Validation of an interphase fluorescence *in situ* hybridization approach for the detection of *MLL* gene rearrangements and of the MLL/AF9 fusion in acute myeloid leukemia

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To validate a 2-step FISH assay for the identification of the t(9;11)(p22;q23), 96 acute myeloid leukemias were studied by cytogenetic analysis, FISH and molecular biology. After a first FISH step using an *MLL* probe, 24/27 cases with 11q23 break showed *MLL* rearrangement. Southern blotting confirmed FISH data. In the second step, 24 cases with *MLL* rearrangement were studied using *MLL* and AF9 probes: 17/18 cases with t(9;11) showed MLL/AF9 fusion. In 6 patients with 11q23/MLL rearrangements other than t(9;11), FISH confirmed MLL involvement and excluded AF9 involvement. This is a reliable method for the identification of MLL/AF9 fusion in interphase cells, allowing for a reclassification of cases with suboptimal chromosome morphology. The frequency of deletion surrounding *MLL* and AF9 breakpoint is low.

Key words: cytogenetics, FISH, *MLL*, acute leukemia

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Rearrangements of chromosomal band 11q23 involving the MLL gene occur at a 3-5% frequency in adult *de novo* acute myeloid leukemias (AML).[1] A number of 11q23/MLL partners were identified[2] and the t(9;11)(p22;q23) involving AF9 (MLLT3) represents the most frequent translocation. AML with 11q23/MLL rearrangements are usually assigned to an intermediate or unfavourable prognostic group and there is evidence that the t(9;11) may characterize a group of AML with a more favourable prognosis among children and possibly adults with AML and an 11q23 translocation.[3] Fluorescence *in situ* hybridization (FISH) was demonstrated to be more sensitive than G-banding analysis in the detection of some rearrangements in acute myeloid leukemias.[4] In order to evaluate the sensitivity and specificity of an interphase FISH approach for the detection of 11q23/MLL rearrangements and of the MLL/AF9 fusion in AML, we set up a 2-step strategy based on the sequential application of two probe combinations. The results of this study are presented and discussed with reference to: a) the efficiency of a noncommercial probe set for the detection of MLL rearrangement; b) the frequency of cryptic deletions surrounding the MLL/AF9 translocation breakpoints; c) the specificity and sensitivity of this approach for the detection of 11q23 rearrangements and of the t(9;11)-MLL/AF9 fusion.

Design and Methods

Design of the study

We studied a total of 96 AML cases including 73 unselected de novo AMLs and 23 additional cases carrying an 11q23 rearrangement by G-banding analysis. As a first screening step we adopted the dual-color (DC) FISH approach for the detection of MLL breaks in all cases using two probe combinations. In the second step we performed a dual-color dual-fusion (DCDF) FISH assay for the detection of MLL/AF9 rearrangement in those cases with an MLL breakpoint identified in step 1. Southern blotting and reverse transcriptase polymerase chain reaction (RT-PCR) were performed to detect MLL rearrangement and MLL/AF9 fusion transcript in all cases in which a discrepancy between FISH and cytogenetics was evident.

Patients and cytogenetics

Seventy-three consecutive AML cases were analyzed. These patients were enrolled in the GIMEMA clinical trial LAM99P and were referred to the Institute of Haematology, University of Ferrara by overnight courrier, for centralized karyotyping. In 4 cases an 11q23 rearrangement was present. Twenty-three additional cases with cytogenetic evidence of various 11q23 aberrations (19 cases with t(9;11), 2 cases with other 11q23 translocations, 2 cases with del(11)(q23)) were selected, in order to be able to test our FISH approach in a large number of potentially positive cases.

Probes selection and FISH studies

FISH studies were performed by personnel unaware (FC) of the result of conventi zonal cytogenetic analysis. In step 1 two different set of probes were used: a dual color break apart system purchased from Vysis Co (distributed in Italy by Abbott, Rome) and a set of two PACs kindly provided by Prof. M. Rocchi from the University of Bari, Bari, Italy (*http://www.uniba.it/*) (Figure 1, panel A). The FISH pattern in germline interphases was represented by two red-green fusion signals (2F), whereas in the presence of MLL break the FISH configuration showed the segrega-

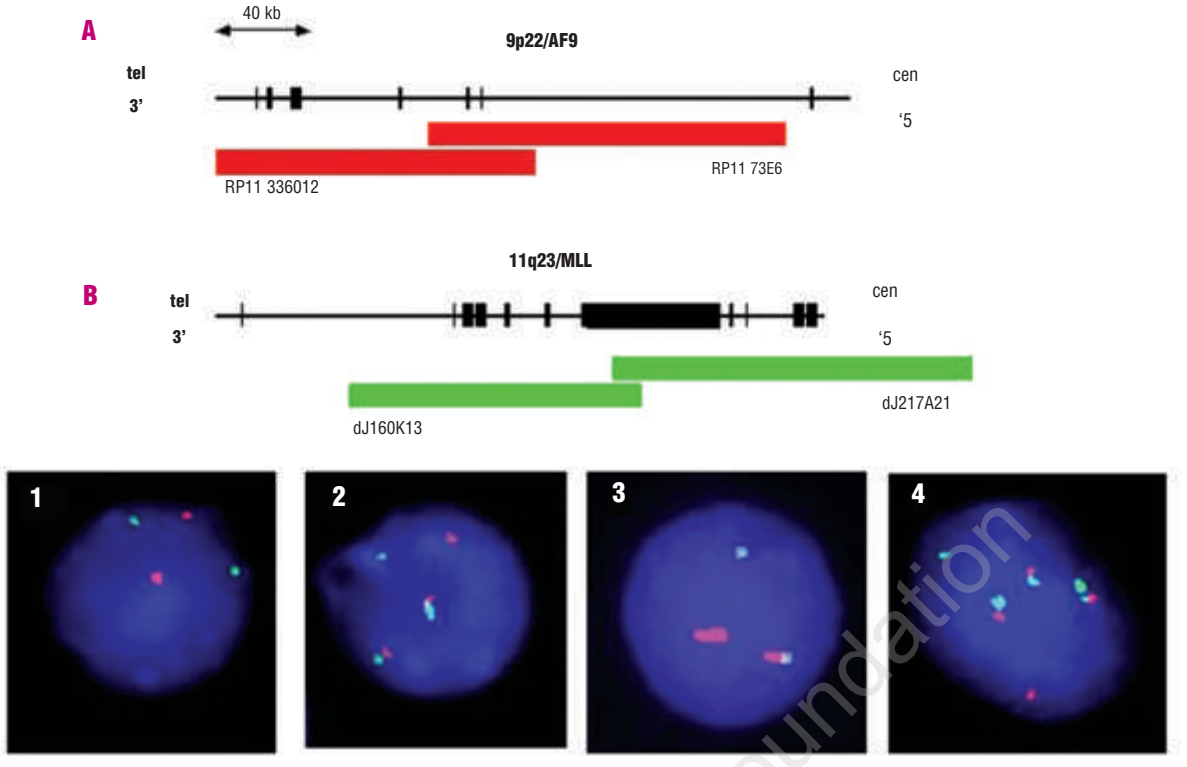

Figure 1. A. Schematic representation of the mapping of *AF9* BACs (RP11-336O12 and RP11-73E6) and MLL PACs (dJ217A21 and dJ167K13) used in this study. Black arrows indicate the location of breakpoints on *AF9* and MLL gene. In the first step the 11q23/MLL PACs were labelled green (dJ217A21) and in red (dJ167K13). In the second step they were labelled in green and used in combination with the *AF9* BACs labelled in red (Dual Color Dual Fusion system). B. DCDF FISH pattern, from left to right: 1. normal interphase with two green signals (MLL germline) and two red signals (*AF9* germline), 2. interphase with translocation t(9;11) and *MLL/AF9* fusion (white arrows indicate fusion signal from derivative chromosomes), 3. interphase with *MLL/AF9* fusion and deletion of one derivative (note the absence of a second fusion), 4. interphase with *MLL/AF9* fusion and duplication of one derivative (white arrow).

Table 1. Cut-off values (average±3SD) for MLL probes and MLL/AF9 probes determined on five bone marrow specimens from patients without clonal defects.

	% Mean value (min-max)	*SD*	*Cut off (mean + 3SD)*
MLL Vysis			
1F1G1R (indicating MLL break)	1.7 (1- 2)	0.4	2.9
1F1G0R (indicating MLL 3' deletion)	2.8 (2- 3.2)	0.1	3.1
MLL PACs			
1F1G1R (indicating MLL break)	2.0 (1.5- 2.5)	0.4	3.2
1F1G0R (indicating MLL 3' deletion)	3.0 (2- 3.7)	0.7	5.1
MLL/AF9 dual color dual fusion			
2F1R1G (indicating MLL/AF9 translocation)	0.4 (0-1)	0.4	1.6
1F1R1G (indicating derivative deletion)	2 (1.5-3)	0.6	3.8
3F1R1G (indicating derivative duplication)	1.3 (1-2)	0.4	2.5

tion of red (R) and green (G) signals and one single fusion (MLL germline) (1F1R1G). Two BACs were selected from the Ensembl database (*http://www.ensembl.org*) to detect the AF9 gene located at band 9p21.3: (Figure 1, panel A). Both BACs were labelled in Spectrum Orange and hybridised together with the MLL PACs labelled in Spectrum Green in order to obtain a dual color dual fusion Break Apart system specific for MLL/AF9 rearrangement (Figure 1). Using these probes the signal configuration in the presence of MLL/AF9 rearrangement was represented by one red signal (germline AF9), one green signal (germline MLL) and two fusion signals (the two derivative chromosomes: 2F1R1G, Figure 1, panel B). DNA labelling and slides hybridization were performed as previously described (6,7). Cut off values for the identification of MLL rearrangements and of MLL/AF9 fusion are shown in Table 1. The FISH data were collected on a fluorescence photomicroscope equipped with a black and white charged couple camera device and run by GenikonTM FISH Imaging Software version 3.6.13 (Nikon Instruments S.p.A. Sesto Fiorentino (FI), Italy).

Molecular biology

RT-PCR and/or Southern blotting studies for the detection of MLL/AF9 fusion and/or of MLL rearrangement were performed as previously described[8,9] in 69 cases with material available, including all cases with discordant cytogenetic and FISH results.

Results and Discussion

Detection of MLL rearrangement

A total of 27 cases had a cytogenetically documented 11q23 aberration; cytogenetic, FISH and molecular data in these patients are presented in Table 2. Of 73 unselected *de novo* AML, 69 cases without 11q23 rearrangement showed the expected germline MLL FISH pattern, (96,9-100% interphase with two fusion signals), a finding confirmed by Southern blotting analysis in all 50

Table 2. Outcome of cytogenetic, FISH and molecular investigations in 27 patients with 11q23 rearrangements.

Pt.	Karyotype	FISH MLL Vysis Signal pattern (% nuclei)	FISH MLL 217a21/167k13 Signal pattern (% nuclei)	FISH MLL-AF9 DCDF Signal pattern (% nuclei)	Outcome of FISH Southern blotting and RT-PCR (*) (comments)
			Cases with t(9;11)(p22;q23) by conventional cytogenetic		
1	47,XY,t(9;11)(p22;q23),+21[18]	1F1R1G (71)	1F1R1E (72)	2F1R1G (69)	*MLL* R/MLL-AF9 FSB+/RT-PCR+
2	46,XX,t(9;11)(p22;q23)[9]/46,XX[16]	1F1R1G (73)	1F1R1G (75)	2F1R1G (77)	*MLL* R/MLL-AF9 FSB+/RT-PCR+
3	46,XX,add(1)(p35),t(9;11)(p22;q23)[5]/46,XX[6]	1F1R1G (80)	1F1R1G (84)	2F1R1G (81)	*MLL* R/MLL-AF9 F/SB+/RT-PCR+
4	46,XX,t(9;11)(p22 ;q23)[10]/46,XX[5]	1F1R1G (81)	1F1R1G (76)	2F1R1G (74)	*MLL* R/MLL-AF9 F/SB+/RT-PCR+
5	46,XX,add(1)(p35),t(9;11)(p22 ;q23)[10]/ 46,XX,idem,del(7)(p14p22)[4]/46,xx[1]	1F1R1G (82)	1F1R1G (87)	2F1R1G (81)	*MLL* R/MLL-AF9 F/SB+/RT-PCR+
6	46,XY,t(9;11)(p22 ;q23)[8]/46,XY[2]	1F1R1G (62)	1F1R1G (58)	2F1R1G (62)	*MLL* R/MLL-AF9 F/SB+/RT-PCR+
7	46,XX,t(9;11)(p22 ;q23)[9]/46,XX[1]	1F1R1G (88)	1F1R1G (89)	2F1R1G (84)	*MLL* R/MLL-AF9 F/SB+/RT-PCR+
8	46,XX,t(9;11)(p22 ;q23) [5]/ 46,XX [2]	1F1R1G (74)	1F1R1G (78)	2F1R1G (76)	*MLL* R/MLL-AF9 F/SB+/RT-PCR+
9	46,XX,t(9;11)(p22;q23)[8]/46,XX[2]	1F1R1G (37)	1F1R1G (40)	2F1R1G (41)	*MLL* R/MLL-AF9 F/RT-PCR+
10	46,XY,t(9;11) (p22;q23) [10]	1F1R1G (85)	1F1R1G (83)	2F1R1G (88)	MLL R/MLL-AF9 F
11	46,XX,t(9;11)(p22;q23)[16]/46,XX[4]	1F1R1G (64)	1F1R1G (81)	2F1R1G (81)	*MLL* R/MLL-AF9 F/SB+/RT-PCR+
12	47-48,XX,t(9;11)(p22;q23),+mar1,+mar2[12]	1F1R1G (95)	1F1R1G (91)	2F1R1G (83)	*MLL* R/MLL-AF9 F/RT-PCR+
13	46,XY,t(9;11) (p22;q23) [10]	1F1R1G (73)	1F1R1G (80)	2F1R1G (76)	*MLL* R/MLL-AF9 F
14	46,XY,t(9;11) (p22;q23) [10]	1F1R1G (91)	1F1R1G (85)	2F1R1G (91)	*MLL* R/MLL-AF9 F
15	46,XX,t(9;11)(p22;q23)[7]/ 46,XX,t(9;11)(p22;q23),+der(9)t(9;11)[2]	1F1R1G (53)/ 1F2R 1G (8)	1F1R1G (47)/ 1F2R 1G (6)	2F1R1G (50)/ 3F1R1G (12)	*MLL* R/MLL-AF9 F/SB+/RT-PCR+ Duplication of der(9)
16	46,XX,t(9;11)(p22;q23) [5]/46XX, idem i(8)(q10)[4]/46,XX [1]	1F1R1G (59)/ 1F2R1G (5)	1F1R1G (53)/ 1F2R1G (5)	2F1R1G (51)/ 3F1R1G (10)	*MLL* R/MLL-AF9 F/SB+/RT-PCR+ Duplication of der(9)?
17	45,XX,del(2)(q33),-7,t(9;11)(p22;q23)[8]/ 45,XX,-7[2]	1F0R1G (85)	1F0R1G (81)	1F1R1G (77)	MLL R/MLL-AF9 F/ Deletion of MLL 3' and AF9 5'[a]
*18	47-49,XY,+3,t(9;11)(p22;q23),i(9)(q10), +add(20)(p13)[8]/46,XY[2]	1F1R1G (69)	1F1R1G (64)	1pseudoF0R2G (71)	*MLL* R/AF9- (see text)/SB+/RT-PCR-
*19	46,XY,t(9;11) (?p22;?q23) [12]	2F (96)	2F (97)	2R2G (96)	*MLL* G/AF9-SB- (M-FISH confirms t(9;11)(p;q) alternative breakpoints)
*20	42-46,XX,t(1;12)(q41;q11),der(2) t(2;?)(q31;?),-5,del(5)(q11),del(7)(q11), t(9;11)(?p22;?q23),+11,-18,+20,+21,+22 [cp20]	2F (90) 3F(5)	2F (93) 3F(4)	2R2G (91) 2R3G (5)	*MLL* G/AF9-/SB- RT-PCR- (Alternative breakpoints)
			Other 11q23 aberrations		
21	46,XX,t(10;11)(p13;q23)[14]/46,XX, idem add(8)(p23) [4] / 46,XX[3]	1F1R1G (65)	1F1R1G (61)	2R3G (66)	*MLL* R/AF9-/SB+
22	46,XY,t(11;22)(q23;q11)[20]	1F1R1G (96)	1F1R1G (95)	2R3G (89)	*MLL* R/AF9-/SB+
23	46,XX,t(6;11)(q26;q23)[12]	1F1R1G (87)	1F1R1G (91)	2R3G (89)	*MLL* R/AF9-
24	46,XY,t(11;17)(q23;q25)[16]	1F1R1G (76)	1F1R1G (81)	2R3G (73)	*MLL* R/AF9 -
25	45,XX,del(11)(q23), +der(13;14)(q10;q10), -14[12]	1F1R1G (30)	1F1R1G (45)	2R3G (47)	*MLL* R/AF9-/SB+
26	44,XY,-5,del(11)(q23), t(15;12;13)(q13,q13;p11) ,del(16)(q22),add(17)(p13),-18[10]	1F1R1G (32)	1F1R1G (41)	2R3G (44)	*MLL* R/AF9-
*27	46,XY, der(11)t(7;11)(q32;q23)[16]/46,XY[2]	3F (81)	3F (77)	2R3G (80)	MLL G/SB- (*MLL* duplication on add(11)(q23); RT-PCR-Inv(16) by FISH; CBFb/MYH11 positive by RT-PCR

Patients with an asterisk are those with discrepancies between cytogenetics and FISH. (*) MLL R/G: MLL rearranged/germline by FISH; MLL-AF9 F: MLL-AF9 fusion by FISH; AF9-: no AF9 break by FISH; SB+/-: MLL rearranged (+)/germline (-) by Southern blotting; RT-PCR+/-: presence (+) / absence (-) of MLL/AF9 fusion transcript; a Frequency of cryptic MLL3' deletion in literature as detected by FISH is 8-16% (Von Bergh et al.;5 Kobayashi et al.;10 Poirel et al. ;11 Bacher et al.)[12]

cases tested. In 3/4 cases with an 11q23 translocation (n° 1, 21 and 22), segregation of green and red signals suggestive of MLL gene rearrangement was observed, whereas in 1 case (n° 27), three fusion signals indicative of trisomy 11q23 were identified in 81% of the interphase nuclei. The karyotype in this case showed an 11q+ chromosome which had been interpreted as der(11)t(7;11)(q32;q23). Southern blotting did not show MLL rearrangement in this patient. Metaphase FISH with a DC MLL probe allowed for the identification of an extra MLL copy on the abnormal 11q, which was reinterpreted as dup(11)(q23q24). Similar data were obtained using the MLL PACs system (Table 2). No patient without 11q23 breakpoint in the karyotype was found to carry cryptic MLL rearrangement by FISH and by Southern blotting. Of the 23 AML with 11q23 rearrangements by cytogenetic analysis, 21 showed segregation of MLL signals, with both commercial and non-commercial probes, indicating that a breakpoint had occurred in the MLL gene. Two cases with 11q23 deletion showed splitting of the MLL probe, with translocation of the distal MLL portion on a group C chromosome. The karyotype in these 2 cases was therefore reinterpreted as t(11;?)(q23;?). In one out of 21 MLL rearranged cases (n° 17), DC FISH showed 1 fusion signal and one green signal (1F1G0R), indicating that deletion of the MLL3' portion had occurred in this patient. Two out of 23 cases (n. 19 and 20) had a germline MLL pattern (2 fusions in >95% interphases): in both patients a reciprocal translocation involving the distal portions of chromosomes 9p and 11q was detected by G banding analysis of metaphases with sub-opti-

mal chromosome morphology and was confirmed by chromosome painting. Southern blotting showed germline MLL in both cases. These data indicate the presence of a 9p;11q translocation without MLL involvement. Thus, FISH confirmed the interpretation of the karyotype in 19 of 23 cases and allowed for a correct reclassification of 4 cases.

DCDF FISH assay

We evaluated the sensitivity of FISH for the detection of t(9;11)(p22;q23)-MLL/AF9 fusion in 24 cases with 11q23/MLL rearrangement identified by DC-FISH, 18 of which had a cytogenetically documented t(9;11)(p22;q23). We detected MLL/AF9 fusion in 17/18 cases with t(9;11)(p22;q23). In 1 case (n° 18, see table 2) with cytogenetic evidence of t(9;11)(p22;q23) one pseudofusion (pseudoF) with green and red signal separated by a distance of three signals (1pseudoF2G) were observed, suggesting that the AF9 gene was not rearranged. Molecular study in this case showed MLL rearrangement and a 9p21 breakpoint distal to AF9, as previously reported.[8] In the remaining 6 patients with documented MLL rearrangement other than t(9;11), FISH showed the presence of 3G signals and 2R signals, confirming the occurrence of MLL break without AF9 involvement. In the first round of interphase FISH experiments we tested a commercial probe (Vysis) and two PACs, previously used in the study of MLL rearrangements.[5] We were able to confirm in our large series that this non-commercial MLL probe set, designed according to the DC break apart system, yielded comparable results in terms of specificity and sensitivity as compared with a commercial probe. The first implication of this study is that this probe can be used safely for the detection of MLL rearrangements in AML samples sent by overnight courier to a central laboratory. Interestingly, in those cases with a discordant karyotype with respect to the FISH pattern, Southern blotting confirmed the interphase FISH result and there was a 100% concordance between FISH and molecular biology in the remaining patients tested by both techniques. A second noteworthy finding in this study is represented by the low incidence of MLL 3'deletion in 11q23 rearranged cases. In this large series, only 1 out of 24 MLL-rearranged cases was found to harbour a cryptic MLL 3' deletion. In this patient (n. 17), FISH analysis with the dual color segregation system and the DCDF system showed an hybridization pattern congruent with deletion of the der(9) involving the translocated MLL 3' and the residual AF9 5'. To our knowledge, this is the first patient in whom a concurrent deletion of MLL 3' and of the 5' portion of AF9 could be documented. Our series was biased in favour of cases with t(9;11) with respect to other 11q23 rearrangements. A review of the literature showed a 16% incidence of MLL 3' deletion (4/24 cases) with two cases of t(9;11) and 2 cases with other 11q23 rearrangements,[5,10,11] thus suggesting that MLL 3' deletion is not peculiar of a particular type of 11q23 translocation. It is worth noting that our patients were studied at diagnosis. Interestingly, a recent paper found that the incidence of MLL 3' deletion in a large series of *de novo* AML was 8%.[12] In a second round of experiments we co-hybridized the MLL probe and the AF9 probe in those patients with a documented MLL break. The advantage provided by this segregation/colocalization approach is documented by the cut-off value for the recognition of MLL/AF9 fusion, which was shown to be very low in 2N interphases, as well as by the absence of false positive and false-negative cases in our series. Indeed, in all cases with discrepant findings between karyotypic analysis and interphase FISH using the MLL/AF9 probes, RT-PCR for the detection of MLL/AF9 fusion confirmed the interpretation achieved by FISH signal screening. In cases n° 19 and n° 20 molecular studies did not show MLL rearrangement and the MLL/AF9 DCDF assay was germline. This may suggest that in these two cases the breakpoints on chromosome 9 and 11 involve other genes. Interestingly, some patients with myeloid neoplasia and 11q23 translocations not involving the MLL gene were previously reported.[13] The DCDF system may have sub-optimal specificity for the detection of MLL abnormalities other than the t(9;11)/MLL/AF9 fusion, if used as a first step probe, because it would not permit the correct identification of cases with MLL trisomy (the resulting 2R3G pattern would lead to an erroneous diagnosis of MLL rearrangement). Likewise, cases with MLL 3' deletion would be erroneously interpreted as carriers of an MLL germline status. In conclusion, we validated a 2-step interphase FISH approach for the detection of MLL rearrangement and of MLL/AF9 fusion in AML enrolled in a clinical trial employing centralized cytogenetic analysis. Specifically, we demonstrated, (i) that a DCDF system is a specific and sensitive method for the identification of t(9;11)-MLL/AF9 fusion in interphase cells, provided that it is used as a second step probe after a preliminary screening of MLL rearranged cases using a DC MLL probe; (ii) that these probes may allow for a correct classification of cases with sub-optimal chromosome morphology; (iii) that the frequency of deletion surrounding the MLL and AF9 breakpoint in patients analysed at diagnosis may be lower than previously reported.

FC, AC, IW, AH and GC contributed to the conception and design of the study. FC performed FISH studies and wrote the paper.FC and AC analyzed hematological data. AB and ET performed conventional cytogenetics. DD and FL performed the molecular biology studies. JMH performed FISH, M-FISH and chromosome painting studies. NC, ARR performed the FISH experiments. AH supervised the FISH experiments and reviewed the manuscript for important intellectual content. The authors declare that they have no potential conflicts of interest. This work supported by MIUR-FIRB, by AIRC, by MIUR 60% and cofin. Part of this work presents research results of the Belgian program of Interuniversity Poles of attraction initiated by the Belgian State, Prime Minister's Office, Science Policy Programming. The scientific responsibility is assumed by the authors. The authors thank the following physicians for referring BM samples for cytogenetic analysis: Amadori S (Rome), Carella AM (S. Giovanni Rotondo), Fioritoni G (Pescara), Longinotti M (Sassari), Nobile F (Reggio Calabria), Peta A. (Catanzaro), Rotoli B (Napoli), Torelli G (Modena), Liso V (Bari). Authors also thank Prof. Mariano Rocchi (Bari) for providing PACs dJ217A21, dJ167K13 and RP11 336O12.

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