Characterization of Complex Chromosomal Rearrangements in Acute Myeloid Leukemia: FISH and Multicolor FISH Add Precision in Defining Abnormalities Associated with Poor Prognosis

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Abstract

Introduction: The identification of specific chromosome abnormalities in Acute Myeloid Leukemia (AML) is important for the stratification of patients into the appropriate treatment protocols. However, a significant proportion of diagnostic bone marrow karyotype in AML is reported as normal by conventional cytogenetic analysis. It is suspected that this karyotype may conceal the presence of diagnostically significant chromosome rearrangements. Complex chromosomal aberrations can be detected in a substantial proportion of AML, which are associated with very poor prognosis. Conventional cytogenetic analysis cannot accurately define the specific alterations in complex chromosomal aberrations. M-FISH allows the comprehensive identification of complex chromosomal aberrations.

Materials and Methods: To address this question, in a series of 321 AML patients, 9 patients showed complex karyotype. Fluorescence in situ Hybridization (FISH) and Multicolor FISH (M-FISH) assay were carried out in 4 patients.

Results: Several rare, novel and recurrent translocations were identified using conventional cytogenetic and molecular cytogenetic techniques. M-FISH analysis identified cryptic chromosomal rearrangements by conventional cytogenetic analysis.

Conclusion: Present study revealed that M-FISH is a powerful molecular cytogenetic tool to characterize complex karyotype in AML.

Keywords

Acute myeloid leukemia; M-FISH; Conventional cytogenetic analysis

Introduction

AML is heterogeneous group of disorders at the cytogenetic and molecular genetics levels. Over the last 30 years, several specific recurrent chromosome aberrations have been described in AML. Acquired cytogenetic aberrations are detected in 55-75% of newly diagnosed patients with AML; the rest show no cytogenetic changes and this masks any clues to their molecular pathogenesis. Cytogenetic study of AML patients represents the most valuable predictor for a poor outcome but it encompasses a heterogeneous patient population who might have diverse pathogenesis and clinical courses [1].

Cytogenetic data significantly contribute understanding of the heterogeneity of AML. In AML, numerous recurrent chromosomal aberrations have been identified and several of them, e.g. t(8;21)(q22;q22), t(15;17)(q22;q11-12), inv(16)(p13q22), are specific for distinct subgroups. The three risk groups and the chromosome abnormalities associated with them are: Favorable risk group: t(8;21), t(15;17), inv(16) or t(16;16); Intermediate risk group: Normal karyotype, t(9;11), -Y (loss of the Y chromosome), +8 (trisomy of chromosome 8), +11, +13, +21, del(7q) [deletion of the long arm of chromosome 7], del(9q), and del(20q); and Unfavorable risk group: Complex karyotype, inv(3) or t(3;3), t(6;9), t(6;11), t(11;19), del(5q), -5 (Monosomy of chromosome 5), -7(5,6,7) [2]. Chromosome analysis using classical cytogenetic banding techniques often fail to completely resolve complex karyotype and cryptic translocations not identifiable by these techniques have been detected using molecular cytogenetic methods. While fluorescence in situ hybridization (FISH) has become an indispensable tool for screening and follow-up of known aberrations, the techniques of spectral karyotyping (SKY) and multiplex-fluorescence in situ hybridization (M-FISH) allow for the simultaneous visualization of all chromosomes of a metaphase in a single hybridization step, and thereby enable screening for the unknown aberrations [3].

In a series of 321 patients, we observed total 9 patients with complex karyotype. Multiplex-FISH (M-FISH) study was possible in 4 patients with Complex Chromosomal Aberrations. Results also documented severe different rare, novel and recurrent chromosomal aberrations. We identified several newer fusions using M-FISH technique.

Materials and Methods

In present study total 321 patients in the range of 1-65 years with different subtypes of AML were enrolled. Cytogenetic studies of the 321 AML patients revealed that 9 patients (5 males and 4 females) were having complex chromosome karyotype. Out of 9 patients 4 patients had AML-M1 subtype, 4 patients with AML-M2 subtype and 1 patient with AML-M5 subtype. Clinical details of patients and cytogenetic and FISH and M FISH results were described in table. Morphological diagnosis was carried out according to FAB criteria. Conventional cytogenetic study was carried out in all patients whereas, FISH and Multicolor FISH study was carried out as and when required.

Conventional cytogenetic study

Conventional cytogenetic analysis was performed using unstimulated Bone marrow (BM) or peripheral blood lymphocytes cells (PBLC) according to standard techniques [4]. The bone marrow cells were cultured overnight without the addition of any stimulating agent to make the cells undergo mitosis. For G banding technique was carried out using Trypsin and Giemsa stain and Karyotyping done according to ISCN2013 [5].
**FISH assay**

FISH on interphase and metaphase nuclei was performed using commercially available probes from Vysis (Vysis Downers Grove, IL). FISH assay were carried out using different Whole Chromosome Paint probes (WCP), Locus Specific Identifier probe (LSI) and Centromeric Enumeration Probes (CEP) according to the standard protocol of the manufacturer. The signals were viewed with a Zeiss Axioskop microscope (Zeiss, Jena, Germany). Analyzing system was used for documentation (metasystems, Altlussheim, Germany).

**M-FISH assay**

M-FISH was performed using a commercially available set of combinatorially labeled whole chromosome paints (SpectraVysion, Vysis), according to the manufacturer’s protocol, with the following minor modifications: (i) pretreatment of the metaphase chromosomes on slides was carried out using pepsin (30 mg/ml in 0.01 N HCl) for 2 to 5 minutes, depending the amount of cytoplast present; (ii) post fixation was carried out using 1% formaldehyde (in 50 mM phosphate-buffered saline [PBS]/MgCl2 solution) for 10 minutes at room temperature; and (iii) the M-FISH probe mixtures were denatured at 72°C for 5 minutes. Separate fluorochrome images were obtained using a CCD camera mounted on an Olympus microscope (Olympus Optical) with an 8-position filter turret containing specific filter sets for DAPI, Spectrum Aqua, FITC, Spectrum Gold, Cy3.5, and Cy5 using a 100-watt mercury lamp. The resultant images were analyzed using BX-61 Olympus fluorescence microscope equipped with CCD camera (Jai, Japan) and cytovision (Applied Imaging, UK) software.

**Results**

Table 1 depicts complex karyotype in 9 patients of adverse risk group. M-FISH analysis was performed in patient No: 1, 2, 7 and 9. M-FISH was not possible in rest of the patients due to insufficient material. The karyotype results were revised after different FISH and M-FISH analysis. Most commonly observed breakpoints in these 9 patients were del(5q), del(6q), add(11p), add(17p), del(Xp), del(8p), add(9q), i(17q), del(1q), del(2q), der(4p), add(4q), del(12p), del(15q), add(11p), der(19), add(9q), del(5p). Several novel and rare translocations found in the current study were t(1;6), t(5;11), t(1;9), t(13), t(7;12). Numerical abnormalities in complex karyotype were +6, +8, +19, +21, -20+10. Trisomy 8 and trisomy 21 was most frequently observed.

**Patient 1:** A 65yrs. old male patient with AML-M1 subtype upon conventional karyotype with G bands metaphases showed translocation 49, XY, del(5)(q), add(6)(q?), +8, -add(11)(p), -20(15). All the metaphases showed presence of 49 chromosomes with del(5) (q), add(6)(q), tetrasomy of 4 and trisomy 10, trisomy 11 with addition in p arm of one copy of chr.11 and monosomy 20 (Figure 1A). The M-FISH analysis revealed, complex karyotype with 49 chromosomes having deletion of 5q, translocation between chr.1 and chr.6, part of chr.1 was observed on q arm of chr.6 (Figure 1B). Tetrasomy of 8, trisomy 10, add11p with trisomy of 11, monosomy 20. FISH analysis was carried out using WCP 11 Spectrum Green (SG) probe and WCP 20 with Spectrum Orange (SO). The results indicated that there was only one copy of chr.20 and three copies of chr.11 (Figure 1C). Hence, any cryptic rearrangements between these two chromosomes were observed. So revised karyotype results after MFISH and FISH analysis were: 49, XY, del(5)(q13q31), der(6)(t(16)(q17;q27)), +8, +8, +10, dup(11p), +der(11)dup(11p), -20(15). This patient underwent standard therapy and survived for 2 months.

**Patient 2:** 65 yrs old female with AML-M1 subtype. G banded karyotype study revealed 49, XX, del(5)(q), +add(11)(p), -17, -18, +mar1, +mar2, +mar3, +mar4[10] (Figure 2A). M-FISH analysis showed translocation between chr.3 and chr.17 and translocation between chr.1 and chr.20. M-FISH results showed translocation between chr.5 and chr.11, Monosomy 17, monosomy 18 and translocation between chr.1 and chr.20. There was pentasomy of chr.22 (Figure 2B). To find out the cryptic rearrangements and genes involved in all these translocations, different LSI probes and WCP probes were applied. LSI BCR-ABL revealed only 1 signal of BCR gene which indicated presence of one copy of BCR gene. However, WCP 22 SG revealed 5 copies of chr.22. The combination of conventional cytogenetic results and FISH analysis with LSI BCR-ABL and WCP 22 SG revealed pentasynteny of chr.22 of which BCR gene was present only on one copy of chr.22 whereas, BCR gene was deleted from rest of the 5 copies of chr.22. So all 4 marker chromosomes were identified as copies of der(22). LSI PML-RARA showed two orange signals of PML on chr.15. One green signal of RARA was observed on chr.17 and the other green signal was on B group chromosome. M-FISH technique, revealed chr.3 involvement (Figure 2C). So t(3;17) was also confirmed using WCP probe 3 SG and WCP 17 SO. M-FISH result was also focused on t(5;11). LSI CSFIR results showed loss of orange signal from one copy of chromosome 5. WCP 5 and WCP 11 signals confirmed t(5;11). So revised karyotype after M-FISH and FISH result was 49, XX, der (3) (t(3;17) (p27;q21), der (5) (t(5;11) (q17;p12), der (11) (t(5;11) (q32;p12), der(11) (t(5;11) (q32;p12), -17, -8, der (20) (t(1;20) (p12), +der (22), +der (22), +der (22), +der (22), +der (22), +der (22), ish der (22) (bcr-) x 4[10]. Patient was expired within a week of diagnosis.

**Patient 3:** A 1 year old male patient with AML-M1 subtype, showed 48, X, -Y, t(1;3)(p13;p22), add(7)(p), +8, +11, +19[10] (Figure 3A) on G banded conventional karyotype results. FISH test was not possible due to sample insufficiency. Patient was lost to follow up hence, further clinical details and cytogenetic studies were not possible.

**Patient 4:** A 55 year female with AML-M2 subtype. Conventional cytogenetic results with G banding showed 48, XX, del(Xp), +6, del(8p), add(9q), i(17q), +21[5] (Figure 3B). FISH and M-FISH were not carried out due to sample insufficiency. Patient was lost to follow up so further clinical details were not available and cytogenetic study was not possible.

**Patient 5:** A 52 year old female with AML-M2 subtype upon conventional karyotype with G banding showed Complex rearrangement 45, XX, del(1q), del(2q), der(4q), -17, der(17q), del(17q)[5] (Figure 3C). WCP 2 SG used to characterize rearrangement. Three green signals of WCP 2 indicated translocation between chr.2 and chr.17. FISH results revealed that green signals of WCP 2 was observed on q arm of chr.17. Patient was expired after fifteen days of diagnosis. So, further cytogenetic studies were not possible.

**Patient 6:** A 35 years male with AML-M2 subtype upon conventional cytogenetic results with G banding showed 46,XYdel(5) (q),del(12p),del(15)(q)[10] (Figure 3D). The patient was lost to follow up so, further clinical details and cytogenetic studies were not available.

**Patient 7:** A 12 years old male patient with diagnosis of AML-M5. The conventional cytogenetic study revealed that there was a complex karyotype and was included in adverse risk group. The karyotype results showed add11q and add17p. Karyotype...
Complex chromosomal abnormalities are associated with rather poor prognosis. CCAs are found in 10-30% of de novo AML and 50% of therapy-related AML and MDS. Conventional cytogenetics has limitations in accurate interpretation of complex chromosomal abnormalities and identification of cryptic translocations. In our study, we found severe structural aberrations including deletions, translocation and additional material of unknown origin attached to chromosome, derivative and maker chromosomes with ambiguous composition. Therefore, we combined M-FISH with conventional cytogenetic to analyze complex chromosomal aberrations.

**Discussion**

AML is a heterogeneous disease in terms of cytogenetic and molecular genetics. It is the most common acute leukemia in adults and its incidence increases with age. Diagnostic cytogenetics is an important prognostic indicator for predicting outcome of AML. Diagnostic karyotype is one of the most powerful prognostic indicators for predicting outcome of AML. Certain chromosome abnormalities are associated with good outcomes while other chromosome abnormalities are associated with a poor prognosis and a high risk of relapse [2].

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Figure 2: G banded partial complex karyotype (B) M-FISH results of complex karyotype (C) FISH results using different LSI and WCP probes.

Figure 3: G banded partial karyotype of Complex karyotype.
In the present study, deletions of q arm of chromosome 5 were observed in 3 patients. Abnormalities of chromosome 5 are common findings in patients with hematological malignancies with poor outcome. Sq has been shown to have a wide variety of breakpoints and it has been reported within complex karyotype [6]. According to Brown et al. [7] t(5;11) patients with an associated Sq deletion were treated on different protocols, had short remission duration who failed to achieve remission and they were relapsed and died after very short survival. In our present study none of the patient achieved remission and expired within a month. We have shown here that the t (5;11) is beyond the resolution of both G-banding and M-FISH. We have previously shown that the der (11), but not the der (5), is detectable using single-color whole chromosome painting. However, the most reliable cytogenetic method for detection of the t(5;11) is FISH with Whole chromosome paint probes according to our results.

Trisomy 8 is reported in 25% of CML cases, with or without simple or complex karyotype changes, in 10-15% of AML patients, in 15-20% of MDS and 5% of ALL. It may be present as a sole aberration or along with other chromosomal aberrations. Trisomy 8 and tetrasomy 8 indicate over expression of gene dosage and suggest its role in neoplastic progression. It is likely to be a disease modulating secondary event with underlying cryptic aberrations as it has been frequently reported in addition to known abnormalities contributing to clinical heterogeneity and modifying prognosis [8].

Monosomy 17 or unbalanced translocation of chromosome 17 is much more frequent and associated with myeloid malignancies [9]. In our study, 5 cases involved 17p deletion, and one case had monosomy 17; all the other cases had unbalanced rearrangements between chromosome 17 and other chromosomes, including chromosomes 3 and 20. We observed a t(7;12)(q7:;p13) which was a rare translocation. 12p region is genetically unstable and fragile, leading to complex genetic events. Survival of patients with 12p rearrangements is very poor approximately less than 6 months [10]. There is a controversy regarding analysis of del7q either balanced or unbalanced translocations [11]. Till date there was only 1 case of t(7;12) in complex karyotype observed in India [12]. In the present study we have come across different translocation of p and q arm of chromosome 1. t (1;6) (?;?) was observed which is a novel translocation. There was a t (1;19)(q7:;p13) which is a recurring chromosome abnormalities generally observed in AML patients. Unbalanced translocations involving chromosome 6, such as der (6) t (1;6)(q21-25;p21.3-23), and other partner chromosomes involving 1q10/1p11 and 1q21-25 breakpoints have been observed. t (1;3)(q?q) was observed. Literature suggests that certain chromosome 1 regions, especially 1q12-1q32 and 1p11-13, might harbor oncogenes or tumor suppressor genes that are pathogenetically relevant to both chronic and advanced phases of myeloproliferative neoplasm. Role of these breakpoints in AML is still unknown [13].

According to Meng [2], balanced translocations tend to be found in younger AML patients, while elderly patients usually have unbalanced aberrations such as complex karyotype. In our study out of 9 patients 5 patients were in older age. Probably different genetic mechanisms are involved in the pathogenesis of AML and these mechanisms might occur at different frequencies as age increases [2].

The emerging nonrandom pattern of abnormalities includes relative paucity, but not absence of balanced rearrangements (translocations, insertions or inversions), predominance of aberrations leading to loss of chromosome material (monosomies, deletions and unbalanced translocations) that involve, in decreasing order, chromosome arms 5q, 17p, 7q, 18q, 16q, 17q, 18p, 12p, and 3p, and the presence of recurrent, even though less frequent and often hidden (in marker chromosomes and unbalanced translocations) aberrations leading to overrepresentation of segments from 8q, 11q, 21q, 22q, 1p, 9p, and 13q. Treatment outcomes of complex karyotype patients receiving chemotherapy are very poor; they can be improved to some extent by allogeneic stem-cell transplantation in younger patients. It is hoped that better understanding of genomic alterations will result in identification of novel therapeutic targets and improved prognosis in patients with complex karyotype [1].

Deletions were actually found to be translocations after M-FISH study. This is very important in cases with aberrations with prognostic significance. In our study loss of chromosomal material was observed much more often than gain in AML with aberrant karyotype. Hence, loss of tumor-suppressor genes may occur which may involved in mechanism of leukemogenesis. Numerical abnormalities in karyotype may affect gene-dosage and may play a significant role in the pathogenesis of AML.

In agreement with literature, we found that the majority of structural aberrations detected by M-FISH were unbalanced. In addition, we also found five kinds of chromosomal abnormalities which had never been reported by other authors. Our findings confirmed that M-FISH was a powerful molecular cytogenetic tool in the clarification of complex chromosomal aberrations. The application of molecular-cytogenetic techniques, such as M-FISH enabled more precise characterization of complex karyotype. This also has led to the identification of several genes whose expression is altered as a result of genomic imbalances in patients in this cytogenetic group. In addition, M-FISH revealed several nonrandom aberrations which are most frequently involved chromosomes 1 (4/9 patients) and 5 (3/9 patients), 17 (4/9 patients) and 11 (4/9 patients).
Fluorescence flaring is a main factor leading to misinterpretations. Some misclassified and missed chromosomal aberrations by M-FISH were corrected by WCP.

In conclusion, we found a good congruence with published literature on the incidence and prognostic value of several well established AML associated genetic aberrations. Complex karyotype is widely considered as a predictor for very poor outcome and frequently is used as an indication for allogeneic transplantation or experimental treatment approaches. It is also concluded that M-FISH is a powerful molecular cytogenetic tool in clarification of complex chromosomal aberrations. Complementary WCP can further identify misclassified and missed chromosomal aberrations by M-FISH. Conventional cytogenetic in combination with molecular cytogenetic techniques including M-FISH and WCP can more precisely unravel complex chromosomal aberrations.

References