



Role of Cytogenetics and Fluorescence In Situ Hybridization in the Laboratory Workup of Acute Myeloid Leukemias

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Abstract

A new understanding of acute myeloid leukemia as a varied group of unique biologic entity has emerged, as a result of the identification of various chromosomal aberrations and their association with clinical prognosis and diagnosis. Following induction treatment, cytogenetic examination can establish the presence of any residual malignant cells, its recurrence, clonal evolution if any, or the formation of novel abnormalities. The G-banded karyotype has been the gold standard method for detecting all of these aberrations for years. The capacity to examine the entire genome through karyotype analysis quickly enabled the detection of deletions, duplications, and structural rearrangements across every chromosome, and the more frequent ones were associated with particular aberrant clinical symptoms. Fluorescence in situ hybridization (FISH) is a sensitive technology that aids in differential diagnosis or therapeutic planning and provides rapid results. Furthermore, the combination of cytogenetic and molecular profiling enables a more precise evaluation of disease prognosis, diagnosis, classification, risk stratification, and patient treatment. Interphase FISH analysis, in conjunction with G-banded chromosomal analysis, can be used as a major testing tool for the evaluation of hematological neoplasms. For accurate and consistent descriptions of genomic changes identified by karyotyping and FISH, a specified terminology is necessary. The International System for Human Cytogenomic Nomenclature is the main source and provides instructions for documenting cytogenetic and molecular findings in laboratory reports. This review discusses the two methods, karyotyping and FISH, their advantages and limitations, sample requirements, various FISH probes that are used, nomenclature for results reporting, and the necessary quality control measures.

Keywords

- WHO classification
- prognosis
- FISH
- cytogenetics
- amplification
- probes

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Introduction

Acute myeloid leukemia (AML) is the most common adult leukemia and is characterized by clonal expansion of immature blast cells in the peripheral blood and bone marrow and is genetically heterogeneous with variable prognosis.¹ AML predominantly affects those over 60 years of age, with progressively dismal prognosis in advanced age, but can develop in children and young adults too.^{2,3} Acquired clonal chromosome aberrations can be seen in the majority of AML patients. The most important initiating step in a significant proportion of adult and pediatric AML is the generation of chimeric fusions arising due to events such as balanced translocations and/or inversions/insertions in hematopoietic stem cells which have been identified as recurrent genetic abnormalities by the World Health Organization (WHO) classification 2008.⁴ These recurrent genetic abnormalities are sufficient for diagnosing AML regardless of blast count in bone marrow and also paved the way for molecular studies that identified genes involved in the process of leukemogenesis. The discovery of specific chromosomal abnormalities and their relationship to cytomorphologic features, immunophenotype, and clinical outcome has led to a new understanding of AML as a diverse group of distinct biologic entities. The clinical significance of cytogenetic findings in AML for classification and understanding of pathogenetic mechanisms is growing, as evidenced by the WHO classification of AML.⁵

Implications of chromosomal aberrations in the determination of clinical outcome and its value as an independent prognostic indicator were established by studies in large series of AML patients.^{6–8} Based on chromosomal abnormalities and gene mutations, guidelines and risk scoring systems have been developed by the National Comprehensive Cancer Network and the European Leukemia Net to help physicians design tailored therapeutic strategies. The disease can be categorized into favorable, intermediate, or adverse-risk groups (→ **Table 1**). For example, t(15;17)(q24.1;q21), [*PML::RARA*], t(8;21)(q22;q22) [*RUNX1::RUNX1T1*], and inv(16)(p13.1q22) [*CBFB::MYH11*] are associated with a favorable outcome,

whereas inv(3)(q21q26.2) or t(3;3)(q21;q26.2) [*RPN1::MECOM*], t(6;9)(p23;q34) [*DEK::NUP214*], and *KMT2A* (11q23) rearrangements fall under adverse risk and are associated with a dismal prognosis.^{9,10} Cytogenetic testing is critical in the selection of targeted therapy for various leukemias. In acute promyelocytic leukemia (APL), novel fusion protein *PML::RARA* is generated by a translocation between chromosomes 15 and 17 and is effectively treated with targeted therapy, ATRA (all-trans retinoic acid).^{11,12} Cytogenetic analysis can confirm the residual disease, relapse, clonal evolution, or the emergence of new anomalies after induction chemotherapy.^{13,14}

In cytogenetic laboratory, the G-banded karyotype is the gold standard method for AML diagnosis and prognosis.¹⁵ Majority of chromosomal rearrangements can be detected using conventional cytogenetics (CC). On the contrary, fluorescence in situ hybridization (FISH) enables higher resolution analysis for cryptic rearrangements not detected by CC, particularly in cases with normal karyotypes, which account for approximately 33 to 50% of AML.¹⁶ The most powerful tools for detecting chromosomal aberrations are CC and FISH. Furthermore, combining cytogenetic and molecular profiling allows for a more accurate assessment of disease prognosis, diagnosis, categorization, risk stratification, and patient management. In this review, we discuss the role of CC and FISH in the detection of recurrent and nonrecurrent aberrations.

Classification

AML was classified according to the French-American-British (FAB) classification system in the 1970s, defining eight major AML subtypes (FAB M0 to M7). This classification system relied on morphology and immunophenotyping criteria to define AML.¹⁷ The WHO 2001, published a Classification of Tumors of the Hematopoietic and Lymphoid Tissues as part of the third edition of the series, WHO Classification of Tumors, and incorporated genetics with morphology, cytochemistry, immunophenotype, and clinical information into diagnostic algorithms for the myeloid neoplasms.¹⁸ The

Table 1 European Leukemia Net (ELN) 2022 prognostic risk stratification based on cytogenetics

Risk category	Cytogenetic abnormality
Favorable	<i>RUNX1::RUNX1T1</i> -t(8;21)(q22;q22) <i>CBFB::MYH11</i> -inv(16)(p13.1q22)/t(16;16)(p13.1;q22)
Intermediate	<i>MLLT3::KMT2A</i> -t(9;11)(p21.3;q23.3) Cytogenetic abnormalities not categorized as favorable or adverse
Adverse	<i>DEK::NUP214</i> -t(6;9)(p23;q34.1)
	<i>KMT2A</i> rearrangements-t(v;11)(?;q23.3)
	<i>BCR::ABL1</i> -t(9;22)(q34.1;q11.2)
	<i>GATA2::MECOM</i> - inv(3)(q21.3q26.2)/t(3;3)(q21.3;q26.2)
	<i>MECOM::?</i> -t(3q26.2;?)
	<i>KAT6A::CREBBP</i> -t(8;16)(p11;p13)
	Monosomy 5/del(5q), Monosomy 7, Monosomy 17/abn(17p)
	Complex karyotypes, Monosomal karyotypes

WHO classification was further updated in 2008 and 2016 to reflect rapidly evolving genetic and biologic information, refining diagnostic criteria, and introducing new disease entities.⁵ The WHO 2016 categorized AML into six main groups: (1) AML with recurrent genetic abnormalities, (2) AML with myelodysplasia-related changes (AML-MRC), (3) therapy-related myeloid neoplasms (t-AML), (4) AML not otherwise specified (NOS), (5) myeloid sarcoma, and (6) myeloid proliferations related to Down syndrome.¹⁹ The recent 5th edition of WHO classification published in 2022 did away with AML NOS and included AML-MRC and t-AML as one single family further narrowing down AML to three major subtypes: (1) AML with defining genetic abnormalities, (2) AML, defined by differentiation, and (3) myeloid sarcoma (► **Supplementary Table S1**, available in the online version).²⁰

Sample Requirement

Bone marrow is the preferred specimen to detect abnormalities in AML. However, in some circumstances, peripheral blood specimens with circulating leukemic blasts can be used as an alternative specimen.²¹ FISH can be performed on formalin-fixed paraffin-embedded (FFPE) tissues for patients with myeloid sarcoma (extramedullary myeloid tumor, granulocytic sarcoma, and chloroma) and bone marrow aspirate smear slides, if fresh, untreated bone marrow is not available. Bone marrow aspirate (1 to 3 mL), from the first or second draw, should be collected in sodium heparin tubes under sterile conditions. During transport, the specimen should be kept at ambient temperature and cultured within 24 hours.²²

Conventional Cytogenetics

CC (karyotyping) is a decade-old technique for a comprehensive evaluation of chromosomal abnormalities in patients with acute leukemia. Karyotyping provides a visual analysis of the whole genome leading to the discovery of recurrent translocations.

Short-term [direct (1 hour), overnight, 24 and 48 hours]] cultures are set up in complete media containing RPMI 1640, fetal bovine serum, and antibiotics. Stimulant is not required as these cells are already dividing. A mitotic inhibitor like colchicine/colcemid is added which arrests the cells at the metaphase stage. These cells are further processed using prewarmed hypotonic solution (KCl) and fixed with chilled Carnoy's fixative (three parts methanol and one part glacial acetic acid). The cells are then washed with Carnoy's fixative until a clear white pellet containing mononuclear cells is obtained. Cell suspension is dropped on a clean glass slide, treated with trypsin and stained with Giemsa producing a series of light and dark bands corresponding to A-T rich and G-C rich regions, on each chromosome. This is the most widespread method known as known as Giemsa-Trypsin G banding.²³ The metaphases are captured using a camera, through a microscope, onto an image analyzer system with karyotyping software. Chromosomes are separated and

depending on their exclusive band pattern are karyotyped and described according to the International System of Human Cytogenomic Nomenclature 2020.²⁴ A minimum of 20 metaphases are karyotyped in each AML case.

CC can detect both structural (translocations, insertions, inversion, duplication, deletions) as well as numerical (monosomy, trisomy, tetrasomy, etc.) abnormalities. The identification of these numerical and structural abnormalities carries prognostic, diagnostic, and therapeutic information in AML.²⁵

Advantages and Limitations of Conventional Cytogenetics

The key benefit of CC, or karyotyping, is that it simultaneously provides a genome-wide overview of all the chromosomes. The ability to examine the complete genome using karyotype analysis has made it possible to identify structural rearrangements and numerical abnormalities on all chromosomes with relative ease. Despite consistently providing valuable and independent prognostic evidence and also a foundation for risk evaluation, CC has major limitations. CC cannot be performed in cases with the absence of actively dividing mitotic cells. Due to limited banding resolution and sometimes poor morphology metaphases, abnormalities lesser than 5 to 10 megabases cannot be detected. Cryptic gene fusions such as *NUP98::NSD1*, *CBFA2T3::GLIS2*, and *MXN1::ETV6*, which indicate a poor prognosis in pediatric AML and cryptic rearrangements of *CBFB* or *KMT2A* cannot be detected by CC.^{26,27} Failure to recognize these aberrations places the patients in wrong risk groups with improper treatment. In 5% APL cases, t(15;17) is produced as a result of complicated rearrangements or insertion events of PML and RARA genes rather than the conventional t(15;17) translocation which can be missed by CC. These patients have a good prognosis and are responsive to targeted therapies that is ATRA and arsenic trioxide.¹¹ Additionally, in AML cases with structural or numerical alterations or, more significantly, those with normal karyotypes or nonproliferation of abnormal clones, cytogenetics does not shed any light on the molecular events. Being a manual procedure, the turnaround time for CC is as long as 7 to 15 days.

Fluorescence In Situ Hybridization

FISH is based on the principle of binding fluorescent probes to complementary sequences of chromosomes with very high specificity. The fluorescently labeled DNA probe and the target DNA affixed to a microscopic slide are codenatured and allowed to hybridize in a preprogrammed humidified chamber, wherein the complementary sequences anneal with each other. Posthybridization washes are done to remove excess, unbound probe and counter-stained with an antifade solution containing DAPI (4',6-diamidino-2-phenylindole). The slides are observed under a fluorescent microscope with specific filters where the signals are enumerated. The FISH technique can be performed on interphase cells doing away with the requirement of mitosis. It can be

applied on fixed bone marrow/peripheral blood specimens, bone marrow aspirate smear slides, and FFPE slides. Quantitative estimation can be done as a large number of interphase cells can be analyzed. A minimum of 200 cells are counted for each probe and described according to the International System for Human Cytogenomic Nomenclature (ISCN) 2020.²⁴

Advantages and Limitations of Fluorescence In Situ Hybridization

For the assessment of hematological neoplasms, interphase FISH analysis can be performed as a primary testing method in combination with G-banded chromosomal analysis. FISH is a sensitive technique and delivers rapid results and can be performed in few hours of time assisting in differential diagnosis or therapeutic planning. It helps in detection of cryptic gene rearrangements in AML with normal karyotypes, poor morphology chromosomes, or inadequate sample. It is useful as an alternative diagnostic method in sample lacking metaphases as FISH can be performed on nondividing interphase cells.²² The main drawback of FISH is that it can only detect abnormalities in genomic regions targeted by the probes applied as the selection of FISH probes is determined by clinical presentation or disease suspicion.

Types of Fluorescence In Situ Hybridization Probes

The choice of the probe is among the most crucial factors in FISH analysis. A variety of probes, ranging from small to whole genome clone probes (1–10 kb) can be used.²⁸ There are essentially three different types of probes with distinct applications: (1) locus-specific probes, (2) centromeric probes, and (3) whole chromosome probes.

1) Locus-specific probes are unique and specific DNA sequences. These probes have wide applications on interphase cells providing information on structural rearrangements like translocations, inversion, duplications, deletions, and amplification. Three different types of locus-specific probes are commercially available.

(a) Dual color dual fusion (DC DF) probes are applied in cases with balanced rearrangements like translocations resulting in gene fusions, for example, t(8;21) (q22;q22) is formed due to fusion of *RUNX1T1* gene present on chromosome 8 and *RUNX1* gene present on chromosome 21. These genes are differentially labeled with red and green fluorochromes which appear as two red and two green signals in normal interphase cells (►Fig. 1A). In the event of a balanced translocation, the partner genes fuse to form overlapping red/green (yellow) fusion signals on derivative chromosomes. Additionally intact one red and one green signals remain on homologous chromosomes (►Fig. 1B).

(b) Break-apart (BA) probes are used for the detection of single-gene rearrangements with multiple unknown partner genes for example, *KMT2A* gene (11q23) and

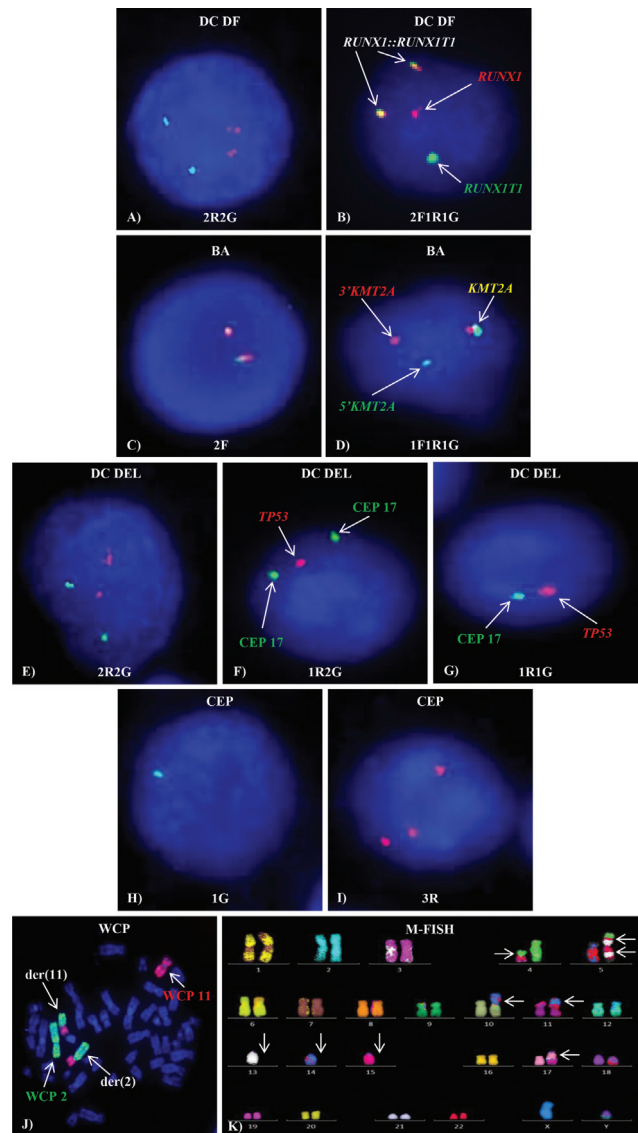


Fig. 1 Different types of FISH probes. Normal (A) 2R2G, (C) 2F, (E) 2R2G and abnormal (B) 2F1R1G, (D) 1F1R1G, (F) 1R2G, (G) 1R1G, (H) 1G, (I) 3R FISH signal patterns on interphase cells (J) WCP showing t(2;11) and (K) M-FISH showing complex karyotype on metaphase.

inversions for example, *CBFB* (16q22). In a BA probe, the 5' and 3' ends of the gene are labeled with two different fluorochromes (usually red and green) producing two yellow (fusion) signals in a normal interphase cell (►Fig. 1C). Rearrangements like translocation or inversion result in separation of 5' and 3' ends resulting in one red, one green, and one fused (yellow) signals. (►Fig. 1D)

(c) Dual/Triple color deletion (DC/TC del) probes are utilized to target-specific genes or disease loci, such as the *TP53* gene at 17p13.1. Control probes are often combined with deletion probes in contrasting colors on the same chromosome. A normal interphase cell displays the presence of both the target and control probes (►Fig. 1E). Loss of fluorescent signal on the targeted region indicates deletion (►Fig. 1F), while loss

of fluorescent signal of the targeted region along with control probe suggests monosomy (►Fig. 1G).

2) Centromere enumeration probes (CEP) are repetitive sequence probes hybridizing to alpha satellite sequences, present in thousands of copies on the centromeres of the chromosomes resulting in very bright fluorescent signals. Centromeric-specific probes are used for detection of numerical abnormalities like monosomy 7 (►Fig. 1H) which has poor prognosis while trisomy 8 which carries an intermediate prognosis in AML (►Fig. 1I).

3) Whole chromosome probes (WCPs) are fluorescently labeled, chromosome-specific hybridizing to entire chromosomes literally giving an appearance of “painting” and thus are also known as whole chromosome painting probes. These probes are generated either by flow sorting or microdissection of aberrant chromosome.^{29,30} A single or two differentially labeled WCPs can be mixed together and applied on metaphase preparation for the identification of marker chromosomes or structural rearrangements like translocations which are difficult to interpret by karyotyping (►Fig. 1J). Multicolor FISH and spectral karyotyping are based on this principle where all 24 chromosomes can be simultaneously visualized in different colors (►Fig. 1K). These techniques further help in the refinement of aberrations found by conventional karyotyping. These probes cannot be used for the detection of inversion or small deletions.³¹

Reporting of Conventional Cytogenetics and Fluorescence In Situ Hybridization Results

A defined nomenclature is essential for the correct and uniform description of genomic alterations discovered by karyotyping and FISH. The ISCN is the primary resource and offers guidelines for describing cytogenetic and molecular findings in laboratory reports. These laboratory reports serve as records that must be understandable, accurate, and should provide all the details necessary for the correct interpretation of the cytogenetic findings to the clinicians. The chromosome aberrations are denoted by a series of symbols and short terminologies. For example, a reciprocal exchange of chromosome segments between two different chromosomes is defined as translocation and is abbreviated as “t.” A karyotype is described as the total number of chromosomes followed by a sex chromosome complement separated by comma, a set of parentheses describing the chromosomes, their bands, and subbands involved in the aberration separated by a semicolon. A male patient with 46 chromosomes with a translocation between chromosomes 9 and 22 is described as 46,XY,t(9;22)(q34.1;q11.2), while a normal male karyotype is described as 46,XY.

Similarly, interphase FISH results begin with “nuc ish” which stands for nuclear in situ hybridization, followed by locus designation in parentheses, a multiplication sign (X) and the number of signals seen. If the number of signals for each probe is the same, the multiplication sign “X” is outside the parentheses. If the number of hybridization signals varies, then the multiplication sign “X” is inside the paren-

theses and the number of cells scored is placed in square brackets “[].” The nomenclature of FISH depends on the type of FISH probe used. As described in the earlier section, for a BCR/ABL1 DC DF probe, a normal cell with two red and two green signals is described as nuc ish(ABL1,BCR)X2[200], while an abnormal interphase cell with a reciprocal fusion between two differentially labeled genes, ABL1 and BCR, produces 2F1R1G signal pattern which is described as nuc ish(ABL1,BCR)X3(ABL1 con BCRX2) [196/200] where “con” stands for connected.²⁴

Conventional Cytogenetics and Fluorescence In Situ Hybridization Strategy in Acute Myeloid Leukemia

In a cytogenetic laboratory, conventional karyotyping and selection of FISH probes targeting specific recurring abnormalities in AML are based on their diagnostic and prognostic utility (►Fig. 2, ►Table 2). Both, CC and FISH should be concurrently applied for workup of newly diagnosed and follow-up AML cases. As FISH has shorter turnaround time than CC, prognostically relevant AML-associated cytogenetic aberrations, cryptic rearrangements, and low-level abnormalities can be reported within 2 to 3 days aiding in therapeutic decisions. At the same time, in the absence of recurrent cytogenetic aberrations, CC helps in the detection of other abnormalities for which FISH probes are not available.

RUNX1::RUNX1T1-t(8;21)(q22;q22.3)

T(8;21), seen in 8 to 10% of de novo AML, involves *RUNX1T1* on 8q22 and *RUNX1* on 21q22 and is associated with a favorable prognosis in children and adults^{4,32} (►Fig. 3A). This aberration can be identified by CC though, in 3 to 4% cases, t(8;21) is formed due to complex rearrangement involving a third chromosome or cryptic insertion. Loss of a sex chromosome (Y in males), followed by del(9q), del(7q), +8, and/or +21, are the most common additional abnormality. Patients exhibit a favorable response to chemotherapy with a high rate of complete remission and disease-free survival, regardless of the existence of other abnormalities.⁴

PML::RARA – t(15;17)(q24.1;q21.2)

PML::RARA fusion is formed due to reciprocal translocation between chromosomes 15 and 17 in APL and is associated with favorable prognosis.³³ Rapid detection of APL by FISH or other techniques is essential due to the high risk of early death and the availability of targeted treatment, ATRA. Unlike the standard t(15;17), complex rearrangements or insertions of the *PML* and *RARA* genes result in t(15;17) in 5% of cases of APL which appear normal by CC. Several variants and cryptic translocations involving *RARA* have been identified in about 10% APL cases which include *ZBTB16::RARA* – t(11;17)(q23;q21), *NPM1::RARA* – t(5;17)(q35;q21), *NUMA::RARA* – t(11;17)(q13;q21), *STAT5B/RARA* – der(17), *PRKAR1a::RARA* – t(17;17)(q21;q24) or del(17)(q21), *BCOR::RARA* – t(X;17)(p11;q21), and *FIPIL1::RARA* – t(4;17)(q12;q21) with differential response to ATRA. FISH with a

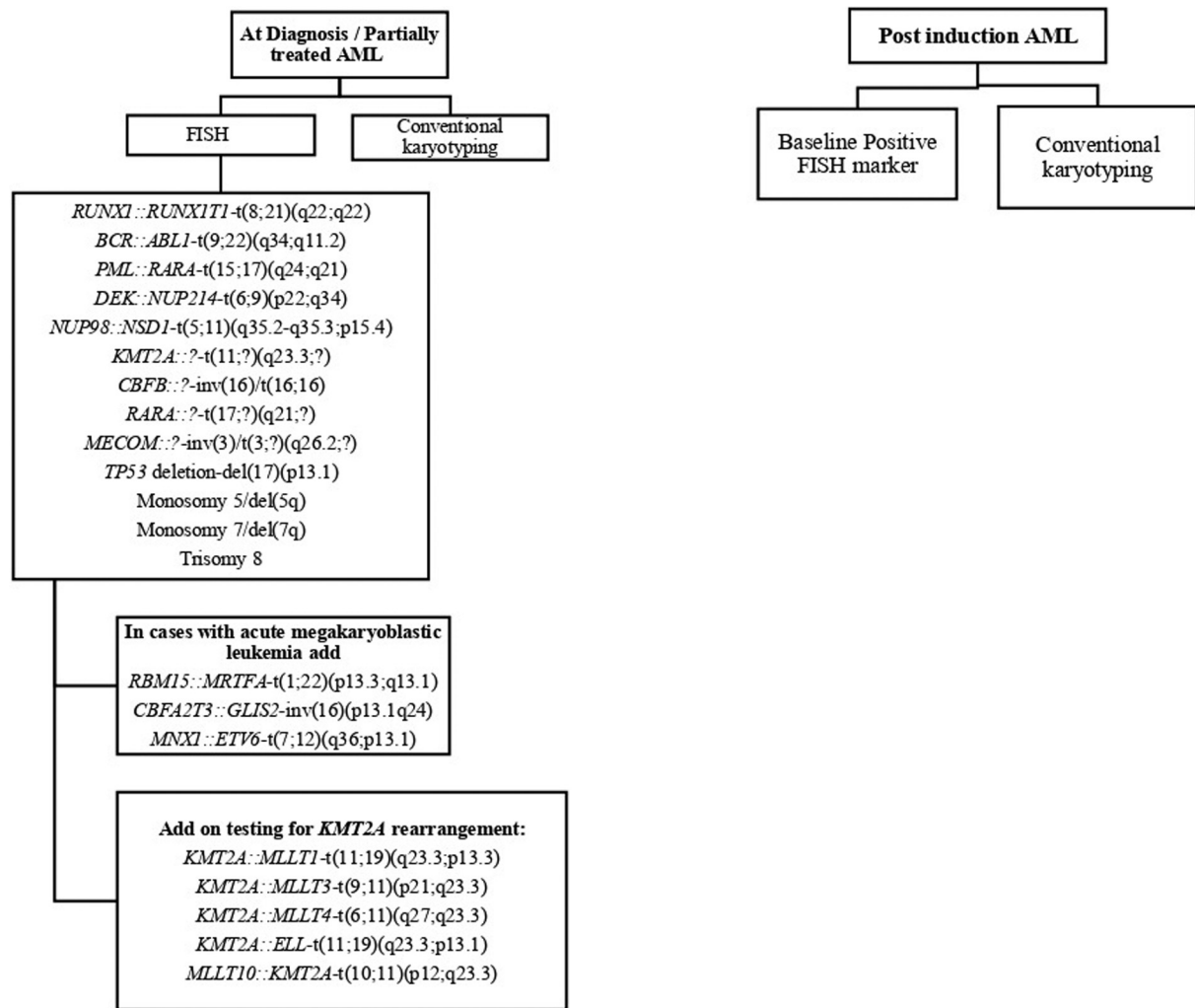


Fig. 2 Flow chart showing strategy of FISH panel in newly diagnosed and postinduction follow-up AML patients.

PML/RARA DC DF and RARA BA probe is useful for rapid detection of *PML::RARA* or *RARA* variants leading to prompt therapy.^{34,35} Good prognosis associated with t(15;17) does not appear to be affected by other additional abnormalities like trisomy 8, del(7q), or del(9q).³⁶

CBFB::MYH11–inv(16)/t(16;16)(p13.1;q22)

Inv(16)(p13.1;q22.1)/t(16;16)(p13.1;q22.1) leads to fusion of *MYH11* at 16p13.1 with *CBFB* at 16q22.1 and is consistent with favorable prognosis as this aberration is associated with complete remission³⁷ (►Fig. 3B). FISH with BA probe is helpful as this rearrangement can be missed by CC in poor morphology metaphases.³⁸ The most common abnormality along with inv(16) is trisomy 22, trisomy 8, and del(7q).³⁹

KMT2A::? – t(11;?)(q23.3;?)

Rearrangements of *KMT2A* (lysine [K]-specific methyl transferase 2A earlier known as mixed-lineage leukemia 1) are considered poor risk markers. Multiple rearrangements (translocations, insertions, inversions) involving *KMT2A* gene on 11q23 are found in 3 to 10% of de novo and therapy-related AML. More than 80 translocation partners and 120 reciprocal

fusion variants have been documented.⁴⁰ Common translocation partner needs to be identified due to variable prognosis, for example, t(9;11)(p22;q23.3) – *KMT2A::MLLT3* is considered to have a better prognosis than t(6;11)(q27;q23.3) – *KMT2A::MLLT4* and t(10;11)(p12;q23.3) – *KMT2A::MLLT10* which predict poor prognosis.⁴¹ Other common translocation partners identified are t(4;11)(q21;q23.3) – *KMT2A::MLLT2*, t(11;19)(q23.3;p13.3) – *KMT2A::MLLT1*, t(11;19)(q23.3;p13.1) – *KMT2A::ELL*⁴² (►Fig. 3C).

BCR::ABL1–t(9;22)(q34.1;q11.2)

Philadelphia chromosome formed due to reciprocal translocation between chromosomes 9 and 22 leads to *BCR::ABL1* fusion, is found in 1% AML cases, and is regarded as high-risk marker.¹⁰

DEK::NUP214–t(6;9)(p23;q34.1)

Fusion of *DEK* at 6p23 with *NUP214* at 9q34.1 results in t(6;9) is frequently associated with basophilia and is seen in both pediatric and adult patients.⁴³ It can present either as a sole or a part of a complex karyotype and is an adverse risk marker (►Fig. 3D).

Table 2 Recurrent and nonrecurrent cytogenetic abnormalities in AML detected by conventional karyotyping and FISH

Cytogenetic abnormality	Genes involved	Prognosis	Conventional karyotyping	FISH	Type of FISH probe
t(8;21)(q22;q22)	<i>RUNX1T1, RUNX1</i>	Good	Yes	Yes	DC DF
t(15;17)(q24.1;q21)	<i>PML, RARA</i>	Good	Yes	Yes	DC DF
t(9;22)(q34.1;q11.2)	<i>BCR, ABL1</i>	Poor	Yes	Yes	DC DF
t(6;9)(p22;q34.1)	<i>DEK, NUP214</i>	Poor	Yes	Yes	DC DF
t(1;22)(p13.3;q13.1)	<i>RBM15, MRTFA</i>	Intermediate	Yes	Yes	DC DF
t(7;11)(?;q23.3)	<i>KMT2A</i>	Poor	Yes	Yes	BA
inv(16)(p13.1q22)/t(16;16)(p13.1;q22)	<i>MYH11, CBFB</i>	Good	Yes	Yes	BA
inv(3)(q21q26.2)/t(3;3)(q21;q26.2)	<i>MECOM</i>	Poor	Yes	Yes	BA
t(11;7)(p15;?)	<i>NUP98</i>	Adverse	No	Yes	BA
inv(16)(p13.1q24)	<i>CBFA2T3, GLIS2</i>	Adverse	No	Yes	BA/ DC DF
t(7;12)(q36;p13.1)	<i>MNX1, ETV6</i>	Adverse	No	Yes	BA/ DC DF
Monosomy 5/del(5q31/q33)	<i>CSF1R, EGR1</i>	Poor	Yes	Yes	DC/TC del
Monosomy 7/del(7q22/q36)	<i>KMT2E, CUL1</i>	Poor	Yes	Yes	DC/TC del
del(17)(p13)	<i>TP53</i>	Poor	Yes	Yes	DC del
Trisomy 8	–	Intermediate	Yes	Yes	CEP

Abbreviations: BA, break apart; CEP, centromere enumeration probe; DC DF, dual color dual fusion; DC/TC del, dual color/triple color deletion.

***RPN1::MECOM*-inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2)**

Inversion(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2) involves genes *MECOM* (*EVII*) at 3q26.2 and *RPN1* at 3q21.3 contributing as an unfavorable prognostic marker with a poor outcome.⁴⁴ Frequently, monosomy 7 accompanies rearrangements of *MECOM* (3q26.2) in 50% cases.⁴⁵

***RBM15::MRTFA* – t(1;22)(p13.3;q13.1)**

T(1;22)(p13.3;q13.1) leads to a fusion of *RBM15* at 1p13.3 with *MRTFA* at 22q13.1 and is seen in 3 to 15% of pediatric AML with median presentation age of 1 to 8 years.^{46,47} This aberration is characteristic of acute megakaryoblastic leukemia with conflicting reports on its prognostic significance, as some series revealed a negative outcome while others suggested a favorable one.^{48,49}

Rearrangements of *NUP98*

NUP98 rearrangements are frequently found in 5% of pediatric AML with an unfavorable outcome. More than 30 *NUP98* fusion partners have been identified in numerous hematological malignancies.⁵⁰ t(5;11)(q35;p15.5) and t(11;12)(p15.5;p13) are cytogenetically cryptic aberrations, found in CN-AML, and cannot be detected by CC, necessitating the use of DC DF FISH probes: *NUP98/NSD1* and *NUP98/KDM5A*, respectively, for the identification of these poor risk abnormalities^{51,52} (– Fig. 3E).

Monosomy 5/deletion 5q

Deletion of the long arm of chromosome 5 [del(5q)] or monosomy 5 can be reliably detected by CC or FISH using deletion probes. Isolated del(5q) is known to have a favorable outcome in MDS; however, in AML, this aberration is regarded as an adverse prognostic marker associated with complex karyotypes and poor response to intensive chemotherapy with 20 to 30% of patients achieving complete remission for a short duration.^{4,53}

Monosomy 7/deletion 7q

Monosomy 7 or deletion 7q, seen in approximately 10% AML cases, is associated as high-risk cytogenetic marker.⁶ It frequently occurs in conjunction with other unfavorable cytogenetic abnormalities like complex karyotype (CK), monosomy 5, or deletion 5q, or inv(3).⁴

***TP53* deletion/del(17)(p13)**

TP53 is a tumour suppressor gene present on short arm of chromosome 17 and its loss is associated with disease progression and dismal outcome. It is found in 3-5% of adult AML patients. *TP53* deletion positive patients have lower WBC counts, are associated with high-risk cytogenetic markers [–5/del(5q), –7/del(7q)], have CKs and poor or no response to standard chemotherapy.^{7,54}

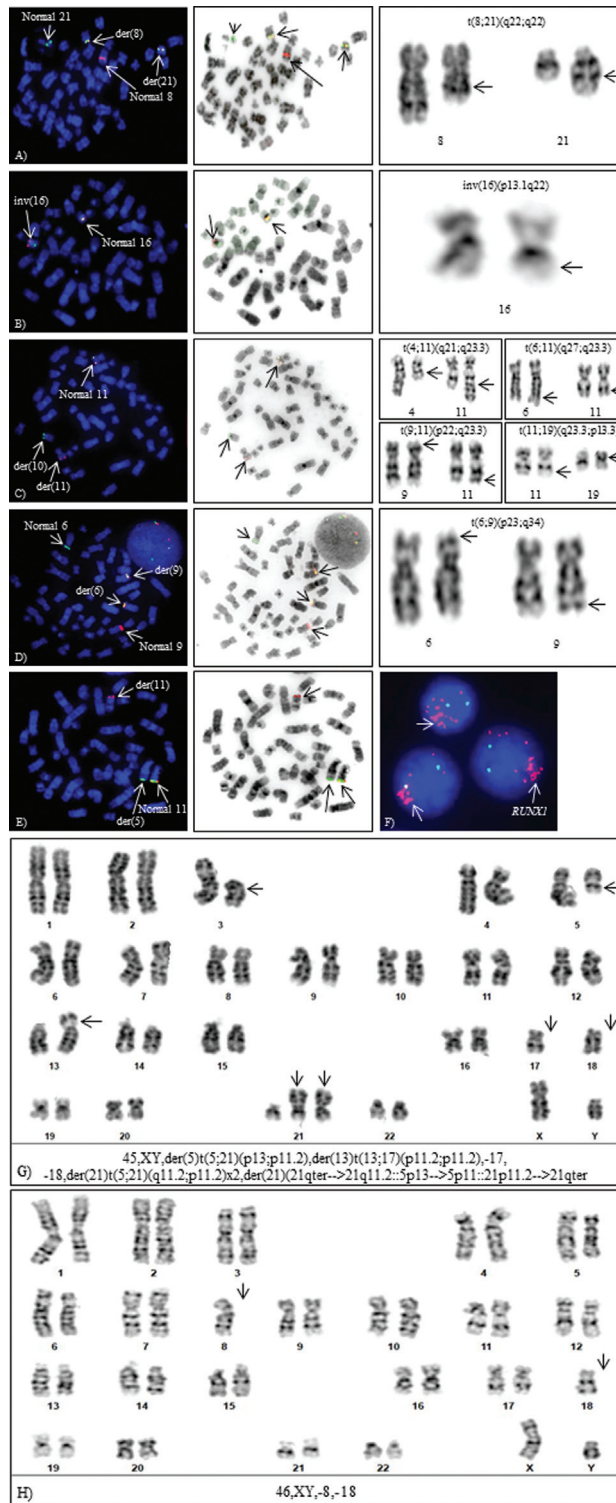


Fig. 3 Cyto-genetic aberrations detected by FISH and CC. Metaphase-FISH, inverted DAPI and G-banded partial karyotypes (A) t(8;21)(q22;q22), (B) inv(16)(p13.1;q22), (C) t(10;11)(p13;q23), (D) t(6;9)(p22;q34.1), (E) cryptic *NUP98* rearrangement: t(5;11)(q35;p15.5) by metaphase-FISH, (F) intrachromosomal amplification of *RUNX1*, (G) Complex karyotype, and (H) Monosomal karyotype.

Trisomy 8

Trisomy 8 is seen in approximately 10 to 20% of AMLs, either as sole or as an additional abnormality with an intermediate

prognosis.⁵⁵ It is seen as a secondary abnormality with t(8;21), inv(16)/t(16;16), or t(15;17) and does not alter its outcome.

Amplification of Oncogenes

Intrachromosomal amplifications of oncogenes like *RUNX1* (21q22) or *KMT2A* (11q23.3) genes, rarely found in AML, are defined more than three copies on a single chromosome and are associated with poor prognosis and inferior outcomes.^{56,57} These oncogenic amplifications either present as a cluster on a single chromosome (homogeneously stained regions-hsr), as double minutes, or are interspersed throughout the genome.⁵⁸ This aberration can be reliably identified by FISH on interphase cells and confirmed on metaphases using LSI *RUNX1*/*RUNX1T1* probe⁵⁹ (►Fig. 3F).

Complex Karyotype

CK is defined as the presence of three chromosomal structural aberrations in the absence of favorable cytogenetic aberrations: t(8;21), inv(16)/t(16;16), and t(15;17)²¹ (►Fig. 3G). AML patients of this subgroup are unresponsive to therapy and have an extremely dismal prognosis.⁶⁰ Numerous chromosome abnormalities, such as unbalanced translocations, gains or losses of chromosomal segments, double minutes, oncogenic amplifications, markers, chromothripsis, and ring chromosomes are frequently present in CKs, necessitating the use of both CC and FISH for their detection.⁶¹

Monosomal Karyotype

Monosomal karyotype features either two autosomal monosomies or one structural abnormality accompanied by one autosomal monosomy (►Fig. 3H). MK affects up to 20% of older populations and accounts for about 10% of all cases of AML and is associated with an unfavorable prognosis with 4-year overall survival of 3% compared to 13% in non-MK patients.⁶² Recognition of MK, is crucial in order to apply alternative therapy to improve the associated poor prognosis.

Quality Control for Fluorescence In Situ Hybridization Probes

Every laboratory employing FISH testing for diagnostic purposes must establish quality control/quality assurance measures by validating each FISH probe utilized for analysis. Determining cut-off values of every probe is also crucial in the application of FISH for AML and other hematological malignancies as these cytogenetic aberrations are clonal in nature and require extremely high sensitivity and specificity to detect minimal residual disease. Using the inverse beta function, confidence interval around the mean, maximum likelihood, or other statistical methods, every laboratory should uniformly establish the cut-off values for FISH probes. Before putting the probe in

use, other factors like probe verification, specificity, and sensitivity should be confirmed and documented for every new lot received.⁶³

Conclusion

Although high-resolution molecular profiling methods like single nucleotide polymorphism array, Next generation sequencing and whole-genome sequencing are crucial for discovering new chromosomal abnormalities, especially fusions, they are impractical for routine diagnostic laboratories due to high cost, long turnaround times, and requirement of expertise in bioinformatics. CC will always be the gold standard, preferred method due to its immense utility in genome-wide evaluations. Application of FISH, as an adjunct to CC, is a powerful strategy used today for identifying recurrent genetic abnormalities that offer valuable information for prognosis, risk stratification, and disease diagnosis in hematolymphoid malignancies.

Authors' Contributions

H.J. was responsible for concept, design, definition of intellectual content, literature search, and manuscript preparation. D.S. was responsible for manuscript editing and manuscript review. The manuscript has been read and approved by all the authors, and the requirements for authorship have been met and each author believes that the manuscript represents honest work.

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Conflict of Interest

None declared.

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