Dual Color FISH on CBF Primary Acute Myeloid Leukemia

Amani Sorour and Dalia Nafea
Departments of 1Clinical Pathology and 2Internal Medicine-Hematology Unit, Faculty of Medicine, Alexandria University, Alexandria, Egypt.

In acute myeloid leukemia (AML), clonal chromosomal aberrations constitute markers of diagnostic value and the molecular characterization of numerous abnormalities has greatly improved the understanding of the biology of distinct subtypes of the disease. Two of the most common recurring chromosomal abnormalities in AML are t(8;21) and inversion of chromosome 16 or its variant which belong to core binding factor (CBF) AML group. We aimed to compare between cytogenetics and dual color Fluorescence In Situ Hybridization (FISH) regarding their sensitivity for detection of CBF AML associated translocations including t(8;21) and inv(16)t(16;16). Fifty five consecutive patients diagnosed as de novo AML were studied by chromosome banding analysis. Among them 32 patients were studied by FISH for the detection of AML1/ETO fusion gene and 11 patients for the detection of CBFβ/MYH11. Four cases of AML (M2) subtype were positive for t(8;21) and 1 (M4) subtype was positive for inv(16) by karyotyping analysis. When FISH was applied 6 cases all of AML (M2) subtype were positive for t(8;21), 2 of them were of normal karyotype, and 5 cases all of M4EO subtype were found to be positive for inv(16)/t(16;16) and 4 of them showed normal karyotypes. In conclusion, FISH can be used as a complementary technique to identify t(8;21) and inv16/t(16;16) in de novo AML as these abnormalities are difficult to diagnose in most cases by conventional cytogenetics alone.

Acute myeloid leukemia (AML) is a complex disease with considerable phenotypical and genotypical heterogeneity. Greater than 100 recurring cytogenetic abnormalities have been observed in AML as well as numerous point mutations. These alterations have been correlated with biologic and clinical features of the disease resulting in delineation of prognostically distinct categories of AML (Look, 1997; Mrozek et al., 1997; Bloomfield et al., 1997; Grimwade et al., 1998; Loewenberg et al., 1999; Caligiuri, 2001). One such category is core binding factor (CBF) AML.

CBF is a heterodimeric transcription factor containing an AML1 (CBFα) and a CBFβ subunit. AML1 directly contacts DNA, and its transactivation potential is enhanced by binding to CBF β that does not contact DNA (Speck, 2002). The transcriptional activation function of CBFα/β and induction of the expression of critical target genes that would normally result, is disrupted by the AML – associated t(8;21), inv(16) and related t(16;16) (Nucifora, 1995). The t(8;21) was cloned in 1991 (Kawano et al., 1997). More than 90 % of t(8;21) is found to be associated with FAB M2; this abnormality is also found in approximately 6 % of AML M1 and rarely AML M4 (Langabeer et al., 1997a; Wong et al., 1993). Two genes are involved in this abnormality, the AML1 gene on chromosome 21q22 and the ETO gene on chromosome 8q22 (Kawano et al., 1997; Downing et al., 1999). AML1/ETO fusion transcript is a transcriptional repressor that aberrantly recruits the nuclear corepressor complex, including histone deacetylase (HD) to CBF sites; it appears to function as dominant negative inhibitor of the native CBF. The fusion appears to confer a subtle of proliferative or survival advantage and properties of self renewal (Yergeau et al., 1997; Okuda et al., 1998). The chromosome 16 abnormalities including inv(16) and its variant t(16;16), which are closely associated with the FAB M4EO, result in the creation of a fusion gene between the smooth muscle myosin heavy chain gene (MYH11) at 16p13 and the core binding factor β (CBFβ) at
16q22. The fusion protein product, CBFβ – MYH11, interacts with nuclear co-repressors, leading to dysregulation of transcription (Ravandi et al., 2003).

Patients with CBF leukemias have a more favorable prognosis. Therefore, accurate identification of these patients at diagnosis is of therapeutic significance (Le Beau et al., 1986). Conventional chromosome banding study allows a comprehensive analysis of the karyotype and is still a fundamental component of modern tumor cytogenetics. Despite continuous improvements in cytogenetic methodology, in almost all studies of patients with AML, substantial number of patients without adequately banded metaphase cells have been reported (Mrozek et al., 1997; Mrozek et al., 2001a). With the increasing availability of a large variety of specific DNA probes, studies that use Fluorescence In Situ Hybridization (FISH) are no longer limited to the detection of numerical aberrations or the identification of certain structural abnormalities in a small number of patients (Gozzetti et al., 2000). FISH is particularly sensitive for detecting the AML specific translocations and inversions, some aneuploidies and deletions. Furthermore, FISH identified chromosome aberrations in certain patients with normal karyotypes (Stefan et al., 2002).

In the present study we aimed to compare the results of cytogenetic analysis with that of dual color FISH with regard to their sensitivity for the detection of CBF translocations including t(8;21), and inv(16) or its variant t(16;16) in de novo AML for selecting risk – adapted post remission therapy.

Patients and Methods

Fifty five consecutive patients diagnosed with de novo AML were enrolled in our study between January 2007, and February 2008. Samples were collected from Hematology Unit- Faculty of Medicine, Alexandria University. Selection criteria for our patients included the following: diagnosis of de novo AML of the French-American-British subtype M0-M7, no history of myelodysplasia or other hematological malignancy; aged 15 years or more, but younger than 60 years; and no previous treatment for leukemia or another malignancy. Leukemias were classified morphologically according to the French-American-British (FAB) Cooperative Group criteria (Bennett et al., 1985) after assessment of Wright’s stained bone marrow, blood smears, or both in addition to analysis of surface markers by flowcytometry when needed. Informed consent was obtained from all patients according to the Ethical committee for human research in Alexandria Main University Hospital.

Cytogenetic and Molecular genetics Studies

Bone marrow (BM) and/ or peripheral blood (PB) samples were taken from 55 patients before treatment. The samples were analyzed cytogenetically by the use of short-term (24, 48 or 72-hour) unstimulated cultures. Chromosomes were G-banded and karyotypes were interpreted according to the International System for Human Cytogenetic Nomenclature (Mitelman, 1995). Fluorescence In Situ Hybridization (FISH) (Mancini et al., 2000) was done on 32 Patients for the detection of t(8;21) and among them 11 patients for the detection of inv(16)/ t(16;16) using the corresponding commercially available probes.

1. Karyotyping

Chromosomal analysis was performed by standard method. Briefly, cells were seeded at 1.2X10^6 cells/ml in RPMI with supplements. Multiple (direct, 24, 48 and/or 72 hour) cultures were set up for each case. Harvesting, fixation, slide preparation and Trypsin – Giemsa banding were done using standard protocols (Mitelman, 1995). Metaphases were analyzed from each culture. The images were captured using Olympus microscope and karyotyped with the cytovision software.

2. Fluorescence In –Situ Hybridization (FISH)

Slides prepared for cytogenetic analyses were used for interphase and metaphase FISH. FISH assay was performed according to Vysis, Inc. (Downer’s Grove, IL) protocol. Briefly, 10 µl of hybridization mixture (1µl probe, 2µl water, and 7µl hybridization buffer) were added to the target area on the slide. The slides were cover-slipped and sealed with rubber cement, and the probe was denatured at 75°C for 5 minute using the Hybrite system (Vysis, Inc.). After overnight incubation at 37°C, the cover slips 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 fluorescence microscope equipped with single band-pass filters for DAPI, FITC.
and Rhodamine and a triple band-pass filter (DAPI/FITC/Rhodamine). Images were captured with a digital camera and digitally recorded using Cytovision software Applied Imaging Corp., Santa Clara, CA). Approximately 200 interphase nuclei and all available metaphase spreads were examined and scored.

2.1. FISH for inv(16)/t(16;16)

Commercially available LSI CBFβ dual-color, break apart rearrangement probe (Vysis, Downers Grove, IL) for detection of inv(16)/t(16;16) was 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.

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. The presence of 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.

2.2. FISH for t(8;21)

Commercially available LSI AML1/ETO dual-color, dual fusion translocation probe (Vysis, Downers Grove, IL) for detection of t(8;21) was used in this study. The probe set consists of a mixture of a spectrum green labeled LSI AML1 (21q22) probe and a spectrum orange labeled LSI ETO (8q22) probe. The approximately 1.3 Mb LSI AML1 probe hybridizes to the 21q22 band containing the AML1 gene.

Evaluation criteria: In a normal cell without the AML1/ETO fusion gene, two orange signals representing normal copies of ETO and two green signals representing normal copies of AML1 are observed whereas an abnormal nucleus showing a one orange, one green and two fusion (1O1G 2F) signal pattern.

Statistical Analysis

The Data was collected and entered into the personal computer. Statistical analysis was done using Statistical Package for Social Sciences (SPSS/version 15) software. A comparison of the overall abilities of the two techniques to accurately classify the patients was performed by a Z test to compare two proportions. Arithmetic mean, standard deviation were used for categorized parameters, Chi square test was used for numerical data, t-test was used to compare two groups while for more than two groups ANOVA test was used. The level of significant was 0.05. We also assessed the respective abilities of cytogenetic and FISH analyses in our patients by calculating the predictive value positive (PVP) and predictive value negative (PVN). The related sensitivity and specificity were also examined.

Results

The study was conducted on 55 patients (31 males and 24 females) with age range of 16-60 years (mean ±SD, 39.83±13.6). There were 3 patients classified as AML M0, 12 with AML M1, 9 with AML M2, 9 with AML M3, 1 with AML M3v, 6 with AML M4, 5 with AML M4 EO, 5 with AML M5a, 3 with AML M5b, and 2 with AML M6, according to FAB classification.

Cytogenetic studies

All the 55 AML patients were studied by cytogenetics; 4 AML M2 patients were positive for t(8;21), 1 AML (M4EO) was positive for inv(16), 5 AML M3 patients were positive for t(15;17), 1 AML (M1) was positive for monosomy 7, 1 AML M2 was positive for deletion – 9q, 1 AML (M4) was positive for addition chromosome 11, 1 AML (M6) was positive for deletion 7, 1 AML (M1) positive for 1p+, among the remaining 39; 30 patients had normal karyotype by conventional cytogenetics whereas 9 patients failed to develop metaphases enough for karyotyping, they were all of other FAB subtypes than (M1, M2 and M4).

Molecular cytogenetics by FISH

BM or PB from 32 patients with AML FAB subtypes M1, M2 and M4 were analyzed by FISH for the detection of AML1/ETO and 11 patients with AML M4 for the detection of CBF β/ MYH11 fusion transcript.
Among the 32 patients studied for AML1/ETO chimeric transcript, only 6 patients (18.75 %) were positive and all were of AML M2 subtype (Figure 1). Among the 11 patients with AML M4, only 5 were positive for CBFB/MYH11 fusion transcript and all were of M4EO subtype (Figure 2).

**Figure 1.** Dual color, dual fusion-FISH using the AML1/ETO probe set showing the typical t(8;21) FISH pattern with one red, one green and two fused signals in the cells with white arrows.

**Figure 2.** Dual color dual fusion FISH using LSI CBFBdual-color, break apart rearrangement probe (Vysis, Downers Grove, IL) probe. Red arrow points at cell with 2 separate green and 2 red signals indicating the presence of inv(16) in both alleles of the CBF β gene. White arrows point at abnormal cells which have one red, and one green signal, indicating separation of the two parts of the CBF β gene by the inversion while the normal allele is seen as red/green Fusion.

Our results demonstrate the high frequency of these fusion transcripts among AML M2 for t(8;21), and AML M4EO for inv16/t(16;16), which showed a statistical significant results. (P<0.002).

**Comparison between conventional cytogenetics and FISH for detection of CBF AML**

Comparing the overall abilities of the two techniques to detect accurately the fusion transcripts for CBF AML, we found that among 55 studied patients by karyotyping 4 patients with AML M2 were positive for t(8;21) and 1 patient with AML M4EO was positive for inv(16). By FISH study, 6 AML M2 patients were positive for AML M1/ETO while 5 AML M4 EO patients were positive for CBFB/MYH11 fusion transcripts.

Our results showed that the ability of FISH study for the detection of CBF chromosomal aberrations was significantly higher than conventional cytogenetics P<0.05 and z=2.097 (Table 1)

We also assessed the respective abilities of cytogenetics and FISH analyses to detect the presence or absence of inv16/t(16;16), and t(8;21) in patients with AML by calculating their predictive value positive (PVP) and predictive value negative (PVN), specificity and sensitivity (Table 2).

For t(8;21), from the 6 positive cases by FISH it was found that there were 4 cases positive by karyotype, while there were 26 cases negative by both FISH and karyotype, i.e. there were 4 cases true positive, 26 cases were true negative. No cases showed false positive results and only 2 cases were false negative.

For inv(16)/t(16;16), from the 5 cases positive with FISH method only one case was detected as a positive by karyotype, while the other 6 negative cases were also detected as negative by karyotype, i.e. there was only one case true positive and 6 cases were true negative. No cases gave false positive results,
while 4 cases were false negative by karyotype.

Table 1. Comparison between FISH and karyotyping regarding Positive cases.

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<th>FISH</th>
<th>Karyotyping</th>
<th>P</th>
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<tr>
<td>5/11 (45.5%)</td>
<td>1/11 (9.1%)</td>
<td>0.013*</td>
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<tr>
<td>6/32 (18.75%)</td>
<td>4/32 (12.5%)</td>
<td>NS</td>
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*P<0.05 is significant; NS= not significant

Table 2. Sensitivity, specificity, accuracy, PVP and PVN, for the detection of both inv(16)/t(16;16) and t(8;21) by conventional cytogenetic assay.

<table>
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<th>t(8;21)</th>
<th>inv(16)/t(16;16)</th>
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<tbody>
<tr>
<td>Sensitivity</td>
<td>66.7</td>
<td>20.0</td>
</tr>
<tr>
<td>Specificity</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Accuracy</td>
<td>93.8</td>
<td>63.6</td>
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<tr>
<td>PVP</td>
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<td>100.0</td>
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<tr>
<td>PVN</td>
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<td>60.0</td>
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PVP= predictive value positive
PVN= predictive value negative

Discussion

The prognostic and therapeutic significance of karyotype and cytogenetics at diagnosis in patients with AML is now fully established. Patients with CBF leukemias including those with t(8;21) and the pericentric inversion of chromosome 16 inv(16) or its variant t(16;16) are associated with favorable prognosis particularly when treated with intensive post remission therapy including high dose cytarabine (Le Beau et al., 1986).

Our results showed that the incidence of CBF leukemias was 20% which has been reported by other authors who found that CBF AML constitute approximately 15 to 20 % of adults younger than 60 years with de novo AML (Ravandi et al., 2003).

In our study, we found that the incidence of t(8;21) or inv(16) among de novo AML were 11% and 9% respectively which was reported previously (Downing, 1999).

de Greef et al. (1996) found that although more that 90% of t(8;21) are found to be associated with AML - M2, this cytogenetic abnormality is also found in approximately 6% of AML- M1, rarely in AML–M4 and other myeloproliferative diseases. The translocation t(8;21) occurs in 30–40% of all M2 subtype (Downing, 1999).

In our results the incidence of AML1/ETO fusion transcript was 66.7 % among AML M2 and this translocation has not been detected among M1 or M4. Our results are similar with those obtained from previous studies showing that the chromosome 16 abnormalities are closely associated with the FAB subtype M4 EO (Mrozek et al., 2001a).

Several recent reports have examined the utility of cytogenetics, FISH and molecular analyses to detect CBFB – MYH11 and AML1/ETO fusion transcripts among patients with AML. In initial reports, patients expressing the CBFB – MYH11 chimeric gene in the absence of chromosome 16 aberrations were reported to account for up to 43 % of all molecularly positive patients (Krauter et al., 1998; Langabeer et al., 1997b). Other studies have reported a much lower incidence of such discordance between cytogenetics and molecular analysis (Mrozek et al., 2001b; Rowe et al., 2000; Poirel et al., 1995; Mitterbauer et al., 2000).

These observations are in agreement with our results where we found that additional cases of AML1/ETO and CBF B- MYH11 fusion transcripts were detected by FISH and were not identified by conventional cytogenetics, demonstrating the high significance of detection by FISH in comparison to karyotyping method (P< 0.05).

The respective PVP and PVN, sensitivity, specificity and accuracy of cytogenetic analysis were lower than FISH as 2 AML1/ETO and 4 CBFB/MYH11 positive patients were considered to be cytogenetically negative for t(8;21) and inv(16) respectively, although they had chromosomal aberrations.

The results from this study demonstrate the applicability of FISH for the detection of CBF
AML specific translocations. This is expected to improve the accuracy of cytogenetic diagnosis within AML in which pretreatment cytogenetics is used for selecting risk adapted post remission therapy. As FISH is a rapid (results are available within 2 working days) and reliable (experiments were successful in all patients) technique, we propose to use FISH as a complementary method for the identification of t(8;21) and inv(16)/t(16;16) in all patients with newly diagnosed AML (FAB subtypes M2 and M4EO) respectively, because these abnormalities may be difficult to detect by conventional cytogenetics alone.

References


