Consultations in Molecular Diagnostics

Fluorescence *In Situ* Hybridization Identifies Cryptic t(16;16)(p13;q22) Masked By del(16)(q22) in a Case of AML-M4 Eo

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We report a patient presenting with acute myeloid leukemia (AML)-M4 Eo, in whom conventional cytogenetic analysis revealed a 46, XY, del(16)(q22) karyotype. Molecular analysis of the bone marrow cells using reverse transcriptase polymerase chain reaction (RT-PCR) identified a *CBFβ*-*MYH11*, "type A" fusion transcript. However, despite a thorough re-evaluation, a balanced chromosome 16 abnormality could not be definitively identified by cytogenetics. Since there exists a small possibility of obtaining a false-positive PCR result, fluorescence *in situ* hybridization (FISH) analysis using dual-color, break-apart probes for *CBFβ* was performed to elucidate the mechanism of fusion gene formation and thus confirm the RT-PCR results. FISH analysis clearly revealed a cryptic t(16;16), which was probably masked by the del(16)(q22). FISH is the preferred diagnostic procedure to elucidate the *CBFβ*-*MYH11* fusion in this situation, and resolves the possibility of both false-positive and false-negative results with RT-PCR technique. Due to the improved prognosis of AML associated with the *CBFβ*-*MYH11* fusion compared to AML generally, we recommend the use of FISH for detection of inv(16)/t(16;16)/*CBFβ*-*MYH11* in patients with failed, complex, or apparently normal cytogenetics, and in whom the cell morphology indicates the strong possibility of this gene fusion. (*J Mol Diagn* 2004, 6:271–274)

disease.¹ Rearrangements of the *CBFβ* gene define one such prognostically distinct group, which is uniquely associated with, but not restricted to, acute myelomonocytic leukemia with abnormal eosinophils (AML-M4 Eo).² Rearrangement of the *CBFβ* gene results in a fusion gene *CBFβ*-*MYH11* produced by the juxtaposition of bands 16q22 (containing *CBFβ*) and 16p13 (containing *MYH11*). At the cytogenetic level, this juxtaposition is brought about most commonly by inv(16)(p13;q22) and less commonly by t(16;16)(p13;q22).²

Patients with *CBFβ*-*MYH11* AML constitute approximately 10% of all *de novo* acute myeloid leukemias and have significantly better prognosis as compared with patients with complex chromosomal aberrations or normal karyotype.³ This is especially true for patients who receive intensive post-remission treatment with high-dose cytarabine.⁴ Consequently, in several treatment protocols, the consolidation therapy of adults with AML has been adapted in a risk-adjusted fashion.⁵ Patients with *CBFβ*-*MYH11* AML may achieve continuous complete remission with chemotherapy, rather than proceeding to autologous or allogeneic peripheral stem cell transplantation.⁵ Therefore, detection of these cytogenetic aberrations is of utmost importance in the risk-adjusted stratification of these AML patients.

Standard karyotypic analysis remains the "gold-standard" for the detection of cytogenetic aberrations. However, both inv(16) and t(16;16) may be subtle, cryptic, or masked by deletions and thus difficult to detect using standard cytogenetic techniques, especially in metaphase spreads showing suboptimal chromosomal morphology. The mRNA transcripts resulting from *CBFβ*-*MYH11* gene rearrangement are amenable to detection by reverse transcriptase polymerase chain reaction (RT-PCR); however, there exists a small possibility of false-positive RT-PCR results, which could theoretically arise

Multiple recurrent chromosomal aberrations resulting in the disruption of the core binding factor (*CBF*) genes have recently been correlated with distinct biological and clinical features of acute myeloid leukemia (AML), resulting in delineation of prognostically important categories of this

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from false-primed sites or carry-over contamination,⁵⁻⁹ although the former explanation is controversial.¹⁰ Conversely, given the marked heterogeneity of breakpoints in (especially) the *MYH11* gene,¹¹ a negative RT-PCR may not exclude the presence of the *inv(16)/t(16;16)*. Hence, in such cases, fluorescence *in situ* hybridization (FISH) studies can be used to elucidate the mechanism of rearrangement and to corroborate the RT-PCR results. We describe one such case of AML-M4 Eo in which, despite thorough reevaluation after obtaining positive RT-PCR results, we were unable to detect *inv(16)/t(16;16)* unequivocally by classical cytogenetics. FISH studies were subsequently required to identify a cryptic *t(16;16)(p13;q22)*, which was masked by an apparent *del(16)(q22)*, thus reconciling the discrepant karyotypic and molecular findings.

Case History

A 52-year-old male presented to his local doctor with recent history of fatigue. Physical examination was unremarkable; no lymphadenopathy or hepatosplenomegaly was palpable. A complete blood count revealed: hemoglobin, 8.5 g/dl (normal: 14 to 18 g/dl); hematocrit, 23.6% (normal, 42 to 54%); platelet count, $13 \times 10^3/\text{mm}^3$ (normal, 150 to $450 \times 10^3/\text{mm}^3$); and white blood cell (WBC) count, $61 \times 10^3/\text{mm}^3$ (normal, 4 to $11 \times 10^3/\text{mm}^3$) with 60% blasts. Bone marrow aspirate confirmed a morphologically classical acute myelomonocytic leukemia with 19% dysplastic eosinophils (Figure 1A). The patient was treated with conventional chemotherapy regimen and went into hematological remission within 1 month. He maintains his remission status 6 months from the time of diagnosis.

Materials and Methods

Cytogenetics

Cytogenetic studies were performed on bone marrow using short-term unstimulated cultures. Metaphases were G-banded by conventional GTW-banding. Karyotypes were described according to the International System for Cytogenetic Nomenclature (1995).

RT-PCR for *CBFβ*-*MYH11* Fusion mRNA

RT-PCR for *CBFβ*-*MYH11* transcript was performed essentially as previously described.¹² Briefly, total RNA was isolated from cells using the RNeasy method (Qiagen, Santa Clarita, CA), according to the manufacturer's directions. One μg of RNA was reverse-transcribed from random hexamers in a total volume of 20 μL containing 50 mmol/L KCl, 10 mmol/L Tris (pH 8.4), 5 mmol/L MgCl_2 , 1 mmol/L dNTP, 50 pmol random hexamer, 1 U/ μL RNasin (Promega, Madison, WI), 5 mmol/L dithiothreitol (DTT), and 100 U Moloney's murine leukemia virus-RT (MMLV-RT; GIBCO-BRL Life Technologies, Grand Island, NY), under the following conditions: 23°C for 10 minutes, 42°C for 60 minutes, 95°C for 5 minutes, and 4°C hold. A first-round PCR amplification was subsequently performed

with 15 μL of cDNA in a total 50 μL reaction volume containing 10 mmol/L Tris, 50 mmol/L KCl, 1.5 mmol/L MgCl_2 , 200 $\mu\text{mol/L}$ dNTP, 20 pmol of each primer, and 2.5 U *Taq* polymerase [Perkin-Elmer/Roche, Branchburg, NJ]. The primer sequences were *CBFβ* (5'-3') GCAGGCAAGG-TATATTGAAGG; *MYH11* (5'-3') CTCTTCTCCTCATTCT-GCTC. These primers amplify the common type A *CBFβ*/*MYH11* fusion with an expected product size of 415 bp; in addition, the less common types D and E transcripts can be detected with these primers (~1.2 kb and 1.4 kb, respectively).¹² Five μL of cDNA were used for control amplification of a segment of $\beta 2$ -microglobulin using primers $\beta 2$ mol/L-f (5'-3') GAAAAAGATGAGTATGCCTG; $\beta 2$ mol/L-r (5'-3') ATCTTCAAACCTCCATGATG. Following an initial denaturation of 95°C for 5 minutes, cycling parameters were three cycles of 95°C for 30 seconds, 59°C for 45 seconds, and 72°C for 90 seconds, then 30 cycles of 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 60 seconds, and a final extension step of 72°C for 5 minutes. PCR products were analyzed by electrophoresis in 1.5% agarose gel (Seakem ME; FMC Bioproducts, Rockland, ME), vacuum-blotted on nylon membrane (Pall Biotryne, East Hills, NY), and UV cross-linked. Membranes were subsequently hybridized with a 5' biotinylated internal *CBFβ* sequence oligoprobe (5'-3') ATAGAGACAGGTCT-CATCGG, stringently washed and detected by chemiluminescence (ECL System; Amersham Life Sciences, Arlington Heights, IL) on autoradiograph film (Kodak XAR; Eastman Kodak, Rochester, NY).

FISH for *inv(16)/t(16;16)*

Probes

Commercially available LSI *CBFβ* dual-color, break-apart rearrangement probes (Vysis, Downers Grove, IL) for detection of *inv(16)/t(16;16)* were used in this study. The probe set consists of a mixture of a 5' *CBFβ* probe labeled with Spectrum red and a 3' *CBFβ* probe labeled with Spectrum green. The 5' *CBFβ* probe is approximately 150 kb and is positioned centromeric to the *inv(16)* breakpoint region. The 3' *CBFβ* probe is approximately 170 kb and is positioned telomeric to the *inv(16)* breakpoint and does not extend over the breakpoint.

FISH Assay

Slides prepared for cytogenetic analyses were used for interphase and metaphase FISH. FISH assay was performed according to Vysis, Inc. (Downers Grove, IL) protocol. Briefly, 10 μL of hybridization mixture (1 μL probe, 2 μL water, and 7 μL hybridization buffer) was added to the target area on the slide. The slides were cover-slipped and sealed with rubber cement, and the probe was denatured at 73°C for 1 minute using the Hybrite system (Vysis, Inc.). After overnight incubation at 37°C, the coverslips were removed, slides were washed in post-hybridization solutions, and counter-stained with 4, 6-diamidino-2-phenylindole (DAPI) in antifade (Vysis, Inc.). Hybridization signals were visualized using a Leitz

A

B

C

Diaplan fluorescence microscope equipped with a triple band-pass filter (FITC/Rhodamine/DAPI). Images were captured with a cooled charge-coupled camera (CCD) and digitally recorded (PowerGene MacProbe software Applied Imaging Corp., Santa Clara, CA). Approximately 200 inter-phase nuclei and five metaphase spreads were examined and scored.

Evaluation Criteria

The probes used result in a red signal on chromosome 16q22 centromeric to the inv(16) breakpoint and a green signal telomeric to the inv(16) breakpoint. Thus a normal cell (with no break at the inv(16) locus) would result in two fused red/green (yellow) signals. A nucleus containing inv(16) would result in separate red and green signals appearing on opposite arms of the inverted chromosome 16, whereas a nucleus containing t(16;16)(p13;q22) would result in one fused red/green signal on the q arm of one chromosome and a green signal on the other arm of the chromosome 16, while the other chromosome 16 will only contain the red signal on one arm.

Results and Discussion

The chromosomal aberrations leading to *CBFβ-MYH11* gene fusion are recognized as indicators of relatively favorable prognosis and are now classified as a distinct subgroup of AML.¹ The detection of these rearrangements is thus not only important diagnostically, but also crucial for treatment stratification and clinical management of these patients.⁵ As both inv(16) and t(16;16) are cytogenetically visible, conventional karyotypic analysis remains the “gold standard” and most institutions rely on conventional G-banding technique for their detection. However, both inv(16) and t(16;16) are subtle abnormalities that could be missed in suboptimal quality metaphases or be masked by deletions or variant translocations.

Cytogenetic analysis of the bone marrow sample in our case of AML M4Eo (Figure 1A) revealed the following karyotype: 46, XY, del(16)(q22)[13]/46, XY. [7] No inv(16)/t(16;16) was discerned at a microscopic level using the conventional GTW banding (Figure 1A, inset). While del(16)(q22) is a recurrent abnormality reported in patients with AML M4Eo, the deletion by itself does not

Figure 1. A: Acute myelomonocytic leukemia with dyplastic eosinophils. Note several myeloid blasts admixed with abnormal eosinophils with large basophilic granules. **Inset:** Partial karyotype showing del(16)(q22) by G-banding (arrow). **B:** RT-PCR for detection of *CBFβ-MYH11* fusion transcripts. Chemiluminescent detection of PCR products hybridized with *CBFβ* internal oligoprobe. Lanes are designated as follows: L, 100 bp size ladder; Pt, patient sample; (-), negative (non-specific RNA) control; W, water blank; (+), positive control for *CBFβ-MYH11* (ME-1 cell line). Arrow demonstrates a 415-bp amplicon type A *CBFβ-MYH11* product. **C:** Dual-color, break-apart FISH: large image of metaphase nucleus showing a fused red/green (yellow) signal on the q arm of one chromosome 16 and a green signal on the other arm, while the chromosome 16 homologue shows only the red signal on one arm, consistent with t(16;16)(p13;q22) [indicated by arrows]. **Inset:** Interphase nucleus showing a yellow fusion signal (normal configuration), as well as one green and one red signal (abnormal signal). See text for further details.

lead to the *CBFB-MYH11* fusion and hence these cases would not be associated with the same prognostically favorable clinical outcome.⁵ In several previous studies, reevaluation of del(16)(q22) after detection of *CBFB-MYH11* transcripts by RT-PCR, led to identification of a masked or cryptic inv(16)/t(16;16).^{5,13} These studies suggest that although true isolated del(16)(q22) exists, many del(16)(q22) can actually occur concomitantly with and therefore mask an inv(16) or t(16;16). Accordingly, RT-PCR analysis performed on our patient's leukemia revealed the presence of the common (type A) chimeric *CBFB-MYH11* gene fusion transcript (Figure 1B). This finding, in turn, prompted careful review of the cytogenetic data, which still revealed only del(16)(q22). To reconcile this major diagnostic discrepancy, FISH analysis was performed using dual-color, break-apart probes for *CBFB*. Of the 200 interphase nuclei examined by FISH, 183 nuclei (91.5%) showed one fusion signal, along with one green and one red signal; analysis of five available metaphases showed a green signal on the short arm with a fusion signal on the long arm of one chromosome 16, and only one red signal on the homologous chromosome 16, confirming the presence of a t(16;16)(p13;q22) (Figure 1C).

RT-PCR is a rapid and specific test for identifying the presence of the *CBFB-MYH11* abnormality.^{6–8,12,13} Several studies report the retrospective identification of a "masked" or missed inv(16)/t(16;16) in leukemias with positive RT-PCR results.^{5,13–17} While these studies suggest a higher detection sensitivity of RT-PCR for identifying the *CBFB-MYH11*, there exists the possibility of false-positive RT-PCR results.^{5,9} Indeed, there are instances where false-positive PCR results have been reconciled as negative on repeat evaluation after finding no evidence for inv(16)/t(16;16) by cytogenetics or FISH analysis.⁵ Although our RT-PCR analysis includes a probe hybridization step to enhance specificity, diagnostic verification by FISH was deemed important in this case, in light of the uninformative karyotype. The complementary FISH analysis in our case clearly revealed translocation of the *CBFB* gene from one chromosome 16, to the short arm (*MYH11* region) of the other chromosome 16, thus elucidating the mechanism of *CBFB-MYH11* gene fusion and confirming the RT-PCR results. The apparent del(16)(q22) probably had masked the t(16;16), making identification of the latter lesion difficult despite good quality metaphases.

In summary, our case presentation reinforces the utility of FISH technique for assessing submicroscopic rearrangements that escape conventional cytogenetics. Given the clinical relevance of these tumor-associated genetic rearrangements, both molecular and FISH analyses retain complementary roles in elucidating failed, complex, or apparently normal cytogenetics in patients in which cell morphology indicates the possibility of a prognostically important gene fusion. It is thus prudent to perform additional molecular diagnostic tests (ie, RT-PCR and/or FISH), particularly in cases with classical morphological features and a discordant or unexpected karyotype (eg, deletion of 16q), in which the inv(16) or t(16;16) may be masked.

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