Dual-color split signal fluorescence *in situ* hybridization assays for the detection of CALM/AF10 in t(10;11)(p13;q14-q21)-positive acute leukemia

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We developed dual-color split fluorescence *in situ* hybridization (FISH) assays to detect *AF10* and/or *CALM* rearrangements. Among nine cases of acute leukemia with translocation breakpoints at 10p13 and 11q14-21, a *CALM/AF10* rearrangement was found in seven and was confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) in all. In 2/7 cases, FISH detected *CALM/AF10* in extramedullary leukemic infiltrations in the mediastinum and breast. As expected, FISH was less sensitive than RT-PCR for disease monitoring of CALM-AF10 positive cases. This new FISH assay reliably discriminates between *MLL/AF10* and *CALM/AF10* genomic rearrangements, identifies variant and complex *CALM/AF10* translocations and detects the *CALM/AF10* rearrangement in extramedullary leukemic infiltrations.

Key words: *CALM*, *AF10*, FISH, acute leukemia.

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In adult and pediatric acute myeloid (AML) and lymphoblastic leukemia (ALL), t(10;11)(p13;q14-q21) is a recurring translocation underlying the molecular lesions from which the MLL/AF10 and CALM/AF10 fusion proteins originate.[1,2] Different clinical-hematologic and morphologic features have been associated with MLL/AF10 and CALM/AF10 fusion. The MLL/AF10 rearrangement occurs mainly in AML with monocytic differentiation and is only rarely reported in B or T cell ALL.[3] The CALM/AF10 fusion is found in 8%-10% of T-ALL, restricted to subgroups expressing TCRγδ and to immature T-ALL, and in CD7 positive leukemias with multilineage phenotype coexpressing T-cell and myeloid antigens.[4] Both rearrangements are predictive of poor prognosis and gene expression profiles suggest that *MLL/AF10* and *CALM/AF10* positive T-cell ALL share common pathways since the *HOXA* cluster genes are upregulated in both leukemias.[5,6] In MLL/AF10 and CALM/AF10 fusion proteins the AF10 carboxy-terminal portion is fused to the amino-terminal part of MLL or CALM. *MLL*, a promiscuous gene with more than 60 different translocation partners, encodes for the human homolog of the *Drosophila trithorax* protein, a transcription factor with repressor and activation domains involved in maintaining the expression pattern of genes which are important in development and cellular differentiation.[7] CALM, an ubiquitously expressed protein, participates in clathrin recruitment and assembly at the internal membrane surface, during endocytosis and in intracellular vesicle-associated protein transport.[8] The *AF10* gene encodes a leucine zipper protein that functions as a transcription factor and both MLL/AF10 and CALM/AF10 retain the AF10 leucine zipper motif which is essential for the transforming properties of the fusion proteins.[9] Molecular investigations and/or fluorescent *in situ* hybridization (FISH) are necessary to assess whether *MLL/AF10* or *CALM/AF10* is involved at the 10p13 and 11q14-q21 chromosomal breakpoints. Moreover, only a molecular approach may demonstrate fusion genes underlying variant or complex chromosomal rearrangements. We developed and validated dual-color split signal FISH assays to detect *AF10* and/or *CALM* involvement in acute leukemia with rearrangements at 10p13 and/or 11q14-21.

Design and Methods

Patients

We retrospectively selected nine patients with acute leukemia and t(10;11)(p13;q14) (eight cases) or t(10;19;11)(p13;p13;q14-21) (one case), from the archives of the Departments of Hematology at the Universities of Bari, Ferrara, Palermo, Perugia, Pescara, "La Sapienza", Rome, and Sassari, Italy; from the Hematology Unit of Catanzaro Hospital, Italy; and from the Service of Hematology of Hospital Sant Pau, Barcelona, Spain. The clinical, hematologic and cytogenetic data of these patients are summarized in Table 1. All patients were studied at diagnosis and two of them during treatment and follow-up (patients 1 and 2, Table 1).

Table 1. Clinical, hematologic, cytogenetic and FISH findings in nine patients with t(10;11)(p13;q14-q21).

Patients	*Age/Sex*	*Diagnosis (FAB)*	*Immunophenotype*	*Karyotype*	*I-FISH MLL*
1	12/F	AML-M1	MPO+,CD45+,CD7+,CD44+,CD11a+,CD11b+,CD33+, CD71+,CD38+,CD34+,HLA-DR+/-,cCD3+	46,XX,t(10;11)(p13;q14)[15]	normal
2	13/M	T-ALL	cCD3+,CD52+,CD99+,CD44+	46,XY,t(10;11)(p13;q14)[10] 46,XY[2]	normal
3	36/M	AML-M2	CD33+,MPO+,CD7+,CD34+,HLA-DR+	46,XY,t(10;19;11)(p13;p13;q14)[17] 46,XY[1]	n.d.
4	27/M	AML-M0	CD34+,CD117+,CD45+,CD33+,CD56+, CD3+,CD7+,CD13+	46,XY,t(10;11)(p13;q14)[1]/46,XY,idem, add(5)(p15)[13] 47,XY,idem,+4[5] 46,XY[1]	n.d.
5	38/F	T-ALL	CD45+,cCD3+,CD7+,CD5+,Tdt+,CD1a+,CD38+	46,XX,t(10;11)(p13;q14)[15]	normal
6	47/M	AML-M1	HLA-DR+,CD34+,CD13+,CD33+,TdT-,CD7-,CD3-,CD20-, CD10-,CD19-	46,XX,t(10;11)(p13;q14)[15]	n.d.
7	19/M	AML-M1	CD11c+,CD33+,MPO+,CD15+,CD7+,CD34+,HLA-DR+,CD71+,CD13-,CD117-,CD14-,CD61-, GpA-,CD19-,CD22-,TdT-,sCD3-,CD5-,CD10-,CD1-	46XY,t(1;7)(q31;q21),t(10;11) (p13;q21)[20]	normal
8	44/F	AML-M1	CD15+,CD33+,CD34+,CD117+,CD7-,CD10-,CD19-, CD13-,CD14-,CD56-	46,XX,t(10;11)(p13;q14)[14] 46,XX,idem,add(8)(p23)[4] 46,XX[3]	1R1G1F
9	2/F	AML-M5	CD13+,CD33+,CD64+,CD117+,CD2-,CD7-,CD10-,CD14-, CD15-,CD19-,CD20-,CD34-	46,XX,t(10;11)(p13;q14)[20]	1R1G1F

FAB: French-American-British; F: female; M: male; +, positive; -, negative; n.d.: not done; Interphase-FISH (I-FISH) with LSI MLL (Vysis) gave a normal hybridization pattern in patients 1, 2, 5, and 7. A 1 red 1 green 1 fusion (1R1G1F) abnormal hybridization pattern, indicating that MLL is rearranged, was found in patients 8 and 9.

Dual-color FISH split assay

Dual-color split signal assays were developed with clones RP11-418C1 (red) for the 5'AF10 and RP11-249M6 (green) for the 3'AF10 to study 10p13 breakpoints, and with clone RP11-12D6 (red) from the 5'CALM side and RP11-90K17 (green) at the 3' CALM side to study 11q14-21 rearrangements (Figure 1, upper panel). With both sets of probes, normal nuclei display a two fusion (2F) signal pattern, whereas nuclei with *AF10* or *CALM* involvement display a one fusion, one red and one green (1F1R1G) hybridization pattern.

FISH was performed as previously described on bone marrow, breast and mediastinal mass tissue taken at diagnosis and/or during disease monitoring.[10] The protocol for extracting nuclei from paraffin-embedded sections has been described previously.[11] Interphase FISH for disease monitoring was performed with the *CALM* probes on 500 nuclei for each patient's sample. Bone marrow samples from 11 healthy donors were used as controls and a total of 20.000 nuclei were scored. The cut-off for the *CALM* 1F1R1G pattern was established at 2.8% (mean ± 2 standard deviations).

The LSI *MLL* probe (Vysis, Olympus, Milan, Italy) was used to study the *MLL*/11q23 gene. FISH analyses were carried out using a fluorescence microscope (Provis, Olympus, Milan, Italy) equipped with a cooled CCD camera (Sensys, Photometrics) controlled by PathVysion software (Vysis, Stuttgart, Germany).

Reverse transcriptase polymerase chain reaction (RT-PCR)

cDNA synthesis and RT-PCR to detect CALM/AF10 and AF10/CALM fusion proteins were performed as described elsewhere.[12] The primers used for CALM/AF10 and AF10/CALM amplification are listed in Table 2. The PCR products were analyzed on agarose gels, gel-extracted using the QIAquick Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany) and sequenced using the same primers used for the amplification (ABI Prism Sequencing Kit).

Results and Discussion

CALM-AF10 and *MLL-AF10* are two translocations that are indistinguishable by conventional cytogenetics. Moreover, these recombinations may be cryptic, associated with normal karyotypes, or masked in complex karyotype with rearrangements involving chromosome regions other than 10p13 and 11q14-21. Thus molecular studies are necessary to distinguish which genomic rearrangement is produced, to ensure accurate molecular characterization, and to correlate genetic findings with morphologic and immunophenotypic features, in order to classify leukemias within specific hematologic-genetic entities.

We set up dual-color split signal FISH assays to diagnose and to monitor the t(10;11)(p13;q14-q21)/*CALM-AF10* in acute leukemias and used RT-PCR to validate the FISH

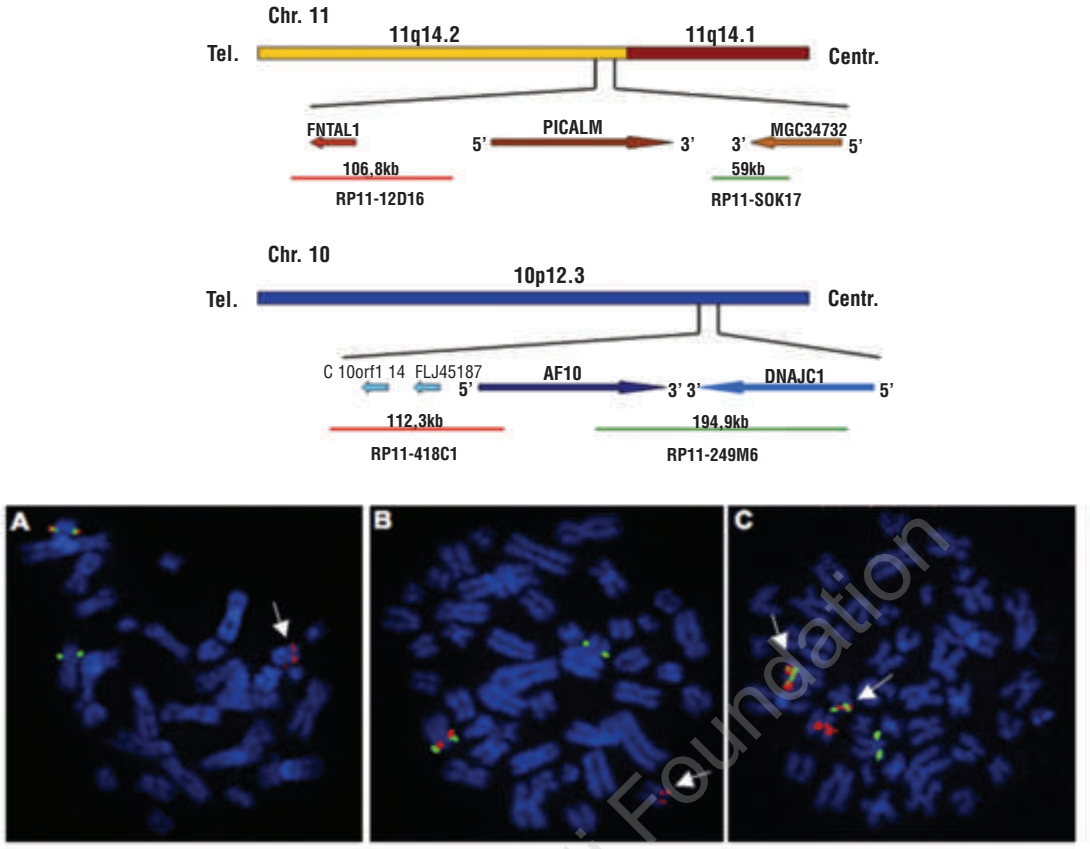

Figure 1. Upper panel: Schematic representation of the short arm of chromosome 10 and the long arm of chromosome 11 and localization/orientation of *AF10* and *CALM*. DNA clones used for the FISH studies are shown with their relative position and size. Lower panel: A, B. Dual-color split signal assay with clone RP11-418C1 (red) and clone RP11-249M6(green) gave a red/green fusion signal on normal 10 and a green signal on der(10). In patient 1 a red signal was present on the der(11) (panel A, arrow), while in patient 3 the red signal was seen on the der(19) (panel B, arrow). C. Dual-color dual-fusion FISH assay in patient 1: RP11-418C1 and RP11-249M6 (green) and RP11-12D16 and RP11-90K17 (red) gave a green signal on the normal 10, a red signal on the normal 11 and a red/green fusion signal on the der(10) and on the der(11) (arrows).

Table 2. Primers used for *CALM/AF10* and *AF10/CALM* amplification.

Primer name	*Nucleotide position in cDNA*	*Sequence*
NG.T 45	CALM(1730-1753)	5' - CCAAACTCCCACCTAGCAAGTTAG - 3'
NA.T 501	CALM(1998-2023)	5' - GGAAGTGTTCCTGTAATGACGCAAC - 3'
ANB 497	CALM(2165-2144)	5'- AAGGATTTTGCTGCTTGAGCAC - 3'
AF10 949B	AF10(949-929)	5' - CCAATGCAGGTGATGGTTCTG - 3'
AF10 1086B	AF10(1086-1066)	5' - GGTGTGTGCAGAGACTTCCTG - 3'
AF10 651B	AF10(651-632)	5' - TGTCATGCAAGCACCAGTGG - 3'
AF10 T288	AF10(288-308)	5'- CGAGAACCCGCTGGTTTATTG - 3'

data. FISH detected the *AF10* rearrangement in all nine patients in this study with clones RP11-418C1 (red) and RP11-249M (green) giving a fusion signal on the normal 10 and a green signal on the der(10) (Figure 1A). In all patients, except patient 3, a red signal was present on the der(11); in patient 3 it was present on der(19) (Figure 1B). *CALM* involvement was demonstrated in patients 1-7. RP11-12D6 (red) and RP11-90K17 (green) gave a fusion signal on the normal 11, a green signal on the der(11) and a red signal on the der(10). In patients 8 and 9, one fusion signal on the normal 11 and one on the der(11) indicated that *CALM* was not rearranged. In these two cases, a rearrangement of *MLL* was demonstrated by FISH (Table 1). Notably, since the dual-color split signal assay proved to be highly specific for both the *AF10* and *CALM* probes, the probes were combined for a dual-color dual fusion assay (probes for *AF10* in green and probes for *CALM* in red). In patients 1, 2, 4-7 abnormal metaphases showed a green signal on the normal 10, a red signal on the normal 11 and a red/green fusion signal on both the der(10) and the der(11) (Figure 1C). RT-PCR detected a CALMnt2091/AF10nt424 fusion transcript in patients 1, 2, 4, 6, and 7 and a CALMnt2091/AF10nt589 fusion transcript in patient 5. In patient 3, the breakpoint was observed at CALMnt 1926 and AF10nt883. In patient 4, a second fusion transcript was observed which had 205 nucleotides inserted between CALMnt2091 and nt424 of *AF10*. These extra nucleotides localize between exons 6 and 7 of *AF10* and a have stop codon 162nt after the breakpoint which is in frame with the *CALM* reading frame.

These two fusion transcripts in the same patient are

most likely due to alternative splicing. The reciprocal AF10/CALM fusion transcript was detected in patients 1, 2, 5-7. In this study FISH detected the first case of a three-way variant translocation resulting in a *CALM-AF10* fusion gene. In patient 3, the t(10;19;11)(p13;p13;q14-q21) gave rise to a *CALM-AF10* fusion on the der(10) with the *5'AF10* translocated to the der(19)(p13) and chromosome 19 material juxtaposed to the *3'CALM* on the der(11)t(11;19)(q14-q21;p13). Whether and which additional molecular lesions contributed to the pathogenesis or development of disease in our patient could not be assessed. As *CALM* and *AF10* have the same 3' centromere- 5'- telomere orientation, the fusion of both genes is almost always the product of a simple t(10;11) due to chromosome breakage at 10p13 and 11q14 and reciprocal exchange. In contrast in *MLL-AF10*-positive leukemias, the transcriptional orientation of *MLL* and *AF10* appears to be the cause of complex chromosomal rearrangements. *MLL* and *AF10* have an opposite centromere to telomere orientation and an *MLL/AF10* fusion can only result after complicated chromosomal rearrangements involving inversions and translocations with often more than two chromosomes.[8] Therefore, systematic analysis of such cases by FISH would be useful to estimate the incidence of additional chromosomal changes associated with t(10;11) and molecular studies are necessary to elucidate the underlying genomic events.

We also showed that our FISH assay is capable of detecting the *CALM/AF10* rearrangement in extramedullary sites of a leukemia. In patient 3, who presented with a mediastinal mass, rearrangement of *CALM* was detected in 84% of nuclei extracted from the paraffin-embedded mediastinal biopsy confirming that these nuclei are part of the leukemic clone. In patient 1 who, after autologous bone marrow transplantation, presented with a left breast mass followed by bone marrow relapse, rearrangement of *CALM* was shown in both breast and bone marrow tissues (80% and 8% of the nuclei with the fusion, respectively).

Interphase-FISH monitoring of the *CALM/AF10*-positive clone was done in patients 1 and 2 and the results compared to those of RT-PCR in patient 1. In this case, interphase-FISH and RT-PCR gave concordant positive results at first relapse after autologous bone marrow transplantation. After allogeneic bone marrow transplantation, RT-PCR detected a residual CALM/AF10 positive clone in two consecutive bone marrow samples (at day +33 and +45 after transplantation) when the patient was in hematologic, cytogenetic and FISH remission. In patient 2, interphase-FISH was negative after induction and at the conclusion of therapy (+24 months after diagnosis). RNA was not available for molecular studies.

In conclusion we developed and validated a sensitive and specific dual-color FISH split signal assay to reliably detect *CALM/AF10* rearrangements in t(10;11)(p13;q14-q21) as well as in complex/variant translocations and to diagnose the *CALM/AF10* fusion in extramedullary leukemic tissues. This FISH assay is as specific as RT-PCR analysis for detecting the *CALM/AF10* rearrangement at diagnosis. As expected, RT-PCR is more sensitive than interphase FISH for monitoring CALM/AF10 positive leukemias and for detecting early relapses.

RLS and BC were the principal investigators. AK, DD, and SKB performed the molecular studies. VP performed the FISH experiments. GS, AB, RN, CA, MN, AA, and MS provided clinical and cytogenetic data of patients from their Centers. MFM was involved in the management of patients. CM was responsible for the conception and supervision of the study. The authors wish to thank Dr Caterina Matteucci for extracting nuclei from paraffin embedded sections, Dr Domenico Magro for providing clinical and hematologic data of patient 4, and Dr Geraldine Boyd for assistance in the preparation of the manuscript. BAC clones belong to the Roswell Park Cancer Institute library RP11 (http://www.chori.org/BACPAC) and were kindly provided by Dr Mariano Rocchi (DAPEG Sez di Genetica, University of Bari, Italy).

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