

Cytogenetics: A Reliable Tool for the Diagnosis and Prognosis of Hematological Malignancies

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ABSTRACT

Aim: The current review aims to provide a holistic overview of the cytogenetic techniques used in diagnosis and prognosis of haematological malignancies.

Background: Since the discovery of the Philadelphia chromosome as a sole abnormality for diagnosing chronic myeloid leukaemia, cytogenetics has been widely used to diagnose malignancies in blood. With the introduction of advanced cytogenetic techniques like Fluorescent In-Situ Hybridization, Spectral Karyotyping and Comparative Genomic Hybridization, identification of clones and monitoring the response to drug therapy has now become easy as these have overcome the challenges of conventional cytogenetics like culture failures, poor morphology of chromosomes and low mitotic index.

Review results: Ample evidence suggests that chromosomal aberrations are critical early indicators of progressing to a hematopoietic disorder. Conventional cytogenetics augmented with molecular cytogenetic techniques, immunophenotyping and real-time PCR not only aids in identifying specific clones but also characterizes haematological malignancies, predicting and assessing the minimal residual disease status during their management.

Conclusion: We conclude that in this era of array diagnostics, cytogenetic techniques are still reliable for accurate diagnoses, characterization and prognosis of haematological malignancies.

Clinical Significance: The present review discusses the role of cytogenetic tools in the diagnoses and prognosis of various haematological malignancies. It also provides an insight into the technical challenges encountered while performing these studies and the considerations to overcome them.

Keywords: Cytogenetics, hematological malignancies, leukemia

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INTRODUCTION:

The discovery of a diploid number of chromosomes as 46 by Tijo and Levan in 1955 marked the beginning of human cytogenetics¹. Subsequently, the discovery of a truncated chromosome, named the Philadelphia (Ph) chromosome, as a sole abnormality for diagnosing chronic myeloid leukaemia (CML) in 1960 by Peter Nowell and David Hungerford revealed for the first time that a specific genetic abnormality causes cancer². Then, as there was a marked improvement in techniques for chromosome preparations, Janet Rowley, in 1973, found that the Ph chromosome was produced due to a translocation between the long arms of

chromosome 9 and 22 chromosome³. Subsequent developments in the field revealed that this translocation leads to a production of the fusion protein (BCR-ABL1) that ultimately causes overexpression of an aberrant tyrosine kinase in leukaemia cells of CML patients, thus proving its pathogenetic role⁴. Strikingly, the discovery of the Ph chromosome leads to a new era of a cancer diagnosis. Since

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then, strong evidence has gathered that cytogenetic changes are crucially associated with the diagnosis and prognosis of many cancers, making cancer cytogenetics studies an essential part of clinical practice.

Based on the association between specific chromosomal abnormalities, particular cytological morphology, and clinical features, World Health Organization in 2008, categorized acute myeloid leukaemia (AML) into four unique subtypes⁵. The vital role of cytogenetic analysis in the detection and characterization of haematological malignancies makes karyotyping of cancer cells compulsory in all newly diagnosed leukaemias. Besides, karyotyping of leukaemias is considered the gold standard for drawing correlations between clonal evolution and disease progression.

Overview of cytogenetic techniques

Conventional cytogenetics

Metaphase chromosomes are crucial for karyotyping. For conventional cytogenetic analysis, bone marrow cells are collected in heparinized vacutainers. Cultures are set up according to standard protocol with slight modifications using specific cell culture media⁶. The cultures are supplemented with fetal bovine serum, L-glutamine and sodium bicarbonate. Parallel cultures are set for non-stimulated cells and incubated in a CO₂ incubator overnight, 24, 48 or 72 hours. Well-spread metaphases are finally karyotyped using Imaging Software on a computer. A minimum of 20 metaphases are evaluated for each case under a 100X objective of a microscope.

Analysis of G-banded chromosomes is followed worldwide for the diagnosis of suspected chromosomal disorders that include children with the intellectual deficit and mental retardation⁷, females with primary amenorrhea⁸, couples with recurrent spontaneous abortions⁹ and male infertility¹⁰. Besides, conventional cytogenetic analysis is the primary genetic test for detecting specific chromosomal alterations in haematological malignancies. Once seen, a typical chromosomal translocation, deletion, or inversion helps a clinician develop the treatment strategy and predict the prognosis. Although, cytogenetic analysis is vital to identify chromosomal regions harbouring putative tumour suppressor genes, and proto-oncogenes¹¹ banding analysis is labour-intensive and time-consuming. Routinely, about 10 to 15 days are needed to obtain the results while many

clinically significant submicroscopic chromosomal changes remain undetected¹². Besides, limited banding resolution and poor chromosome morphology make the correct interpretation of complex and cryptic chromosome alterations and their characterization challenging to ascertain.

Technical challenges of conventional cytogenetics and overcome measures

Conventional cytogenetic studies of haematological malignancies are technically challenging. As their outcome is unpredictable, laboratory to laboratory variations in the basic protocol must be adopted to achieve consistent and reliable results. Peripheral blood cultures can be set up in addition to the bone marrow cultures of leukaemias if the blast cells are greater than 10%¹³. Evidence suggests that the cell cycle duration varies significantly in malignant cells of patients. The success in cultures was obtained after an incubation of 16 hours to 292 hours in a series of 37 AML patients¹⁴. Therefore, setting up multiple cultures is a key to obtaining successful results as it maximizes the chances of getting optimal cell divisions. This can be achieved by following direct treatment of the bone marrow cells by colcemid followed by harvesting, culturing bone marrow cells overnight, and blocking at the S-phase of the cell cycle known as synchronization¹⁵.

For identifying commonly found subtle structural chromosome aberrations of malignant cells, high-resolution banding technique of good quality long chromosomes can also be achieved by synchronization techniques¹⁵. However, fluorodeoxyuridine synchronization cultures have been reported inferior compared to short-term cultures in ALL¹⁶. For most cytogeneticists, ALL is a frustrating disease as it has poor chromosome morphology and low mitotic index and samples with a marked tendency to clot during harvest pose significant technical challenges.

Cytogenetic heteromorphisms

Heterochromatin present on 1, 9, 16 and long arms of the Y chromosome are heteromorphisms that vary in size in different individuals and should not be taken as an abnormality. Although, most of the reports would not consider these polymorphic variants of chromosomes, such as the pericentric inversion (inv(9)(p11q13)) is regarded by many cytogeneticists. However, its role in malignancy remains controversial. We have earlier reported pericentric

inversion of chromosome 9 in infertile men¹⁰, and recurrent spontaneous abortion cases⁹ due to constitutional pericentric inversions of chromosome 9 are the commonest chromosomal inversions result in the formation of asymmetric bivalents in meiotic metaphase-I spreads and structural chromosome aberrations in sperm studies^{17,18}. However, in leukaemia cytogenetics, we never came across a case with pericentric inversion of chromosome 9.

Molecular cytogenetics

To overcome the shortcomings of conventional cytogenetic analysis, molecular cytogenetic techniques like fluorescence in situ hybridization (FISH), spectral karyotyping (SKY), and comparative genomic hybridization (CGH) have come up as advanced diagnostic tools. These are now extensively used as an addition to conventional cytogenetics for detecting and validating chromosomal alterations¹⁹⁻²⁴. Furthermore, varied molecular cytogenetic techniques have been identified as a critical matched alternative to G-banding. They develop a detailed interpretation of numerical and complex chromosomal abnormalities by bridging the gap between conventional cytogenetic and molecular cytogenetic analysis studies²⁵⁻²⁷.

FISH

In FISH, chromosome region-specific and fluorescently labelled DNA probes are used to detect a specific chromosomal abnormality. The probe is a short fluorescently labelled fragment of DNA complementary to a particular sequence of the test DNA. The sample DNA in the metaphase chromosomes or interphase nuclei is co-denatured with the fluorescently labelled probe of the region of interest and, with the use of a hybridizer, is hybridized at the target sequence of the DNA as it re-anneals into a double-stranded DNA. The specific signal can be detected with the help of a fluorescence microscope using an appropriate set of filters. In addition, the slides can be screened for the presence or absence of the signal, making them ideal for testing²⁸. The vital feature of FISH is that it can be performed using metaphases and interphase cells²⁹. Besides, fresh tissue sections, formalin-fixed and paraffin-embedded tissue sections and cytology preparations can also be used for FISH analysis, as in the case of breast cancer. Secondly, the FISH procedure is time-saving, and the result turnout time is just 24 hours. FISH probes are of

many types depending upon the type of chromosomal abnormality under consideration, like locus-specific probes, chromosome enumeration probes, dual-colour translocation probes and dual-colour break-apart probes. These are commercially available and prepared from bacterial artificial chromosome (BAC) clones. FISH probes can vary, ranging from 10 kilo base pairs to 100 kilo base pairs (kb) of DNA in length.

FISH has been used not only for detecting aneuploidies in prenatal samples but also in chromosomal rearrangements in leukemia's and lymphomas. Detection of submicroscopic deletions in microdeletion syndromes and subtelomeric rearrangements of chromosomes has also been made possible with the advent of this advanced cytogenetic technique³⁰. Furthermore, the use of multicolor FISH probes has given birth to yet another FISH technique known as Spectral Karyotyping (SKY), which has remarkably improved the stratification of complex chromosome aberrations³¹. In this technique, whole chromosome painting probes give each chromosome a different colour, making it easy to identify chromosomal rearrangements.

Technical considerations in FISH

We have used FISH in leukemia cytogenetics routinely to detect deletions using locus-specific probes (Figure 1a) and aneuploidies using chromosome enumeration probes (Figure 1b), translocations using dual-colour translocation probes (Figure 1c), and inversions using break-apart probes (Figure 1d). However, there are some technical challenges that we come across while reporting FISH slides.

- (a) The light emitted by fluorochromes on a probe is often minimal, lasts for a short time, and fades. In the case of small probes, it sometimes becomes difficult to observe them clearly under a microscope. Therefore, it is essential to capture the image with an attached microscope camera to record the observations before the fluorescence fades away.
- (b) Choosing an area on a FISH slide is another factor that may affect your interpretations. For analysis, areas with low background, well-spread cells and brighter signals should be considered. Using interphase nuclei, at least 200 cells are analyzed to conclude.
- (c) The slides should be screened independently by two well-trained persons to avoid bias.

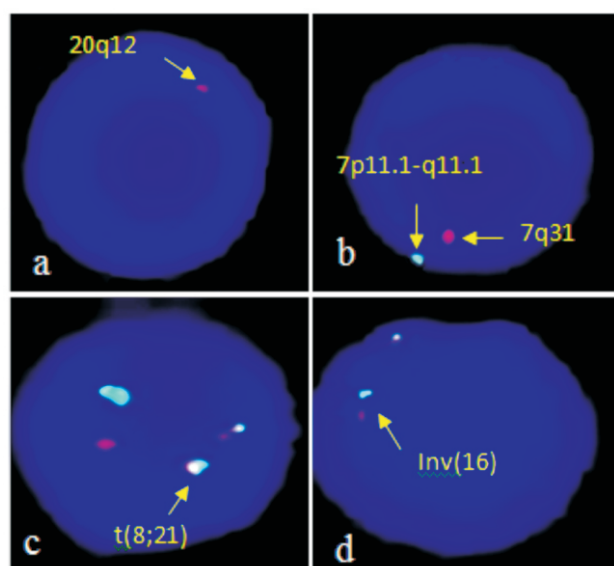


Fig 1: FISH images of interphase nuclei showing (a) deletion (20q12), (b) Loss of chromosome 7, (c) translocation (8; 21) and (d) inversion (16) using Vysis LSI D20S108 del (20q12) probe, Vysis LSI CEP7 Dual Color Probe, Vysis RUNX1/RUNX1T1 Dual Color Dual Fusion Probe and CBFB Break Apart 16q22 Rearrangement Probe respectively.

CGH

Advancing further in molecular cytogenetic technology, Comparative Genomic Hybridization (CGH) facilitates the screening of an individual's entire genome for detecting chromosomal alterations and represents a variation of FISH technology with the advantage of finding out cytogenetic imbalances, particularly copy number variations throughout the genome³². However, this method cannot detect whole-genome ploidy, mosaicism, balanced chromosomal translocations and inversions due to the limited resolution (5-10 Mb) of metaphase chromosomes³³.

Array CGH

Array comparative genomic hybridization (aCGH) is a specific molecular cytogenetic method that combines CGH and DNA microarrays and enables whole molecular cytogenetic profiling. It is proved to help identify primary tumors, thus contributing to more efficient therapy protocols³⁴. This technique is advanced in terms of using fluorescently labelled DNA targets throughout the genome in the form of an array as an alternative to the conventional

metaphase chromosome spreads used in CGH and therefore provides a microarray platform to compare the content of DNA in differentially labelled genomes of a test and a reference genome. The microarray platform facilitates the simultaneous use of thousands of DNA sequences of interest throughout an individual's genome. In addition, it provides specific information about chromosomal locations of aberrations identified in a single experiment^{35,36}.

Cytogenetics of hematological malignancies

Chronic myeloid leukemia

Chronic myeloid leukemia is a bone marrow disorder wherein mature granulocytes, and their precursors proliferate at an increased speed. Reciprocal translocation of chromosomes 9 and 22 resulting in the BCR/ABL fusion is an established cause of this disease. The diagnosis of CML is primarily based on cytogenetic analysis. The t (9; 22), once detected, forms the basis of further management of the disease³⁷. Although karyotype analysis can be repeated during the treatment as well as the post-treatment intervals, however, the follow-up cytogenetic analysis becomes difficult as the bone marrow as well as the peripheral blood cells often turn out to be notorious and don't respond to the hypotonic and other treatments, thereby resulting in no or poor morphology chromosomes.

Moreover, about 1% of CML cases have been found to have a masked chromosome Philadelphia³⁸. In that case, there is a standard structure of chromosomes. In such situations, it is mandatory to carry out a FISH analysis to detect the fusion of ABL and BCR genes and establish the diagnosis.

Apart from the classical t(9:22) translocation, these patients can have other secondary chromosomal abnormalities and additional chromosomal rearrangements (complex karyotypes) that can only be detected by conventional cytogenetics. In addition, further cytogenetic changes have been reported in 60 to 80% of CML cases. These changes forecast progression to an accelerated disease phase or a terminal blast crisis³⁷.

Acute myeloid leukemia

Over the past several decades, cytogenetics has become a fundamental part of acute myeloid leukemia (AML) diagnosis and management. As per the World Health Organization (WHO) classification, the cytogenetic

analysis must be carried out to diagnose AML to categorize a sequence of recurrent genetic markers based on which AML is subcategorized³⁹. AML patients can be stratified into three different prognostic groups: good, intermediate and poor, using cytogenetic methods⁴⁰. inv(16)(p13.1q22)/t(16;16);CBFB-MYH11;+/-other abnormalities, t(8;21)(q22;q22); RUNX1-RUNX1T1+/-other abnormalities, t(15;17)-PML-RARA, Normal karyotype + mutated NPM1 (without FLT3-ITD), normal karyotype + mutated CEBPA karyotypes feature in the favorable prognosis group. In the intermediate-risk group characteristic feature of karyotype include normal karyotype: mutated NPM1 + FLT3-ITD, normal karyotype: wild-type NPM1 + FLT3-ITD, normal karyotype: wild-type NPM1 + no FLT3-ITD, -Y, del7q, del9q, t(9;11), del11q, isolated trisomy 8, +11,+13,+21 and del20q. Karyotypes with adverse prognosis are inv(3)(q21q26)/t(3;3); RPN1-EVI, t(6;9)(p23;q34); DEK-NUP214, t(v;11)(v;q23); MLL rearranged (except t(9;11), del(5q)-5, -7, abnormality of 17p and complex karyotype⁴¹. Besides, about 40-50% of AML cases show a normal karyotype with an intermediate prognosis⁴². Further molecular genetic testing like FISH and reverse transcriptase-polymerase chain reaction are increasingly important.

Acute lymphocytic leukaemia

Acute lymphoblastic leukaemia is characterized by the abnormal immature proliferation of lymphoid cells⁴³. The most frequent hematologic malignancy in children accounts for about a quarter of all cancer diagnoses in children below the age of 15⁴⁴. In pediatric ALL, cytogenetic findings such as t(9;22), t(12;21), hyperdiploidy, hypodiploidy, 11q23 rearrangement and der(21)t(21;21), 21 trisomy/polysomy, have been reported. Detection of these cytogenetic abnormalities is pivotal for predicting the disease's progress and prognosis. The most common chromosomal abnormality in pediatric ALL patients is the t(12;21) translocation that results in the fusion of ETV6/RUNX1 genes and has been related to a favorable prognosis^{45,46}.

From cytogenetic factors the Translocation t(9;22), MLL gene rearrangements and hypodiploidy less than 45 chromosomes are known to be wrong prognosticators. A standard cytogenetic profile is an indicator of intermediate prognosis⁴⁷. Short-time survival and low proliferation ratio of blast cells are characteristic features of ALL, and

therefore, it is impossible to establish a karyotype in about 16% of ALL cases⁴⁸.

Myelodysplastic syndrome

The myelodysplastic syndromes (MDS) are a heterogeneous group of pluripotent hematopoietic stem cell malignancies. Except for the unbalanced der(1;7)(q10;10), translocations of chromosomes are rarely observed in MDS. Therefore, it has become crucial to find numerical chromosomal abnormalities, inversions and deletions^{49,50}. The interstitial deletions, namely del(5q), del(7p) and del(20q) in MDS, are clinically crucial as these deletions are subtle, and the FISH technique is helpful in the identification of these abnormalities^{51,52}. 5q deletions are present in 10% to 15% of MDS patients, and the prognosis is good^{53, 54}. (5q prognosis) Loss of 7/del7q predicts a poor prognosis, and no targeted therapies are available for patients having this abnormality. Patients with del(20q) as a sole cytogenetic aberration (favorable subgroup) live longer than patients with del(20q) and other chromosomal changes (poor prognosis subgroup). Cytopenia is the most common difficulty in MDS as very few cells are obtained to analyze. Therefore, one needs to be meticulous in getting the best out of the minimum.

Chronic lymphocytic leukaemia

Chronic lymphocytic leukaemia (CLL) is a heterogeneous disorder in which immunologically incompetent B lymphocytes build up in the blood; bone marrow and lymphoid organs.⁵⁵ about 80% of CLL have chromosomal anomalies as an etiology. The most common deletion with a favorable prognosis is 13q deletion, found in 55% of patients. Deletion of 11q23, especially with the deletion of the ATM gene, is located in 18% of CLL patients and is an indicator of adverse prognosis. Trisomy 12 and normal chromosomal profile have no known risk prognosis, while 17p deletion that results in p⁵³ gene deletion is an indicator of the worse prognosis.⁵⁶

CONCLUSION

Cytogenetic abnormalities are a significant aetiology for the progression of leukemia's. Varied chromosomal deletions, translocations, and amplification can be detected in most hematological malignancies. Cytogenetic tools like karyotyping and FISH are reliable in finding these aberrations. Furthermore, cytogenetic techniques

augmented with molecular techniques are suitable not only for detecting mutations but for the quantitative measurement of pathogenic transcripts of interest. Recent advances in cancer cytogenetics have led to remarkable improvement in identifying chromosomal alterations, further expanding the diagnostic potential of cytogenetic studies in leukemia diagnosis and related research areas.

Although single-cell karyotyping is still the easiest way to understand clonal evolution and its relationship with the disease progression, advanced cytogenetic techniques help identify chromosomal abnormalities that remain unresolved by conventional cytogenetics. In addition, advanced molecular cytogenetics technology like CGH and Array CGH provides a view of the whole genome at a higher resolution which may generate a lot of new information about cancer genomics. Strikingly, in this era of array diagnostics, cytogenetic techniques still have a pivotal role in the accurate diagnosis, prognosis and management of hematological malignancies.

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