Detection of Minimal Residual Disease in Acute Myelomonocytic Leukemia With Abnormal Marrow Eosinophils by Nested Polymerase Chain Reaction With Allele Specific Amplification

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Acute myelomonocytic leukemia with bone marrow eosinophilia (AML-M4Eo) as it is not found in other FAB subtypes of AML, including AML-M4. To assess the presence of type A CBFB/MYH11 fusion transcripts in five AML-M4Eo patients in remission, we designed a sensitive assay combining nested PCR and allele-specific amplification (NPASA). This article must therefore be hereby marked in accordance with U.S.C. section 1734 solely to indicate this fact.

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24 AML-M4Eo patients at presentation are summarized in Table 1. Diagnosis was established according to standard FAB criteria. CR was defined by clinical and cytological criteria. Mononuclear cells obtained from BM or PB samples were isolated by Ficoll-Hypaque centrifugation and viably frozen in liquid nitrogen until use.

### Cytogenetic Analysis

Cytogenetic analysis was performed on PB or BM cells after 24 and 48 hours in unstimulated culture. RHG bands and G bands with trypsin or with Wright staining were obtained. Karyotypes were reviewed and designated according to ISCN criteria (Table 1).

### RNA Preparation

Total RNA was extracted from cryopreserved cells according to the method of Chomczynski and Sacchi. RNA extraction was performed on at least two different remission samples from patients with AML-M4Eo. The quality of RNA was controlled on an ethidium bromide–stained 1% agarose gel containing 2.2 mol/L formaldehyde.

### RT-PCR Amplification

Experimental precautions recommended by Kwok and Higuchi were taken to avoid contamination.

First-step PCR. One microgram of total RNA was denatured at 95°C for 3 minutes and added to 10 μL of a mixture containing 1× PCR buffer (10 mmol/L Tris-HCl, pH 9.0, 50 mmol/L KCl, 0.1% Triton X-100, 0.01% gelatin), 5 mmol/L MgCl₂, 10 mmol/L DTT, 1 mmol/L of each dNTP, 2.5 μmol/L random hexamers, 20 U RNase inhibitor (Boehringer Mannheim, Meylan, France), and 200 U Moloney reverse transcriptase (Bethesda Research Laboratory, Gaithersburg, MD). Reverse transcription was performed at 37°C for 45 minutes. For amplification, 10 μL of this cDNA was used in a total volume of 50 μL containing 1× PCR buffer, 1.5 mmol/L MgCl₂, 200 μmol/L of each dNTP, 0.3 μmol/L primers C1 and M1, and 1 U Taq DNA polymerase ATGC (ATGC Biotechnologies, Noisy-le-Grand, France).

After an initial denaturation step of 3 minutes at 95°C, 35 cycles consisting of 30 seconds at 95°C, 30 seconds at 57°C, and 1 minute at 72°C followed by a final extension of 8 minutes at 72°C were performed on a DNA thermal cycler (PTC-100 Programmable Thermal Controller; MJ Research Inc, Cambridge, MA). Cycling parameters have been optimized to allow a reliable detection of types A, C, and D transcripts (Fig 1).

Nested PCR and allele-specific amplification. The first round of amplification was performed as described above. In the second round of amplification, 5 μL of the first PCR products were added in a 50-μL total volume containing 1× PCR buffer, 1.5 mmol/L MgCl₂, 200 μmol/L of each dNTP, 0.3 μmol/L of inner primers C2 and M2, and 1 U Taq DNA polymerase. Cycling parameters included an initial denaturation step of 3 minutes at 95°C, followed by 25 cycles of denaturation at 95°C for 30 seconds and annealing at 57°C for 30 seconds.

For all samples, amplification of β-actin cDNA was performed on the same cDNA used to identify CBFB/MYH11, as a control. Negative controls were included in all PCR experiments. Four negative controls were added during the study of CR samples by the nested PCR reaction. The sequences of the primers used are as follows: C1, 5'CGAGCGAAGTTATTTGGAAGG3' (nt 427 to 448 of CBFB); C2, 5'ACACCGGAATTTGAGATAGA3' (nt 427 to 448 of CBFB); C3, 5'TCCTCTCTCATCTGGTC3' (nt 427 to 448 of CBFB); C4, 5'ACATGCGCTCTTCGACACTGG3' (nt 427 to 448 of CBFB).
Tris borate EDTA (TBE) buffer and visualized by ethidium bromide staining.

For hybridization, C1M1 amplification products (10 µL) were run on a 1% agarose, 1% NuSieve gel in 1× TBE buffer, transferred to a Hybond N+ membrane (Amersham International, Buckinghamshire, UK), and hybridized to 30 ng of a junction-specific oligonucleotide probe labeled with 10 mCi γ-32P-adenosine triphosphate (ATP) by T4 polynucleotide kinase (New England Biolabs, Beverly, MA). The sequences of oligonucleotide junction specific probes are as follows: type A, 5’CGGGAGGAAATGGAGGTCCATGAG3’; type C, 5’GGAAATGGGAAATGAAGTTG3’; and type D, 5’GAGGA-AATGGAGCAGAAGGC3’.

RESULTS

Correlation of CBFB/MYH11 Fusion Transcripts With AML-M4Eo and inv(16)(p13q22)

Twenty-four patients with AML-M4Eo were included in this study. Morphologic examination of BM aspirates at di-

![Fig 1. Agarose gel electrophoresis and Southern blot hybridization of the three types of C1M1 PCR products. Blot was successively hybridized to radiolabeled junction specific probes and autoradiographed. MV, DNA molecular weight marker V (Boehringer Mannheim); ΦX174, ΦX174 DNA-Hael1 digest (New England Biolabs); arrows point to measured sizes of PCR products: type A, 420 bp; type C, 1.2 kb; type D, 1.4 kb. (PCR products are named according to Claxton et al[9]).](image)

TTGGGTG3’ (reverse sequence of nt 3364 to 3385 of MYH11 cDNA); M4, 5’ATCTCTGGAGGCACGCCATCT3’ (reverse sequence of nt 2328 to 2349 of MYH11 cDNA); M5, 5’TTCCTAG- TTCCGTCTCTACTT3’ (reverse sequence of nt 2141 to 2160 of MYH11 cDNA); M7, 5’TTCGGCAGCTATGGGACCTCC3’ (Fig 2); AC11, 5’CCTCATGAAGATCCTCACCG3’ (5’ β-actin primer in exon 4); and AC2, 5’TTCGTGGATGCCACAGGAC3’ (3’ β-actin primer in exon 5).

Dilution Experiments

To assess the sensitivity of RT-PCR, 1 µg of total RNA isolated from BM cells of patient no. (PN) m20 was serially diluted in RNA extracted from normal PB mononuclear cells. The resulting cDNA was then submitted to 35 cycles of amplification with primers C1 and M1, followed by 25 cycles of amplification with nested primers C2 and M7.

Analysis of PCR Products

C1M1 and C2M7 amplification products were run on a 5% Long Ranger gel (Bioprobe Systems, Montreuil-sous-Bois, France) in 1×
agnosis always showed the typical abnormal eosinophilic cells. These cells were easily detected in all but one case (PNm02). In this case, the percentage of abnormal eosinophilic precursors (chloroacetate esterase-positive) was less than 5% of all nucleated BM cells. These results were confirmed by review of the slides.

Expression of CBFB/MYH11 fusion mRNA by tumor cells was assessed by a RT-PCR assay, using primers C1 and M1 designed by Liu et al. Fusion mRNAs were found in 22 of 24 patients with AML M4Eo (Table 1). Twenty of these 22 patients have a pericentric inversion of chromosome 16 as the sole karyotypic change or in association with additional chromosomal abnormalities. Cytogenetic analysis was not performed at diagnosis in the two remaining cases. We have characterized the fusion mRNAs in our 22 positive cases. Using C1 and M1 primers, a type A fusion product (420 bp) was found in 20 of these 22 patients. Among the two other positive cases, one (PNm10) has a type C (1.2 kb) and the other (PNm02) has a type D product (1.4 kb). Morphologic features of these two cases were not different from those of patients with type A PCR product. The specificity of these amplified bands was verified by hybridization to oligonucleotide probes directed against the three fusion sequences published by Liu et al. Results for the three types of PCR products are shown in Fig 1.

No fusion product was found in two of 24 patients (PNm09 and Pnm24). Cytological examination of these cases did not show any characteristic distinct from those usually seen in AML-M4Eo. One patient (PNm09) had a t(8;16)(q11;q22) and the other (PNm24) had five metaphase cells, probably with an inv(16), but only RHG bands were performed in this case (Table 1). To demonstrate that adequate cDNAs had been synthesized in these cases, 25 cycles of amplification of β-actin were performed. Positive samples with fusion products type C and D were also tested simultaneously as controls. Results were confirmed on a second RNA sample. Therefore, the presence of a type A, B, C, or D product in these two cases seems unlikely. Negative PCR results were also obtained with other MYH11 primers (M2, M3, and M4) or using the sensitive assay we have designed for the detection of minimal residual disease (see below).

To correlate the presence of CBFB/MYH11 fusion transcripts with the FAB subtypes of AML, 47 patients with other types of AML (M1 [nine cases], M2 [nine cases], M3 [five cases], M4 [13 cases], M5 [nine cases], and M7 [two cases]) were studied. No CBFB/MYH11 PCR product was detected.

These results show that the expression of CBFB/MYH11 transcripts is strongly associated with AML-M4Eo and with inv(16) and that the type A fusion product is found in a large majority of AML-M4Eo patients.

Study of Minimal Residual Disease by Nested PCR and Allele-Specific Amplification

We have performed dilution experiments to test the sensitivity of the RT-PCR assay for the CBFB/MYH11 fusion transcripts using primers C1 and M1. Unfortunately, as shown in Fig 2, a weak sensitivity has resulted from these experiments. The sensitivity was not greatly improved either by hybridization or by a seminested PCR (outer primer C1, inner primer C2, and primer M1) or a nested PCR (outer primers C1 and M4, and inner primers C2 and M5) (data not shown).

To improve this level of sensitivity, nested PCR and allele-specific amplification (PASA) of the type A junctional sequence were combined (Fig 2). Using this approach, five AML-M4Eo patients with a type A CBFB/MYH11 fusion product at diagnosis were evaluated in CR. Four patients are in continuous complete remission (CCR). One patient (PNm18) developed a secondary leukemia (AML-M5) with chromosomal abnormalities, including band 11q23 rearrangement, but without inv(16). RT-PCR results are shown in Fig 3. A CBFB/MYH11 fusion product was detected in four patients who were in CR from 4 to 22 months after induction chemotherapy. One case (PNm18) had a positive result that converted to negative after an autologous BM transplant.

Fig 3. RT-PCR results of five patients with AML-M4Eo in remission. All had a type A CBFB/MYH11 fusion product at diagnosis. (A) Longitudinal follow-up: (○) RT-PCR-positive result; (●) RT-PCR-negative result; * chemotherapy treatment; (□) autologous BM transplantation. Arrow indicates the time of diagnosis of secondary leukemia. (B) Polyacrylamide gel electrophoresis (ethidium bromide-stained) of RT-PCR products after the second round of amplification with primers C2 and M7: positive control, 0.1 pg of Pnm20 RNA diluted in 1 μg of RNA from normal PB mononuclear cells; (a) 10 months; (b) 21 months; MV, DNA molecular weight marker V (Boehringer Mannheim); black arrow points to 65-bp C2M7 PCR products.
transplantation for secondary leukemia. One patient (PNm06), tested after 5 years of CR, was negative. It should be noted that the first-step PCR has allowed the detection of CBFB/MYH11 fusion mRNA only in one patient (PNm16) (data not shown).

DISCUSSION

These results extend the data recently reported in the study by Claxton et al. and add important information regarding the evaluation of minimal residual disease in inv(16) acute leukemias. Our analysis of a large number of patients confirms that CBFB/MYH11 transcripts are found in the great majority of inv(16) AML and that one type of fusion product (type A) is more frequent. Inv(16) is a subtle cytogenetic alteration that can be difficult to detect on preparations with insufficient or poor-quality metaphase cells. In these cases, RT-PCR appears to be a useful additional technique for the identification of CBFB/MYH11 transcripts.

In this study, the detection of CBFB/MYH11 transcripts was closely associated with the presence of abnormal BM eosinophils, as it was found only in AML-M4Eo but not in other FAB subtypes of AML, including AML-M4. However, no distinct morphologic characteristic was found in the two cases with less frequent fusion transcripts (type C and D) or in the two RT-PCR-negative cases.

We did not detect CBFB/MYH11 chimeric transcripts in two patients, including one with a variant translocation. This absence of CBFB/MYH11 transcripts detectable by RT-PCR might be caused by the involvement of other breakpoints on CBFB or MYH11. Alternatively, different genes could possibly be rearranged in these cases. Claxton et al. also reported two patients with negative amplification. One case with inv(16)(p13q22) possibly had an inadequate RNA and the other had a different breakpoint on 16q (16q24).

PCR evaluation of chromosomal rearrangements is an established tool for the detection of minimal residual disease as recently shown in AMLs for the t(15;17) and t(8;21) translocations. For a clinical purpose, such a strategy needs to be specific and sensitive. The low level of sensitivity found when using RT-PCR assay for the detection of CBFB/MYH11 transcript is reminiscent of that found by others for the PML/RARα gene detection. It should be noted that sensitivity was evaluated by serially diluting RNA from BM cells of an inv(16) patient in RNA extracted from normal PB cells instead of water.

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Detection of minimal residual disease in acute myelomonocytic leukemia with abnormal marrow eosinophils by nested polymerase chain reaction with allele specific amplification

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