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Acute Leukemias

Detection of inv(16) and t(16;16) by fluorescence *in situ* hybridization in acute myeloid leukemia M4Eo

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ABSTRACT

Background and Objectives. **It has been established that cytogenetic findings at the time of diagnosis of acute myeloid leukemia (AML) are powerful prognostic indicators. Pericentric inversion of chromosome 16 and translocation t(16;16) resulting in chimeric fusion of CBFB and MYH11 genes are typically seen in the M4-Eo FAB classification subset of AML and are associated with low-risk disease. These subtle chromosomal abnormalities may be difficult to detect in poor-quality metaphase preparations and if missed could lead to incorrect assignment to risk groups and influence the therapy decision-making process.**

Design and Methods. **We prospectively studied, at diagnosis, 10 patients with AML-M4 Eo by cytogenetics and fluorescent *in situ* hybridization (FISH) with two cosmids (36 and 40). As a control group, 7 patients (5 with a diagnosis of AML other than M4 Eo and two cases of reactive eosinophilia) were analyzed. In addition reverse transcriptase chain reaction (RT-PCR) studies were carried out in 6 cases.**

Results. **Karyotypic analysis detected the inv(16) in all but one of the patients with M4-Eo while none of the control cases showed any abnormality on chromosome 16. FISH studies showed that all 10 patients had abnormalities on chromosome 16; the patient with normal karyotype showed an inv(16) by FISH, while a case with inv(16) by cytogenetics had a t(16;16) by FISH. RT-PCR demonstrated amplification of the CBFB/MYH11 product in all cases analyzed.**

Interpretation and Conclusions. **In patients with M4Eo and rearrangements of chromosome 16, FISH studies may afford more complete information than conventional cytogenetics and can be an alternative to RT-PCR studies.**

Key words: cytogeneetics, FISH, RT-PCR, inv(16), t(16;16), CBFB-MYH11, acute myeloblastic leukemia, M4Eo

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Rearrangements of chromosome 16 are frequently found in patients with *de novo* acute myeloblastic leukemia (AML) of myelomonocytic subtype with increased bone marrow eosinophils (M4Eo).[1,2] This type of leukemia represents 5% of all AML[3,4] and is usually associated with either inv(16)(p13q22) or t(16;16)(p13;q22). The presence of inv(16) or t(16;16) has been associated with a high cure rate with standard chemotherapy, including high-dose Ara-C, and a relatively good prognosis.[3,5] At molecular level the inv(16) or the t(16;16) generates a CBFβ-MYH11 fusion gene that can be detected by reverse transcriptase polymerase chain reaction (RT-PCR) in over 90% of cases.[6-8] These studies have demonstrated the existence of a marked molecular heterogeneity and at least eight types of fusion transcripts (A-H) have been reported, with the A-type being predominant (88%).[9] Abnormalities on chromosome 16 are difficult to detect by routine cytogenetics, and thus the use of additional methods is desirable.[10] Recently, several probes to analyze the rearrangements of chromosome 16 by fluorescent *in situ* hybridization (FISH) have been reported,[11,12] but none of them has explored the value of cytogenetics, FISH, and RT-PCR for detecting abnormalities on chromosome 16.

The present paper reports on a series of 17 patients (10 AML-M4Eo; 5 AML-M4 and two cases with chronic eosinophilia) who were simultaneously investigated by conventional cytogenetics, FISH and RT-PCR for comparative assessment of abnormalities of chromosome 16.

Design and Methods

Ten patients diagnosed with M4Eo between 1996 and 1999 were included in the study together with a control group of 7 patients: 5 patients with the diagnosis of AML-M4 (without eosinophilia) and two cases of reactive eosinophilia. The diagnosis and classification of patients were based on morphologic and cytochemical examination of peripheral blood (PB) and bone marrow (BM) aspirate according to criteria proposed by the FAB co-operative group.[13]

Cytogenetics

Bone marrow specimens were cultured according to standard methods. When possible, 20 or more metaphases were analyzed to detect clonal abnor-

malities. These were defined by the presence of two metaphase cells with identical structural rearrangements in accordance with ISCN guidelines (1995).[14]

FISH

Fluorescence *in situ* hybridization (FISH) studies were carried out as previously described.[15] Chromosome spreads were treated with RNAase (100 mg/mL) and pepsin (0.1 mg/mL), and fixed in formaldehyde 1%. The hybridization mixture containing 5 ng/IU labeled cosmid was denaturated at 75ºC (7.5 minutes) and allowed to reanneal with unlabeled human Cot-1DNA. The slides were incubated overnight at 37ºC. After the coverslips had been removed, the slides were washed in the post-hybridization solutions. The biotinylated probes were detected with avidin-FITC and the digoxigenin-labeled probes were visualized using a mouse antibody to digoxin. Hybridization signals were analyzed by at least two independent observers on an Olympus BX60 coupled to a Cytovision Ultra system (Applied Imaging, Sunderland, UK) using a cooled, charge-coupled camera.

Probes

The chromosome 16 cosmids C36 (D16S79), proximal to the 16p13bp, and C40 (D16S257), distal to the 16p13bp, were obtained from a library of partially *Mbo*I-digested (Y18 DNA).[16] Both probes were kindly provided by H.G. Dauwerse, Department of Human Genetics, Leiden, The Netherlands. Hybridization signals were interpreted as follows: metaphases were considered normal when both signals from cosmid C36 and C40 were present on 16p; inv(16) when the signal from C40 moved to 16q while C36 remained on 16p; and t(16;16) when the C40 signal was translocated to the other chromosome 16 (that contained two normal signals on 16p). Probes C36 and C40 did not allow for the detection of del(16p). They are present in both the inversion and the translocation of chromosome 16 although the derivative chromosome 16 is deleted.[16]

RT-PCR

RNA was extracted, from washed bone marrow mononuclear cells by the guanidium thiocyanate method described by Chomczynski and Sacchi.[17] Reverse transcription (RT) was performed on 1-2 µg of total RNA in a 20 µL volume using random hexamers as primers and 200 U of Superscript™ II. Subsequently, 5 µL of RT products were used for two-step PCR analysis according to the guidelines proposed by Claxton *et al.*[18] Primers used to study the different *CBFB/MYH11* fusion transcripts are shown in Table 1. The PCR was performed in a Gene-Amp PCR System 9600 thermocycler. Seminested-PCR was carried out with primer 1 and primer 2 in the first round, and primer 3 and primer 2 in the second round. When a large PCR product was obtained (size not consistent with the most common transcript type A), a seminested-PCR using primers 1 and 3, as 5' primers and primer 4 as 3' primer was carried out. Finally, 15 µL of the PCR product were electrophoresed on an agarose gel stained with ethidium bromide. Two negative controls (one with HL60 RNA and one without RNA) and one positive control sample were included in each experiment. The integrity of the RNA was assessed by amplification of the ubiquitously expressed *ABL* gene following the recommendations of the European *BIOMED 1 Concerted Action for standardization of MRD studies in acute leukemia*.[19]

Table 1. Primers used for the detection of *CBFβ/MYH11* fusion transcript.

Primer	*Sequence 5'-3'*
1	CAGGCAAGGTATATTTGAAGG
2	CTCCTCTTCTCCTCATTCTGCTC
3	GTCTGTGTTATCTGGAAAGGCTG
4	CGTACTGCTGGGTGAGGTTCT

Table 2. Clinical features at diagnosis of patients with AML-M4Eo and abnormalities of chromosome 16.

Case	*Sex*	*Age (yrs)*	*Hb (g/dL)*	*WBC (x10⁹/L)*	*Plts*	*BM eosinophils*	*Resp.*	*Relapse*	*Survival (mos.)*
1	M	63	130	55	56	18%	CR	No	35+
2	M	51	66	54	16	75%	CR	No	22+
3	F	10	99	70	16	13%	CR	No	20+
4	F	29	106	25	25	16%	NE	NE	1
5	M	29	90	185	40	8%	CR	Yes	10+
6	F	56	98	106	11	9%	CR	Yes	2+
7	M	29	81	64	34	15%	CR	No	29
8	M	52	97	5	84	12%	CR	No	39
9	M	52	93	51	21	19%	NE	NE	1
10	F	29	58	125	15	19%	CR	No	2

Hb: hemoglobin; WBC: white blood cells; male; F: female; CR: complete remission; NE: not evaluable.

Results

Patients' characteristics

The most relevant clinical and hematologic characteristics of the 10 patients with AML-M4Eo included in this study are summarized in Table 2. Their median age was 29 years (range 10 to 63). Most patients displayed marked anemia (median hemoglobin was 93 g/L, range 58-130 g/L) and all had platelet counts below 100×10^9/L (range 11-84). All but one case (#8) had leukocytosis (median WBC count was 55×10^9/L, range 5-185). The median percentage of eosinophils in the BM was 18% (range 8-75). All cases were treated with idarubicin or daunorubicin and Ara-C. Eight out of the ten cases achieved a complete remission (the other two cases died during the induction therapy); of the 8 achieving complete remission, 2 have subsequently relapsed (Table 2).

Cytogenetics

Cytogenetic results of the 10 cases with a diagnosis of AML-M4Eo are summarized in Table 3. An inv(16)(p13q22) was observed in 9 out of the 10 cases while case #5 showed a normal karyotype. Additional abnormalities to the inv(16) were present in

Table 3. Cytogenetics, FISH and RT-PCR of patients with AML-M4Eo.

No.	Cytogenetics	FISH	CBFβ–MYH11
1.	46,XY,inv(16)(p13q22) [18]/ 46,XY [2]	inv	A
2.	46,XY,inv(16)(p13q22) [20]	t	A
3.	46,XX,inv(16)(p13q22) [23]	inv	A
4.	46,XX,inv(16)(p13q22) [17]/ 46,XX [4]	inv	C
5.	46,XY [25]	inv	A
6.	46,XX,inv(16)(p13q22) [20]/ 46,XX [4]	inv	A
7.	45,X,-Y,del(2)(q32q36),inv(16)(p13q22) [21]	inv	ND
8.	46,XY,inv(16)(p13q22) [20]/ 46,XY [3]	inv	ND
9.	46,XY,inv(16)(p13q22) [20]	inv	ND
10.	46,XY,t(3;10)(p22;p14),inv(16)(p13q22) [24]/ 46,XY [2]	inv	ND

FISH: inv: inv(16)(p13q22)(C36 st, C40 mv); t: t(16;16)(p13;q22)(C40mv). A: A-type amplification; C: C-type amplification; ND: not done.

two cases (#7 and 10). Case #7 had a loss of chromosome Y and a del(2)(q32) while case #10 had a t(3;10)(p22;p14) (Table 3).

In the control group, no chromosomal abnormalities were found in any of the 7 cases analyzed.

FISH

Double color FISH studies with specific probes C36 and C40 were carried out in the 10 patients with the diagnosis of AML-M4Eo and in the 7 control cases. A minimum of 15 metaphases were analyzed in the M4Eo group and 25 in the control group. The results of the FISH studies in the patients with AML-M4Eo are summarized in Table 3. All patients showed rearrangements of chromosome 16. Interestingly case #2, with an inv(16) by conventional cytogenetics, had a t(16;16) in the FISH analysis (Figure 1A). Moreover, case #5, displaying a normal karyotype, had an inv(16) by FISH (Figure 1B). In the control group both cases with chronic eosinophilia and all cases with AML-M4 showed normal chromosomes 16 in all 25 metaphases analyzed for each patient. In cases #1, 2, 3, 5 and 7 BM was studied by FISH at time of complete remission. No evidence of either inversion (cases #1, 3, 5 and 7) or translocation (case #2) was observed by FISH studies.

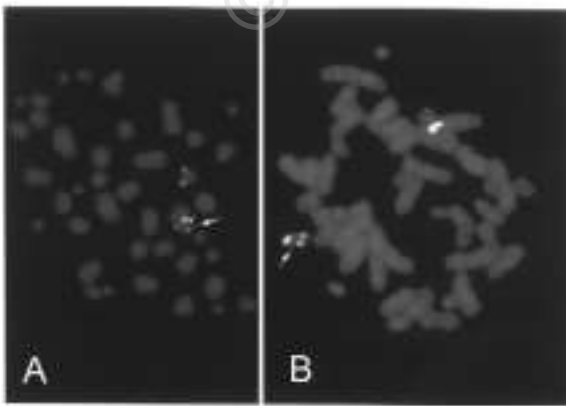

Figure 1. FISH studies on case #2 (A) and on case #5 (B) showing a t(16;16) and an inv(16) respectively. C36 is labeled in green and C40 is labeled in red. Yellow arrows show the abnormal localization of C40.

RT-PCR

Amplification of *CBFβ/MYH11* hybrid transcripts was present in all six cases studied. Five out of the six cases had the classical A-type fusion transcript, including case #2 with t(16;16) and case #5 with a normal karyotype. Case #4 showed a C-type or D-type fusion transcript according to the nomenclature proposed by Claxton *et al.*[18] or Liu *et al.*,[9] respectively (Table 3, Figure 2).

Discussion

We used FISH and RT-PCR to study the rearrangements of chromosome 16 in a series of patients with a diagnosis of AML-M4Eo and in a control group with related hematologic disorders (AML-M4 without eosinophilia and reactive eosinophilia). The results showed that FISH and RT-PCR analysis are

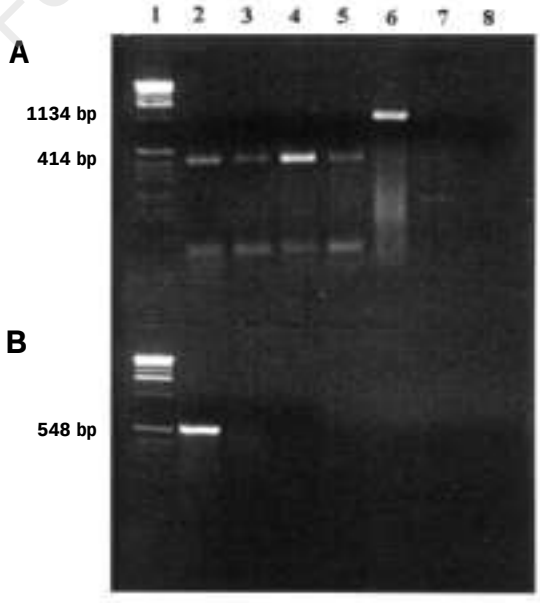

Figure 2. Detection of *CBFβ-MYH11* fusion transcripts by RT-PCR. (A) Agarose gel electrophoresis of external primer set amplification products. Lane 1 is the 1 Kb DNA ladder molecular weight marker. Lanes 2, 3, 4 and 5 show the expected 414 bp fusion fragments from the common type A transcript. Lane 6 shows the 1,134 bp fragment expected from type C. Lanes 7 and 8 are negative controls from a patient with AML-M4 without eosinophilia and without RNA, respectively. (B) Amplification products with inner primers used for larger chimeric fragments. Lane 2 shows a 548 bp product which corresponds to a type C fusion. Lanes 3 and 4 are negative controls from a patient with AML-M4 without eosinophilia and without RNA template, respectively.

highly specific and have a good correlation. Both techniques allow refinement of the results obtained by conventional cytogenetics.

The clinical and hematologic features of the patients included in the present study are similar to those referred to other series of AML.[3,5] All the evaluable cases achieved a complete remission with standard AML chemotherapy; two (cases #5 and 6) relapsed. All these data are in accordance with those from large series in which the AML patients with t(8;21), t(15;17) or rearrangements of chromosome 16 have the best prognosis.[3,4]

Regarding cytogenetic results, most of the cases included in our study had an inv(16) as shown by conventional cytogenetics. In two cases abnormalities additional to the inv(16) were found, with a frequency similar to that previously described.[20] Interestingly, the two cases with additional abnormalities have died but neither of them due to leukemia relapse: case #5 in complete remission after a bone marrow transplantation and case #10 during consolidation therapy after achieving complete remission with induction therapy. It has been reported that the presence of changes additional to the inv(16) does not affect the response to therapy or the survival.[3,20] In our study we did not find variant translocations, recently described by others.[21]

FISH studies using a set of cosmids hybridizing proximally and distally to the 16p13 breakpoint demonstrated the presence of an inv(16) in 9 of the 10 cases while the remaining case showed a t(16;16). Interestingly case #5, with a normal karyotype and a low percentage of mature eosinophils in the bone marrow showed an inv(16) by FISH. This cytogenetic abnormality may sometimes be difficult to find only by conventional cytogenetic analysis.[10,22] Moreover case #2, with an inv(16) in the cytogenetic analysis, displayed a t(16;16) by FISH study (Figure 1B). This case showed pronounced bone marrow infiltration by mature but morphologically abnormal eosinophils. It has been demonstrated that this population of mature eosinophils also has the pericentric inv(16).[23] Translocation t(16;16) is less frequent than inv(16) in AML-M4Eo and is not associated with specific clinical or hematologic characteristics.[12]

RT-PCR studies showed the *CBFβ/MYH11* fusion transcript in all cases analyzed with a heterogeneous distribution in the fusion point in the two genes, particularly in the *MYH11* gene. At present, eight different *CBFβ/MYH11* fusion transcripts have been reported.[9] In accordance with previous reports,[6,9,18,22,24,25] most of our cases had the clasical A-type fusion transcript while only case #4 had a C-type fusion transcript. So far, no clear differences in clinical outcome or type of chromosomal aberrations (inversion or translocation) have been reported upon comparing the different transcript types.[9]

In summary, the present study supports the usefulness of FISH and RT-PCR studies, with specific probes, in the study of rearrangements of chromosome 16. Both methodologies have a good correlation and should be used at the time of diagnosis in the study of acute myeloblastic leukemias with abnormal eosinophils.

Contributions and Acknowledgments

JMH: design and writing of the paper. MBG: FISH studies. IG: cytogenetics. NCG: cytogenetics and FISH. CC: molecular studies. FR: clinical data. JMR: Design of the paper and clinical data. MG: molecular studies. EF: review of the paper. JFSM: review and final approval. We thank Carl Hilliker, Juan Luis García, Pilar Fernández, M. Angeles Hernández and Mark Anderson for technical assistance.

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Disclosures

Conflict of interest: none.

Redundant publications: there is a less than 25% overlapping with another paper published by some of the authors. The reference is: Calvo C, Ribera JM, Milla F, et al. Leucemia aguda mielomonocítica con eosinofilia e inversión del cromosoma 16. Estudio de 6 casos. Med Clin (Barcelona) 1997; 108:182-5. Patients #7, 8, 9 and 10 included in our study were described in this paper. This study includes more patients and reports the not previously performed FISH studies.

Manuscript processing

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Potential Implications for clinical practice

- FISH is clinically useful on detecting rearrangements of chromosome 16 in patients with M4Eo.

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