)
November 2008	2 nd medullary relapse	ND	ND

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BM: bone marrow
GS: granulocytic sarcoma
M: metaphase
I: interphase
ND: not done
BMT: bone marrow transplantation

LEGENDS TO THE FIGURES

Figure 1. Conventional cytogenetics

A. Partial R-banded karyotype at diagnosis showing der(7)t(7;11)(q31;q14)

B. R-banded karyotype at first relapse following the first BMT:

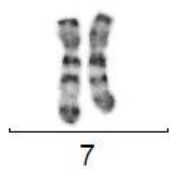
46,XY,t(3;11;22;21;3)(p13;q14;q12;q22;q26)

Figure 2. Fluorescence *in situ* hybridization

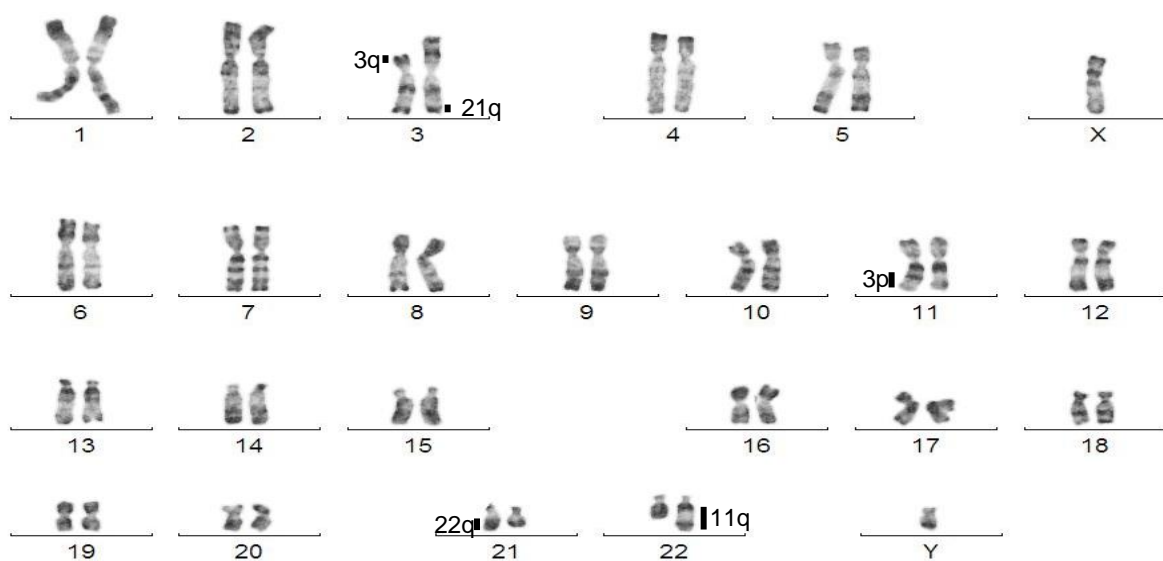
A. FISH at diagnosis with LSI MLL dual color and TEL/AML1 ES dual color (Green/Orange) revealing two distinct clones (left nucleus is trisomic for 11q and right nucleus is deleted for *RUNX1*).

B. FISH at first relapse following the first BMT with BAC RP11-13M6 (orange) and BAC RP11-77G18 (green) showing disruption of *AML1* by t(3;21).

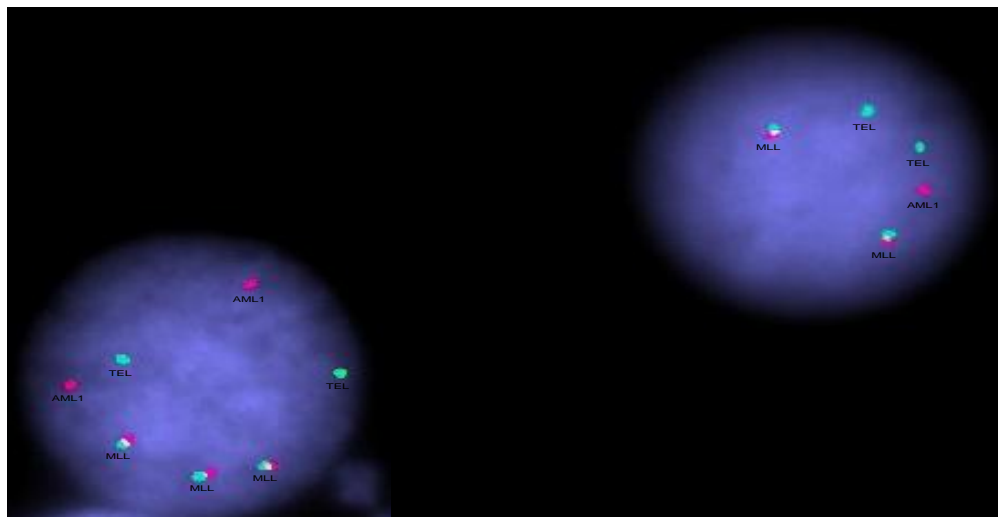
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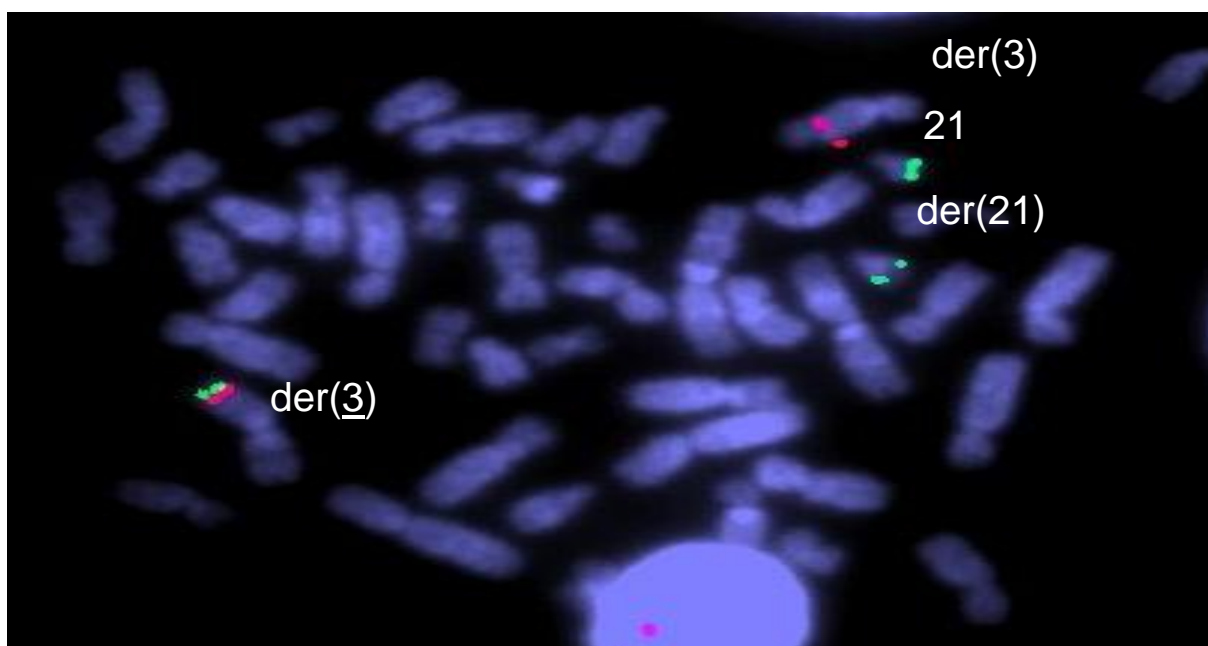
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