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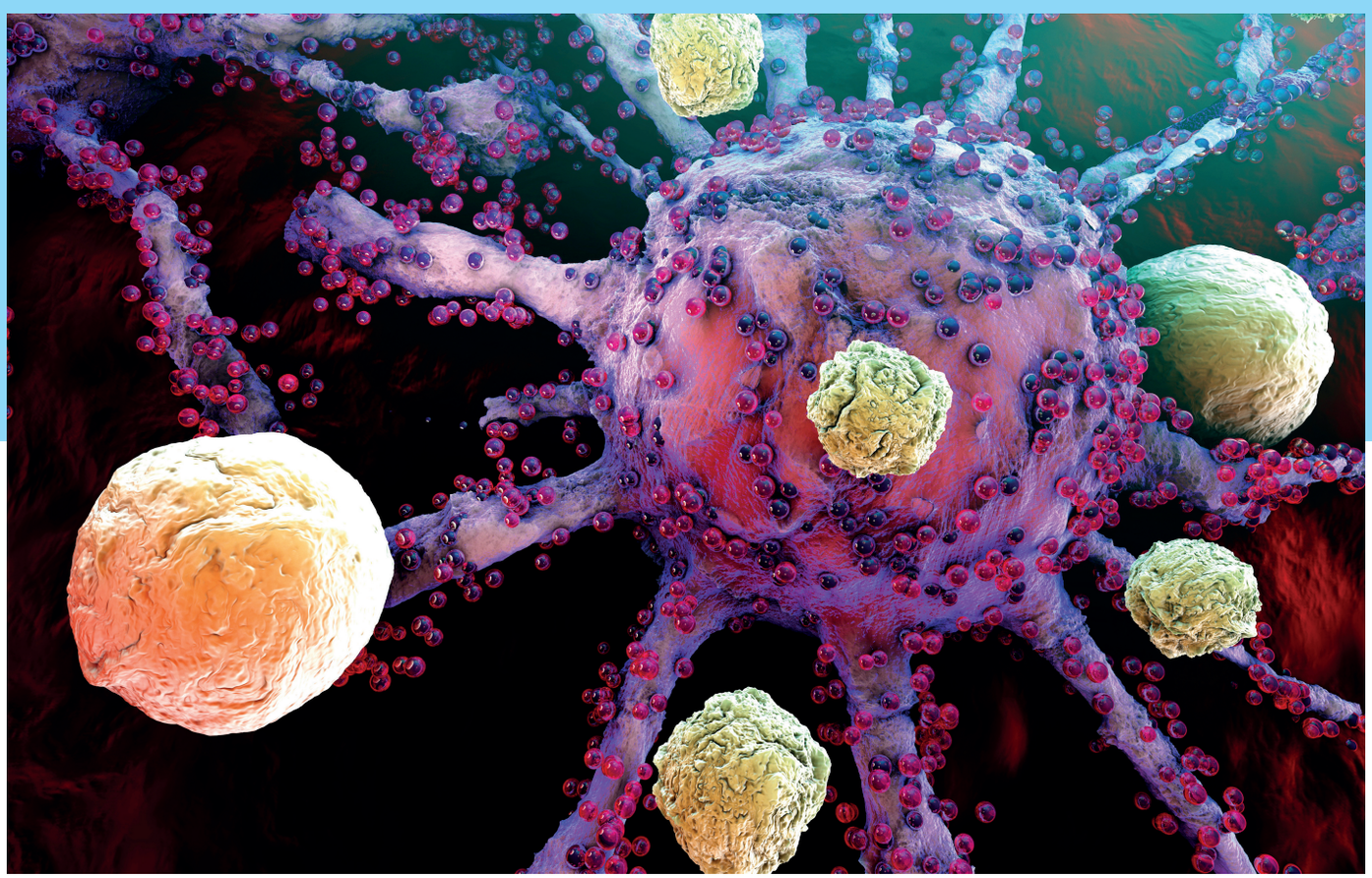

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Volume 44, Number 5/2023

Indian Journal of Medical and Paediatric Oncology

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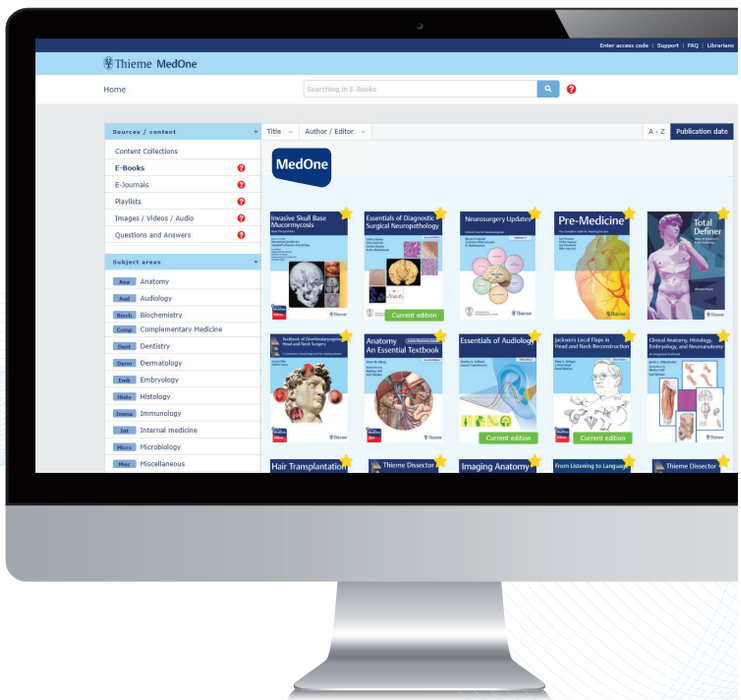

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Editorial

Editor's Note on Hematopathology Special Issue

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The field of hematopathology is rapidly evolving. Along with advancements in the fields of flow cytometry and molecular diagnostics, the assessment of minimal residual disease (MRD) has progressively become a cornerstone in the clinical management of hematologic malignancies. The idea to have a special issue on hematopathology in the *Indian Journal of Medical and Pediatric Oncology* (IJMPO) was conceptualized by Dr. Padmaj S. Kulkarni (Editor-in-Chief at that time) and was further materialized under the current Editor-in-chief Dr. Seema Gulia. I feel honored to have been given an opportunity to be the Editor for this special issue in my field of interest and expertise.

In this special issue, we focus on the importance of dialogue between clinical hematologists and hematopathologists and the role of flow cytometry and molecular genetic techniques in the diagnosis of hematologic malignancies. This special issue also discusses the technical aspects, clinical implications, advantages, and pitfalls of MRD monitoring in patients being treated for acute leukemias.

I would like to express my sincere gratitude to all authors for their quality time in shaping up their respective manuscripts. Without the cooperation of the authors, it would not have been possible to complete this issue within its stipulated time frame. All the manuscripts in this issue underwent double-blinded review by two to three expert reviewers and I am thankful to all these reviewers for their constructive inputs in moulding up the manuscripts to their current form. This special issue is also the fruit of the tireless hard work put up by our editorial staff and publishing team.

Hope that I have done justice to the job entrusted on me and I am thankful to IJMPO for this opportunity. I expect the readers to relish each manuscript in this special issue and make the most use of it in their day-to-day practice.

Conflict of Interest
None declared.

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What Does an Adult Hemato-Oncology Physician Expect from a Hematopathologist?

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Abstract

Recent advances in the field of hemato-oncology have significantly improved outcomes for patients. However, these changes have also increased the complexity of investigations required at the time of diagnosis and during the follow-up of these patients. Close interaction and exchange of information between the pathologist and the clinician is important for sucessful management of patients. This article briefly discusses the advances in the field and the impact of these changes on the management of patients. A clinician's perspective of what is required from a hematopathologist while managing patients in the current era is presented. An attempt is made to classify the requirements as to what is expected in ideal as well as in resource-limited settings.

Keywords
- hematological malignancies
- cancer
- pathology
- expectations
- advances

Introduction

The last two to three decades have seen an explosion of research in hematological cancers, including biological and targeted therapies.[1–5] The classic example is the use of breakpoint cluster region-Abelson leukemia (BCL-ABL) tyrosine kinase inhibitors (TKIs) in chronic myeloid leukemia (CML).[5] The use of imatinib had converted a fatal malignancy into a chronic illness. Less dramatic, but equally significant are the targeted therapies now used in chronic lymphocytic leukemia (CLL; Bruton's tyrosine kinase [BTK] inhibitors), anti-CD20 monoclonal antibodies, several agents in multiple myeloma, and all-trans retinoic acid (ATRA) in promyelocytic leukemia.[6–8] We have also learned to risk-stratify cancers using biological markers. The intensity of chemotherapy can be tailored, and toxicities reduced with minimal residual disease (MRD) stratified treatment in acute lymphoblastic leukemia (ALL).[9,10] ►**Table 1** shows a few selected examples of how advances in the understanding of disease have affected diagnosis and management of hematological cancers.

Expanding knowledge has helped evolve the classification, prognostication, and treatment of hematological malignancies. The role of the hematopathologist has also grown in this period. Most pathologists in the 1970s to 1980s had access to hematoxylin and eosin (H&E) stained specimens and relied on their experience and expertise for the limited subclassification of lymphomas. For leukemias, additional tests like cytochemistry were used for basic differentiation. In the current era, a pathologist has a tremendous array of investigative tools available. These include immunohistochemical studies, conventional cytogenetics, flow cytometry (FCM), molecular techniques like polymerase chain reaction (PCR), in situ hybridization (ISH), and next-generation sequencing (NGS). The modern hematopathologist must wear many hats and plays a vital role in the optimal management of patients. In this article, the role of the hematopathologist in various cancers is discussed. The additional investigations currently available and often pursued also bring with them an enormous cost burden, which can be significant in the Indian context. To help clinicians and pathologists to optimize the use of expensive tests, these are classified as essential, optional, and optimal as per ►**Table 2**. This is similar to the classification used in the National Cancer Grid (NCG) guidelines for hematological cancers and is designed to help optimize patient assessment in Indian situations.[11]

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Table 1 Examples of how changes in understanding of the biology of hematological cancers affect management

Biology of cancer	Remarks/examples
Prognosis	● Minimal residual disease as a prognostic factor and as an endpoint for trials in ALL, myeloma, and CLL[40,44,45]
Identification of newer targets of treatment and development of tailored drugs	● *BCL-2* targeted by venetoclax has improved survival in AML and CLL[30,46] ● Anti-CD38 monoclonals in myeloma[47] ● Bispecific antibodies in acute lymphoid leukemia[48]
Classification of hematological cancers	● Regular review and revision by the WHO have resulted in identifying newer entities with different prognoses and treatments ● It has changed from a one-size-fits-all approach to specific treatment for individual entities[33,49]
Response adapted therapy	● Minimal residual disease-based treatment tailoring (intensification/allogenic transplant) in acute lymphoid leukemia[44] ● Interim PET-based response-adapted therapy in Hodgkin's lymphoma[50]

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloblastic leukemia; CLL, chronic lymphoblastic leukemia; PET, positron emission tomography; WHO, World Health Organization.

Table 2 Classification of usefulness and impact of investigations in current practice

Classification	Explanation
Essential	They are considered mandatory for current practice. Even with resource constraints, efforts must be undertaken to get this information. All pathology laboratories must endeavor to establish these tests in-house
Optimal	This would be considered a basic standard in most of the tertiary centers currently managing hematological cancers in India. It would be preferable to have this established in-house in most centers
Optional	These investigations are ideal and may affect a particular patient or situation. Still, for the most part, they may not be considered part of routine care or have a prohibitive cost, which is not justifiable in the Indian context. It may be done rarely. Sample numbers may not be sufficient to justify in-house establishment of these facilities

Acute Lymphoblastic Leukemia

Recent Advances

Pediatric ALL survival has increased to 80 to 90% due to optimized chemotherapy, better supportive care, risk-adapted treatment (especially with MRD assessment), and baseline molecular characterization.

Adult ALL: Survival gains are modest except in the adolescent and young adult (AYA) population.[12] More intense therapies based on pediatric protocols are preferred in the AYA population. About 15 to 25% of adults would be Ph+, where targeted therapy with TKIs is incorporated. Molecular characterization has identified early T precursor ALL (ETP-ALL), and Philadelphia-*like* (Ph-like) ALL, with worse prognosis.[13] Identification of cell surface antigens helps decide targeted therapy, like anti-CD20 (rituximab), anti-CD19 (blinatumomab) and anti-CD22 (inotuzumab ozogamicin) monoclonal antibodies. Anti-CD38 antibody daratumumab has shown efficacy in relapsed T-cell ALL (T-ALL). Hence, the pathologist must have a close liaison with the treating team to make markers relevant and tailored to the requirement. MRD assessment is crucial in determining survival and decisions for allogeneic consolidation transplants. Thus, there is a demand for more complex investigations during workup, which have had varying degrees of impact on the outcomes (►**Table 3**).

Expectations from Hematopathologist

- FCM for diagnosis and subtyping is currently considered standard work up for ALL. In addition, most current protocols expect stratification based on MRD assessment. The most popular and established method for MRD assessment would be multiparameter FCM.
- Conventional cytogenetics is a well-established technique for the characterization of leukemia. Alternatively, fluorescence in situ hybridization (FISH) and PCR may help look for specific abnormalities like the *BCR::ABL1* translocation.
- During therapy, there are situations where there are cytopenias, and the clinician is unsure about the cause (persistent disease vs. chemotherapy effect). Bone marrow examinations done during these conditions need careful interpretations with close interaction between the pathologist and clinician (information on the duration of cytopenias, diluted aspirates, and the use of growth factors).
- Assessment of MRD using NGS shows high concordance with quantitative PCR while being more specific and is a potential alternative for frontline MRD evaluation.[14]

Acute Myeloid Leukemia

Recent Advances

The initial treatment of AML depends entirely on the risk category (National Comprehensive Cancer Network [NCCN], European LeukemiaNet [ELN] guidelines).[15,16] The primary investigations needed for this would be conventional cytogenetics and molecular studies to establish the status of *NPM*, *FLT3*, and other molecular markers. Patients classified as high risk would be eligible for considering allogeneic transplants in the first remission. FCM-based MRD is not as well established in AML as in ALL, but data is emerging on its usefulness

Table 3 Investigations in adult ALL and how they impact management

Investigation	Implications	Usefulness/ application
Flow cytometry for phenotyping and subclassification	Accurate diagnosis of acute leukemia subtype; helpful information for future MRD studies (not mandatory). In addition, entities like ETP may be identified.[51] FCM can also assess ploidy	Essential
BCR::ABL1 assessment by FISH and PCR	Survival improvement by 20% with the addition of TKI. The decision of transplant in CR1[52]	Essential
MLL status (break apart probe *KMT2A*) A mixed phenotype is suggested by morphology and immunophenotyping, and require molecular studies for confirmation[53]	The presence of MPAL has variable implications on prognosis but is generally considered to have poor outcomes Demonstrated 11q23 abnormality is a definite poor prognostic factor. Many protocols recommend CR1 allogenic transplant in these patients	Optimal
Minimal residual disease assessment by FCM (most common) or molecular studies	Risk-stratified therapy. Intensifying therapy in MRD-positive patients may improve outcomes. Persistent MRD positivity may indicate allogeneic transplantation	Optimal
Ph-like ALL assessment	May have a poorer prognosis. It may be represented in up to 25–30% of patients in AYA age groups. However, testing is complicated and expensive, and treatment implications are few at this point[13]	Optional
Conventional cytogenetics	It may help detect the Ph chromosome. Ploidy may be assessed and may have a prognostic impact (less in adults than in the pediatric age group). More than five chromosomal anomalies may have a poor prognosis	Optimal
CSF cytology	Involvement of CSF needs additional CNS-directed treatment. This is the standard workup for all ALL patients. The sensitivity of assessment may be increased by adding FCM	By spin and cytology: essential By FCM: optimal

Abbreviations: ALL, acute lymphoblastic leukemia; AYA, adolescent and young adult population; BCR-ABL, breakpoint cluster region-Abelson leukemia; CNS, central nervous system; CR1, complement receptor type 1; CSF, cerebrospinal fluid; ETP, early T-cell precursor; FCM, flow cytometry; FISH, fluorescence in situ hybridization; MLL, mixed lineage leukemia; MPAL, mixed-phenotype acute leukemia; MRD, minimal residual disease; PCR, polymerase chain reaction; TKI, tyrosine kinase inhibitor.

(►**Table 4**). Mutation profiling using NGS provides information regarding the type and number of gene mutations, variant allele frequency, and amenability to targeted therapeutics. In addition, it helps us to understand the ontogeny of the disease, underlying germline predisposition, clonal hematopoiesis, and resistance-causing mutations.[17]

Expectations from Hematopathologist

- FCM for diagnosis and subtyping is standard.
- Conventional cytogenetics is mandatory in AML. It remains the cornerstone of risk stratification in AML, classifying the disease into favorable, unfavorable, and

Table 4 Investigations in adult AML and how they impact management

Investigation	Implications	Usefulness/ application
Flow cytometry for diagnosis	Diagnosis of acute leukemia and subclassification	Essential
FCM for MRD assessment	Prognostic as of now. No therapy indications established[54]	Optional
Conventional karyotyping	Risk stratification and treatment. Can pick complex anomalies missed by FISH or PCR	Essential
Molecular studies: *NPM* mutations and *FLT3* mutations/duplications	To help in risk stratification	Essential
Other molecular tests (*CEBPA, c-kit, p53*)	These also have prognostic, and treatment implications but are rarer	Optimal
FISH/PCR for *PML::RARA* translocation	Important implications: either technique must be available in suspected patients to confirm diagnosis	Essential
NGS	MRD assessment	Optimal

Abbreviations: FCM, flow cytometry; FISH, fluorescence in situ hybridization; MRD, minimal residual disease; NGS, next-generation sequencing; PCR, polymerase chain reaction; PML, promyelocytic leukemia; RARA, retinoic acid receptor alpha.

intermediate-risk categories.[18] FISH and PCR may help look for specific abnormalities and further refine prognostication, especially in those with intermediate cytogenetics.

- As in ALL, cytopenias need careful evaluation and interaction between the clinician and hematopathologist during therapy. Hematogones are normal precursors in the bone marrow, recognized by their characteristic morphological features and immunophenotype. The pathologist must help distinguish the residual disease from hematogones.
- In cases of suspected acute promyelocytic leukemia (APML), urgency is required in the diagnosis. Early treatment in these patients can improve survival to >90 to 95%. It is also essential to detect variant translocations in APML as they have therapeutic implications. For example, *NPM1::RARA* [t5,17 (q35;q21.1)] and *NuMA::RARA* are responsive to retinoids [t11,17(q13;q21.1)], while *PLZF::RARA* [t11,17(q23;q21.1)] is resistant to retinoids.
- Bone marrow examination is done to assess response to AML induction therapy. The initial response to therapy is assessed using a bone marrow examination done on day 14 of treatment. However, the D14 marrow is not always practiced due to inherent challenges in its interpretation. Specific centers prefer to repeat induction or to escalate therapy for persistent disease. Another bone marrow examination is performed on day 28 of induction or after white blood cell (WBC) and platelet count recovery to assess achievement of remission.
- The role of MRD assessment in AML is established as a potent prognostic factor. However, the impact of treatment modification based on MRD is unclear. The commonly employed techniques for MRD assessment are PCR and multiparameter flow cytometry (MFC) and, less typically, NGS. NGS has been used for MRD assessment in patients with *RUNX1, NPM1, FLT3* mutations, and other novel targets.[19] However, what is unclear is the best modality to adopt and how to incorporate the findings in therapy alterations. Each center would do its best to evolve its own MRD technique and standardize these until more data develop in this space. Leukemia-associated aberrant immunophenotypes (LAIPs) present on the blasts are identified at diagnosis using MFC and are later used to assess MRD. MFC can be performed in 80% of AML patients and has a sensitivity of one blast cell per 10^3 to 10^4 nucleated cells. MRD assessment of core binding factor AML (t(8,21) or inversion 16) using quantitative reverse transcription PCR (RT-PCR) allows the identification of patients at high risk of relapse. It is now being studied to predict the role of risk-directed/preemptive therapy.

Chronic Myeloid Leukemia

Recent Advances

The advent of imatinib in the last two decades and subsequent generations of TKIs have made CML a chronic disease. A chronic phase CML patient can expect a life span similar to an age-matched person.[20] However, optimum treatment needs close monitoring of *BCR-ABL* by quantitative PCR.[21] When patients develop resistance, mutation analysis helps decide the subsequent line of TKIs.

Expectations from Hematopathologist

- *At diagnosis:* From bone marrow studies, we need to understand the blast percentage to know the risk group and the phase of CML. Conventional karyotyping helps diagnose additional chromosomal abnormalities and variant translocations and understand clonal evolution. Additional cytogenetic abnormalities, including trisomy 8, an additional Ph translocation, isochromosome 17q, and trisomy 19, are seen in the bone marrow aspirate of 3% of patients, which may have prognostic implications.[22]
- Up to 15% of CML patients may be negative on cytogenetic analysis and can be diagnosed by FISH testing. These are referred to as Philadelphia chromosome (Ph)-negative CML. FISH also helps diagnose patients with complex (e.g., Chr 9;14;22) and cryptic translocations. Commonly used RT-PCR assays will only detect the p210 and p190 variants of *BCR::ABL1*. Hence, if a patient with suspected CML is negative for *BCR::ABL1* transcripts by RT-PCR, it is essential to rule out *BCR::ABL1* fusion using FISH testing.[23] Bone marrow fibrosis is less relevant in the TKI era. However, this is valuable information and may predict cytopenias during early treatment.[24]
- For a patient presenting with blast phase CML, knowing the type of blast crisis is helpful—lymphoid versus myeloid. In lymphoid blast crises, we can use vincristine, steroids, and TKIs. In myeloid crises, TKIs are often used with or without cytarabine-based chemotherapy.
- *Molecular studies:* At baseline, we need to confirm the diagnosis by demonstrating the *BCR::ABL1* transcript. Qualitative PCR is preferred at baseline to know whether the transcript is p190, p210, or others like p230, which are rare. It is essential to see if it will be a quantifiable transcript during follow-up (as most of the qPCR assays are kit based and can detect only known transcripts). A qPCR may be appropriate at baseline as the follow-up evaluations are normalized based on the International Standard (IS).[21]
- During follow-up: Current follow-up standard for CML is quantitative PCR for *BCR::ABL1* transcripts. This is important to define treatment landmarks and to change therapy in those without optimal response or treatment failure. Well-defined cutoffs are available from the ELN guidelines that most centers worldwide follow. The availability of cheaper generic second-generation TKIs have made these results relevant in India. For patients wanting to attempt treatment-free remission (TFR), qPCR needs to be done once every 4 to 6 weeks, and the turnaround time should be less than 2 weeks. The laboratory needs to mention the sensitivity of the testing, the *ABL* copies, and the sensitivity of the PCR machine itself in these reports. The reporting must conform to standard guidelines.[25] The kinetics of *BCR::ABL1* transcripts predict treatment response and overall prognosis. It has been found that the time taken

for the *BCR::ABL1*value to halve was a strong independent predictor of sustained TFR. A quicker *BCR::ABL1* decline increased the likelihood of achieving TFR eligibility.[26]

- Analysis for kinase domain (KD) mutations is necessary for patients who progress to accelerated and blast phases. PCR and Sanger's sequencing are the commonly employed techniques. Sanger's sequencing has limited sensitivity and has limited ability in diagnosing polyclonal and compound mutations. NGS is a promising technique for KD mutation analysis.[27] The advantages of NGS are its higher sensitivity, ability to scan the entire KD for any mutation, and the ability for clonal analysis in case of multiple compound and polyclonal mutations falling within the same sequence reads.[28]

Chronic Lymphoid Leukemia

Recent Advances

From conventional treatment with rituximab and chemotherapy (fludarabine, cyclophosphamide, rituximab [FCR] or bendamustine, rituximab [BR]), the current standard of care in CLL management are BTK inhibitors, and venetoclax.[29,30] In India, BTK inhibitors like ibrutinib and acalabrutinib are available as generics at lower costs. Although these are expensive, access has increased in the last 2 to 3 years.

Expectations from Hematopathologist

- Apart from establishing the diagnosis, which is straightforward in the majority of the cases (lymphocytosis with typical morphology with FCM demonstration of CD5+ expressing B cells, which are negative for cyclin D1 and positive for CD23), pathologists would need additional tests to confirm the diagnosis in situations where the typical markers are not expressed. These include differentiating rare cyclin D1 negative mantle cell lymphoma (MCL) cases, CD23 negative CLL, etc. Additional markers that help in these situations are available.
- Though BTK inhibitors can be considered superior to chemoimmunotherapy for all patients with CLL, they are still expensive and require lifelong therapy. Hence, many Indian hemato-oncology physicians prefer chemoimmunotherapy in their patients and reserve the BTK inhibitors for later lines. However, it becomes crucial to check for 17p deletion. FISH can usually do this from the peripheral blood. While detecting *TP53* mutation using NGS increases sensitivity, it may also slightly increase the false-positivey rate.[31] 17p deleted patients do poorly with BR or FCR and should be treated with BTK inhibitors. FISH can also detect other cytogenetic abnormalities, which correlate with prognosis, namely, deletion 13q (favorable) and deletion 11q (adverse).
- Multiple markers like molecular studies for 17p, β-2 microglobulin, and various FCM-based markers are available for prognostication. The mutational status of the variable region of the immunoglobulin heavy chain (*IGHV*) gene using PCR is a powerful predictor of response duration and overall survival.[32] Although not standard, increased expression of zeta-associated protein 70 (ZAP-70) and CD38 are imperfect surrogates for *IGHV* unmutated status, which correlates with higher clinical stage, increased chances of disease progression, and poorer overall survival. Although it would be helpful to have this information, these are not mandatory for patient care. The decision on whether to start treatment in a given patient can be made based on simple clinical parameters and the Rai stage.[29]
- In patients with anemia, a Coombs test would be needed to look for hemolysis.
- A bone marrow aspirate and biopsy is not routinely recommended for CLL diagnosis. The only indications for bone marrow examination in the contemporary era is to look for factors that might contribute to cytopenias other than leukemia cell infiltration. It can also be helpful in patients who have persistent cytopenias after treatment to establish disease versus therapy-related causes.
- Acquired Bruton tyrosine kinase/phospholipase C gamma 2 (PLCG2) mutations have been associated with resistance to BTK inhibitors resulting in CLL progression and Richter transformation. Longitudinal, targeted, deep multigene sequencing is an emerging modality to diagnose these mutations. As the usage of BTK inhibitors expands in India due to the availability of generic molecules, these tests will become relevant very soon, and laboratories will benefit from developing these additional capabilities.

Lymphomas

Recent Advances

In the last two decades, there have been multiple studies on diffuse large B-cell lymphoma (DLBCL) biology. Prominent ones are the cell of origin classification (including gene expression–based study and later histochemistry-based substitution), and the use of FISH to subclassify high-grade B lymphomas (HGBL) into DLBCL/HGBL with MYC and BCL2.[33] The subclassification has led to better prognostication, but the impact on treatment has been limited. One exception is the double-hit lymphoma, where more aggressive regimens may be warranted but not proven to have benefits beyond the R-CHOP (Rituximab, Cyclophosphamide, Vincristine, Doxorubicin, and Prednisolone) regimen.[34] Despite several studies, most patients with DLBCL continue to be treated with R-CHOP as other regimens have failed to demonstrate superiority.[35]

Expectations from Hematopathologist

- The World Health Organization (WHO) classification in 2016 brought a clear pathway for distinguishing various entities called high-grade B-cell lymphomas.[36] This differentiation is essential in the clinic as the treatment varies. So, a specific classification can help the clinician, especially when FISH testing needs to be optimized because of cost constraints.
- **Testing for DHL by FISH:** DLBCLs that show rearrangements of the *C-MYC* and *BCL2* (DHL) and/or *BCL-6* respond poorly to the R-CHOP regimen. Hence, it is advisable to check for these aberrations in patients with germinal center

B (GCB) subtype with advanced clinical stage, extranodal involvement, bone marrow and central nervous system (CNS) involvement, and high International Prognostic Index (IPI) score. Since this entity constitutes only 5 to 10% of all DLBCLs, routine testing for all cases is not financially viable. Hence, it would be preferable to have an algorithmic approach to decide when to go for FISH testing.[37]

- There are entities within the B-cell lymphoma category that are CD20 negative, like ALK + DLCBL and plasmablastic lymphomas. These need to be flagged as they are not eligible for rituximab.
- In situations where tissue availability is limited (sick patient, only fine-needle aspiration cytology [FNAC] is available, or a small needle biopsy material), the crucial information required from the pathologist is whether it is high grade or low grade and whether CD20 is expressed or not. This will help in important treatment decisions. The other important information is whether a high-grade lymphoma has a blastic morphology. This can be very challenging in situations with limited tissue, and markers like TdT may help out. Cell blocks prepared from the FNAC specimen can be used for immunohistochemistry and molecular testing.
- WHO classification of lymphoid malignancies: Arguably, this is one area of hematological cancers with maximum changes and subdivision of entities. Again, it is helpful for pathologists to consider subtyping as much as possible. Although everything may not change treatment immediately, the information about newer subtypes may inform future understanding.
- Regarding Hodgkin's lymphomas (HLs), tissue diagnosis is crucial. Subtyping into individual entities has become less relevant from treatment and prognostic points of view with modern therapies. R-CHOP is considered the preferred treatment for the entity of nodular lymphocyte predominant HL.[33]
- Identifying surface molecules has helped in the evolution of targeted therapy in lymphomas. A few examples include anti-CD20 (rituximab, obinutuzumab, ofatumumab, and ibritumomab in B-NHLs), anti-CD22 (moxetumomab pasudotox in hairy cell leukemia), anti-CD30 (brentuximab vedotin in HL, anaplastic large cell lymphoma, and CD30+ T cell lymphomas), anti-CD 79b (polatuzumab vedotin in DLBCL), and crizotinib in ALK + ALCL.
- In patients with marginal zone lymphomas, detection of t(11,18) using RT-PCR helps identify those who show resistance to *Helicobacter pylori*–based antibiotic therapy.
- Analysis of T-cell receptor (TCR) gene rearrangements using PCR helps differentiate between lymphomas and benign pathologies like reactive lymphoid hyperplasia. This is particularly useful in samples where small quantities of DNA are available, such as skin biopsies in cutaneous T-cell lymphomas or cell suspensions[38]

Multiple Myeloma and Plasma Cell Disorders

Recent Advances

The diagnostic criteria for myeloma have been updated, and the high serum-free light chain (SFLC) ratio and high plasma cell proportion in the bone marrow (>60%) are considered myeloma defining (even without symptoms).[39] Besides multiple myelomas, the SFLC assay helps diagnose and monitor oligo-secretory and nonsecretory myelomas, light chain myelomas, and light chain amyloidosis. An abnormal SFLC kappa/lambda ratio has prognostic significance for monoclonal gammopathy of unknown significance, smoldering myeloma, and solitary plasmacytomas. MRD assessment has been recognized as an essential prognostic factor in myeloma.[40] Posttransplant D-100 is the preferred timepoint for assessment of MRD. Although clinical trials have assessed MRD as an endpoint, it is yet to find favor as a clinically relevant endpoint. It would be good to have this information, especially from academic large-volume centers treating myeloma. The methodology for assessing the MRD status for myeloma has been standardized, and guidelines are available. The most common techniques used for MRD assessment in myeloma are multiparameter FCM and NGS. In centers with limited resources for MRD assessment, a stringent complete response is taken as a treatment endpoint, as demonstrated by the absence of clonal cells by immunohistochemistry or immunofluorescence in the bone marrow. In terms of treatment, several new drugs have been approved for treating myeloma in the last few years.[4] These do not change the diagnostic requirement for the most part. However, there are unique challenges in interpreting reports, as detailed below.

Expectations from Hematopathologist

- In situations of monoclonal gammopathy of undetermined significance (MGUS)/smoldering myeloma, when there are minimal plasma cells in the bone marrow, it becomes critical to know whether they are clonal. Appropriate IHC would be required for the final decision. When the flow laboratory is well developed, FCM-based assays can also demonstrate clonality. These are unique situations and may not be routinely required in practice.
- Current classification recognizes >60% plasma cells in the marrow as myeloma-defining criteria.
- FISH studies from the bone marrow for del 17p, t(4,14) t(14,16). These are the commonest cytogenetic abnormalities in myeloma and are part of current prognostic scores. Gain or amplification of chromosome 1q is a poor prognostic factor and has been included in the second revision of the international staging system (R2-ISS). These are desirable to have in all patients. Certain treatment decisions, like the continuation of proteasome inhibitors in maintenance, may be considered in patients with high-risk disease. Plasma cell enrichment of the bone marrow specimen improves FISH sensitivity.
- Hyperdiploidy, which occurs due to trisomy of odd-numbered chromosomes, can be detected using cytogenetics. It is a favorable prognostic factor associated with improved overall survival.
- There are challenges in interpreting monoclonal spikes when treating with monoclonal antibodies like daratumumab. The clinician must convey this information to the pathologist. Similarly, challenges exist while blood

grouping these patients, which the clinician and hematopathologist must be aware of.

- The percentage of circulating plasma cells using peripheral smear helps diagnose plasma cell leukemia and is a prognostic factor in multiple myeloma. A study showed that the median overall survival of patients with $<5\%$ plasma cells was 50 months compared with 6 months in those with $\geq 5\%$ circulating plasma cells.[41]
- Identifying solitary/multiple plasmacytomas using immunohistochemistry on tissue samples for CD138 and CD38 and establishing monoclonality by kappa/lambda light chain restriction helps identify cases that could have been labeled as nonmyeloma as per bone marrow examination showing the limited number of plasma cells.[41]

Myeloproliferative Neoplasms

Myeloproliferative neoplasms (MPNs) other than CML (polycythemia vera, myelofibrosis, and essential thrombocytosis being the three common ones) have been recognized by specific gene alterations such as the *JAK2* mutation. The WHO classification specifies diagnostic criteria for each of these entities.[42] Besides standard histomorphology, molecular testing for mutations (such as *JAK2)* is essential. However, since these are usually rare conditions, with most institutions seeing 5 to 10 cases per year, it may not be financially viable to develop in-house molecular testing capabilities in all except the few very high-volume centers. Cytogenetics is vital for the prognostication of primary myelofibrosis.[43]

Conclusions

- Several advances in understanding biology and newer therapeutic agents have led to significant alterations in the diagnosis, classification, and treatment of hematological cancers.
- A modern unit treating hematological cancers as well as the supporting hematopathology laboratory needs to adapt to the rapid changes to stay updated with the best treatment practices.
- However, many of the advances are expensive and may not be available in all centers. Hence, a balance needs to be struck between what is practical and achievable and what is desirable. These requirements need to be tailored toward individual center requirements and capabilities.
- A close interaction between the treating physician and the hematopathologist is ideal to realize the best utilization of resources and ensure optimal outcomes.

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None declared.

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The Symbiotic Relationship between a Clinical Hematologist and Hematopathologist in the Management of Children with Cancer

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Introduction

Hematological malignancies, namely, leukemias and lymphomas comprise the major part of clinical practice for most centers that manage children with cancer.[1] Survival of children with common pediatric malignancies such as acute lymphoblastic leukemia (ALL), Hodgkin's lymphoma, and Burkitt's lymphoma has improved by leaps and bounds over the last few decades. This can be largely attributed to superior diagnostic techniques and risk-stratified approaches that tailor the therapy for different patient subgroups. The pathology laboratory, by performing indispensable tests ranging from cytology and histomorphology to immunohistochemistry (IHC), flow cytometry, and molecular methods, has veritably paved the way for this success story.

The evolution of hematology as a specialty can be attributed to doctors who played the dual role of managing patients at the bedside and examining their peripheral smears and bone marrow aspirates in the laboratory.[2] It is only in the last 20 years that clinical and laboratory hematologists are emerging as distinct specialists. The dichotomy is necessitated by the growing number of children being diagnosed with hematological disorders and the multitude of investigations that are now mandatory for their standard of care. Yet, it is noteworthy that contemporary courses that train clinical and laboratory hematologists ensure the rotation of the trainees in the laboratory and clinical areas, respectively. In this perspective, the authors illustrate the importance of a healthy relationship between the two groups of professionals with the help of some real-life scenarios.

Case 1: A Smear in Time Saves Nine

A 9-year-old boy presented with high-grade fever and vomiting for 1 week and altered sensorium for 1 day. Neuroimaging revealed intraparenchymal hemorrhage. A complete blood count (CBC) showed a hemoglobin (Hb) level of 5.8 g/dL, white blood cell (WBC) count of 93,200/mm^3, and platelet count of 35,000/mm^3. Prothrombin time and activated partial thromboplastin time were both prolonged. The clinician suspected the possibility of acute promyelocytic leukemia (APML) and called his hematopathology colleague to review the peripheral blood smear, and the smear was promptly reported to show blasts and abnormal promyelocytes. The child was immediately initiated on all-trans retinoic acid and administered platelet and plasma transfusions. By the next day, a bone marrow test was performed, which corroborated the diagnosis of APML on morphology and flow cytometry. After 3 days, a polymerase chain reaction (PCR) test for PML-RARα decisively established the diagnosis. Timely initiation of therapy based on good clinical suspicion and reliable smear findings while waiting for the confirmatory diagnostic results enabled complete recovery of the child.

The importance of the peripheral blood smear cannot be undermined. A simple Romanowsky smear seen by experienced eyes can reliably demonstrate atypical cells in leukemia and enable the clinician to plan further investigations and treatment. In a typical case of leukemia with pallor, bleeding, lymphadenopathy, and hepatosplenomegaly, the smear can confirm the clinical suspicion. The smear findings can raise the possibility of leukemia in atypical situations too. A child with severe musculoskeletal pains due to

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leukemia may be misdiagnosed as juvenile rheumatoid arthritis, and an astute hematopathologist can call the clinician after seeing the blood smear to prompt a bone marrow test before starting steroid therapy. A smear can also rule out a malignant etiology and redirect the diagnostic pathway. For example, an infant with transfusion-dependent β-thalassemia can present with pallor and hepatosplenomegaly. WBC may be spuriously elevated due to nucleated red cells (NRBC) and the platelet count may be low due to untreated Vitamin B12/folic acid deficiency. An unnecessary and invasive bone marrow test can be prevented and the clinician can be directed to obtain a hemoglobin variant analysis to confirm the diagnosis. Even in today's age of the automated hematology analyzer giving CBC reports in most laboratories, abnormal cells are flagged and smears are prepared for review by the pathologist.[3] No machine is a replacement for the trained eyes of a hematopathologist! As the adage goes, "The eye cannot see what the mind does not know." Therefore, it is also important that the clinical differential diagnoses are effectively communicated to the hematopathologist.

Case 2: A Good Bone Marrow is a Bull's Eye Hit by an Arrow

A 14-month-old boy presented with fever and easy bruisability for 6 months. He had been treated for immune thrombocytopenia (ITP) elsewhere and had undergone bone marrow test twice with inconclusive reports. The trephine was reported as myelofibrosis. At the time of presentation to our center, the CBC showed Hb level of 5.1 g/dL, WBC of 7,600/mm^3, and platelet count of 10,000/mm^3. The disproportionate anemia and presence of hepatosplenomegaly alerted the clinical hematologist that this was not ITP. The hematopathologist reported the smear to have NRBC, leukoerythroblastic blood picture with 11% blasts in the differential, which morphologically resembled megakaryoblasts. The bone marrow aspirate was dilute yet again. The trephine biopsy was reported as fibrotic with some atypical megakaryocytes. The flow cytometry of the peripheral blood was able to immunophenotype the circulating blasts as megakaryoblasts. The colleague in the cytogenetics laboratory was alerted to look for t(1;22), which was promptly identified in a week, confirming the diagnosis of acute megakaryoblastic leukemia. The child was treated with intensive chemotherapy for acute myeloid leukemia (AML) and is now in remission 6 months post therapy.

A bone marrow investigation done by an experienced person is indispensable to the diagnosis of hematological malignancies. Extra samples of the aspirate must be sent for genetic analysis and to the flow cytometry laboratory in all cases with suspicion of malignancies. In the above case, it was a combination of morphology, flow cytometry, and genetic techniques that led to the establishment of a diagnosis. In the absence of the cytogenetic aberration identified, it would have been arduous to label the child as AML based on peripheral blood flow cytometry alone. A cytogenetics report that normally takes a minimum of 2 weeks was expedited by the laboratory consultant at the request of the clinician, leading to the initiation of chemotherapy within a week of presentation to our center.

Case 3: Laboratory Milestones on the Clinical Road to Remission

A 4-year-old boy was admitted with fever, pallor, hepatosplenomegaly, and pancytopenia. A bone marrow test was performed with aspirate samples sent to the flow cytometry, molecular hematology, and cytogenetics laboratories. Six hours later, the flow cytometry laboratory confirmed the diagnosis of pre-B ALL to the clinician. He was assigned to the "standard risk" category based on his age and a presenting white cell count of 5,600/mm^3 (<50,000/mm^3). After 1 week of the oral prednisolone prephase, an absolute blast count in the blood of 200/mm^3 suggesting a good prednisolone response (<1,000/mm^3 on day 8 after the steroid prephase) and the absence of blasts in the cytospin preparation of the cerebrospinal fluid (CSF) corresponded to a CNS1 stage. By 2 weeks of therapy, the molecular hematology laboratory reported a PCR positivity for ETV6-RUNX1, which carries a good prognosis in ALL. The cytogenetics laboratory reported a normal karyotype. At the end of induction chemotherapy, a repeat bone marrow showed no residual leukemic blasts, and a flow cytometry-based minimal residual disease (MRD) was reported by the hematopathologist to be 0.15% (positive MRD taken as ≥0.01%). The chemotherapy protocol was then escalated to "high risk" based on the positive MRD. The child completed his intensive and maintenance chemotherapy successfully and remains in remission 5 years after treatment.

ALL is the poster child for how a clinician and pathologist work closely together to establish the diagnosis, prognosis, and final risk group for treatment. In the past 50 years, ALL has transformed from an almost uncurable cancer to cancer that is cured in almost 90% of children in the developed world.[4] Flow cytometry has revolutionized the diagnosis of leukemia and establishes the immunophenotype for deciding treatment. From the first report of Philadelphia chromosome (Ph) positivity in ALL 60 years back to the recent discovery of novel genetic entities such as Ph-like ALL with advanced molecular techniques, the concept of a blanket diagnosis of "ALL" or "AML" has changed to a genetically heterogenous wide spectrum of leukemias.[5,6] The latest classifications of hematological malignancies incorporate both immunophenotype and characteristic genetic aberrations in defining the categories.[5]

Cytotoxic treatment–related mortality is a major hurdle to the successful treatment of cancers.[7] Factors such as the presenting blood counts, absolute blast count after prephase, CSF cytology, genetics, bone marrow morphology, and MRD are now used for optimal risk stratification.[8–10] MRD measurement by flow cytometry or genetic techniques has superseded morphological assessment of remission in ALL and emerged as an independent predictor of outcome.[9] Risk stratification permits escalating intensities of cytotoxic therapy for different patients. This has significantly reduced toxic morbidity and mortality. AML is stratified completely based

on the genetic aberrations detected and the MRD response post-induction chemotherapy.[11] The decision of offering a hematopoietic stem cell transplant in the first remission is also based on the same factors.

Akin to flow cytometric immunophenotype, IHC is used for the diagnosis of lymphomas. The simple classification of Hodgkin's lymphoma (HL) and non-Hodgkin's lymphoma (NHL) is now replaced by a multitude of types of lymphomas that have been defined by contemporary IHC and genetic tests.[12] The nodular lymphocyte-predominant HL, for example, is a distinct entity that has a unique morphology and IHC, which is treated with different protocols from classical HL.[13] The term NHL is expanding to include more and more entities with molecular advances. Despite the mind-boggling array of lymphoid neoplasms that are now described, the basic diagnostic approach stands firm and unshaken. For instance, in an older child who is operated on for intussusception, the clinical hematologist would suspect a Burkitt lymphoma and obtain a metabolic profile to demonstrate tumor lysis syndrome with hyperuricemia. The hematopathologist would then review the histopathology for the classically described "starry sky appearance." The rapidly proliferating lymphoma requires rapid initiation of chemotherapy and supportive care while awaiting further immunohistochemical confirmation of a mature B-cell phenotype and a *C-MYC* translocation by a fluorescence in situ hybridization. The concept of MRD in leukemias has led to the prospective evaluation of minimal disseminated disease in lymphomas as a tool for prognostication and risk stratification.[14]

Case 4: Putting Pieces Together with NGS

A 4-year-old girl was brought by her family to the oncology clinic. Her 27-year-old mother had recently succumbed to a disseminated gastric adenocarcinoma, and the family was anxious about the risk of cancer occurring in her. On examination, she was observed to have premature graying of hair. Her CBC and liver and renal function tests were all within normal limits and she had no other clinical concerns. A focused next-generation sequencing (NGS) for inherited marrow failure syndromes was performed on her peripheral blood, which detected a heterozygous mutation in the RTEL1 gene identified in dyskeratosis congenita. The family was counseled on the need for surveillance for the development of mucocutaneous features, bone marrow failure, and malignancies.

NGS has revolutionized the diagnostic machinery in the field of hematology. It has a wide range of uses including the identification of inherited disorders that predispose to malignancy and fine molecular characterization of malignancies such as ALL and AML. In pediatric ALL, NGS is now being used at diagnosis for identifying mutations that are prognostic and potentially targetable (e.g., Ph-like ALL), accurately quantifying MRD, identifying resistance to therapy (e.g., *CREBBP* mutations that induce glucocorticoid resistance), and pharmacogenomics (e.g., *TPMT* and *NUDT15* mutations that indicate a need to reduce the doses of 6-mercaptopurine).[15–17]

Conclusion

In this era of rapid advancement in medicine, it is vital for clinical and laboratory health professionals to work in constant liaison. In the absence of a complete clinical database and a list of differential diagnoses in the request form, the pathologist cannot be expected to give an accurate diagnosis on any test. Similarly, the clinical hematologist is heavily dependent on the details provided in each report from the laboratory for his or her practice. Any discrepancy between the clinical expectation and the pathology report must prompt a conversation to discuss the case in detail. Multidisciplinary discussions must be held regularly to discuss challenging cases. Further, research is the way forward to achieving the ambitious dream of curing all children with cancer in the future. Quality research in hematology mandates an active corroboration between clinical and laboratory-based researchers. Last but not least, in low- and middle-income countries, where the availability of advances lags behind high-income countries, the clinician and pathologist must make use of all modalities available to provide optimal care to children with hematological malignancies.

Author Contributions
The manuscript has been read and approved by all the authors that the requirements for authorship have been met and that each author believes that the manuscript represents honest work.

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Role of Morphology in the Diagnosis of Acute Leukemias: Systematic Review

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Abstract

The role of hematopathologists in the diagnosis of acute leukemia (AL) starts with the morphological examination of either peripheral blood smear or bone marrow. The morphological hallmark for the myeloblast includes "Auer rods" and "Phi bodies." The addition of cytochemical stains such as myeloperoxidase, Sudan Black B, periodic acid-Schiff stain, nonspecific esterase, and Perls' stain acts as an important adjunct to the morphological classification in the resource-constrained settings. The recent World Health Organization classification still endorses the utility of morphology which requires the presence of either $\geq$ 20% lymphoblasts or myeloblasts/or its equivalents (monoblasts, promonocytes, or megakaryoblasts) and integrates it with the clinical features, immunophenotyping (IP), and molecular genetics for making the diagnosis of AL. Morphology can give clue to the specific diagnosis of acute myeloid leukemia (AML) with t(8:21), t(15:17), t(16:16), or inv(16) and this diagnosis can be made irrespective of blasts count if such translocations are demonstrated by molecular tests. There are some interesting findings such as blasts with "hand-mirror" morphology, nuclear cleavage, prominent cytoplasmic vacuoles, pseudo-Chediak-Higashi granules, cup-like nucleus, and other dysplastic features helping in differentiating lymphoid and myeloid leukemias. Transient abnormal myelopoiesis in Down syndrome and hypoplastic AL can be picked up on morphological examination. Bone marrow biopsy would be greatly beneficial and complementary to aspirate smears and is required for diagnosing exact cellularity, topography of cells, dyspoiesis, myelonecrosis, gelatinous marrow transformation, myelofibrosis, and IP can be performed using immunohistochemistry. Morphological examination in AL is not only helpful for diagnosis but also useful for predicting the prognosis in posttherapy cases, AML with myelodysplasia-related changes, therapy-related myeloid neoplasms, and mixed phenotype AL. Hematogones, blastoid mantle cell lymphoma, high grade B cell lymphoid with blastoid morphology, Burkitt leukemia, prolymphocytes in prolymphocytic leukemia, hairy cell leukemia variant, plasmablasts especially in plasmablastic leukemia, or plasma cell leukemia can mimic AL and IP is useful in these situations. Hence, morphology should be considered as a kind of "gold-standard" starting point for the analysis of AL cases. Morphological examination cannot be replaced and advanced tests cannot be used as surrogate for morphology.

Keywords

- acute leukemia
- lymphoblasts
- myeloblasts
- cytochemistry
- blast equivalents
- bone marrow biopsy
- acute leukemia mimics

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History and Evolution of Morphological Classification for Acute Leukemia

The credit for the discovery of acute leukemia (AL) goes to the ancient Greeks, who recognized this disease in the 4th or 5th century BC. The term "leukemia" is derived from the Greek words "Leukos" meaning white and "Haima" meaning blood was first proposed by Rudolf Virchow in 1846.[1]

The earliest report was by the famous French physician Alfred Velpeau (1795–1867).[2] Later on, for the first time, pathologist at the Royal Infirmary Edinburgh, John Hughes Bennett, recognized AL as a distinct blood-related disease and published his 35 cases of suspected AL in 1852.[1,2] Few years later, Henry Fuller, a physician at St George's Hospital in London described the first case of childhood AL and the first reported use of the microscope to diagnose AL.[3] Virchow (1856) was credited with concluding that the disorder was not the result of an infectious process but rather was caused by the tissue that produced the white blood cells.[1–3]

Neumann stated in 1872 that AL was a disease of the bone marrow (BM). Mosler introduced BM puncture as a means of antemortem diagnosis of AL. In 1900, the Swiss hematologist Naegeli described the presence of myeloblasts or lymphoblasts in the circulating blood which formed a basis for the classic diagnosis of AL. Monocytic leukemia was first described by Reschad, and leukemic cells recognized as unusual forms of myeloblasts.[1,2]

The French-American-British (FAB) classification of hematological malignancies, which was first put forth in 1976, is mostly based on morphology with a few cytochemical stains.[4] The categorization was further improved by the changes put forth by the FAB group in 1985, including the suggestions made by a committee of the U.S. National Cancer Institute in 1990.[5–7]

In the year 2001, the diagnostic paradigm shifted based on the principles of the Revised European-American (REAL) classification of lymphoid neoplasm, the introduction of the World Health Organization (WHO) classification for hematological and lymphoid cancers (3rd edition) was made along with the incorporation of clinical features, morphology, immunophenotyping (IP), and molecular genetics for the categorization of AL.[8,9] Prior to 2001, there were two editions of the WHO Classification of Tumors: the first (1967–1981) contained a list of acceptable terminology with brief histologic descriptions, and the second (1982–2001) included histologic and immunohistochemical (IHC) features as well as one image for each histologic type.[10]

Even though with the evolving classification of AL based on molecular genetics, still morphological examination and cytochemical staining of air-dried BM and peripheral blood smears (PBS) continue to form the basis for diagnosis and classification of AL. Recently, in 2017, revised WHO 4th edition was introduced and the upcoming WHO 5th edition expected to release in 2023 has still incorporated morphology in the classification of AL along with defining genetic abnormalities.[11]

Samples Encountered in Clinical Practice for Diagnosing AL

The complete hemogram sample received in ethylenediaminetetraacetic acid (EDTA) along with PBS is often the starting point, followed by a BM aspirate (BMA) or an imprint smear, or a trephine BM biopsy (BMB). The staining with Romanowsky stains such as Leishman- or Giemsa-based stains give excellent morphological details.[12] In most of the centers in India, either one of the stains is used, but they are complementary to each other if they are used in combination. It is always recommended to make extra smears from BMA or for imprint smears for IP using immunocytochemistry.[13] The multicolor flow cytometry (MFC) is an another commonly employed investigation for IP in AL because of its quicker turn-around-time and higher sensitivity.[14]

Both heparin and EDTA samples can be utilized for IP; however, EDTA has the advantage for morphological analysis of the sample prior to IP but antigenic preservation will be diminished when the sample is stored for more than 24 hours. Heparin samples, on the other hand, create background staining on blood smears that make morphological evaluation difficult, but these samples can be stored up to 48 to 72 hours still retaining antigenicity for IP using MFC and also used for cytogenetics evaluation. It is also generally recommended to perform BMB at the same time because both these techniques are complimentary to each other not only for morphological evaluation but also for ancillary studies such as special stains and IHC.[12,15]

Role of Morphology in the Diagnosis and Classification of AL

ALs are characterized by the uncontrolled proliferation of either lymphoblasts or myeloblasts or both which are classically defined as $\geq$ 20% in either PBS or BM.[1,13] Classically, the complete hemogram and PBS in most of these cases show normocytic normochromic anemia, leukocytosis, and thrombocytopenia with the presence of $\geq$ 20% circulating blasts. "Leucoerythroblastic blood picture" is the terminology quite often used when there is a presence of blasts along with polychromatophils, nucleated red cells, and left shifted myeloid cells. It is not uncommon in the clinical practice to come across AL cases where there are $<$ 20% blasts in PBS; however, BMA would show $\geq$ 20% blasts. This is called as "subleukemic leukemia." On the other end of the spectrum, one might not be able to find the blasts in the PBS, but BMA or imprint smear would show $\geq$ 20% blasts which is called as "aleukemic leukemia."[13,16]

There are few instances where exception to this general rule applies because of the advances in the molecular genetics and WHO have defined certain cases of acute myeloid leukemia (AML) irrespective of blast count based on their molecular findings. It includes cases with translocations such as t(8:21), t(15:17) or inv(16) or t(16:16) and/or their respective transcripts demonstrated either by fluorescence in situ hybridization or molecular tests such as polymerase chain reaction.[17]

The starting point for the classification of AL is to differentiate whether the blasts are of either lymphoblasts or myeloblasts. The only morphological hallmark to identify the myeloblasts is the presence of "Auer rods," which are the condensation of needle-like crystals comprised of azurophilic granules seen in the cytoplasm. "Phi bodies" are also considered specific for the myeloblasts which are unique fusiform or spindle-shaped structure seen in the cytoplasm with demonstrable myeloperoxidase (MPO) activity.[3,13]

Blasts and its Equivalents in AL (►Fig. 1)

Lymphoblasts are typically composed of small to medium sized blasts with high nuclear-cytoplasmic ratio, moderately condensed to dispersed chromatin, inconspicuous nucleoli, and scant basophilic and agranular cytoplasm. However, the presence of fine or coarse azurophilic granules, inclusions, or vacuoles in the cytoplasm does not exclude it as lymphoblasts if the cytochemistry or IP is compatible with their diagnosis. Auer rods are never present.[18]

For therapeutic purposes, acute lymphoblastic leukemia (ALL) is the terminology used if either PB or BM demonstrates $\geq$ 25% lymphoblasts. On the other hand, if the clinical presentation is predominantly the involvement of lymph nodes with or without the involvement of extranodal sites with $<$ 25% lymphoblasts in either PB or BM, then it is called as lymphoblastic lymphoma (LBL) according to the WHO classification.[17]

Myeloblasts are characteristically large-sized blasts with high nuclear-cytoplasmic ratio, opened up nuclear chromatin, and presence of 2 to 3 prominent nucleoli. The cytoplasm is scant but shows the presence of azurophilic and secondary granules. The characteristic "Auer rods" or "Phi bodies" are seen confirming the myeloid nature of the blasts morphologically.[3,17] There are few cells which are considered as "blasts equivalents" other than lymphoblasts or myeloblasts to make AL diagnosis and are described below as follows.[13,19]

Abnormal promyelocytes often show either kidney-shaped or bilobed nucleus. The cytoplasmic granules may be so large and/or numerous that they totally obscure the nuclear-cytoplasmic margin. "Faggot cells" are bundles of

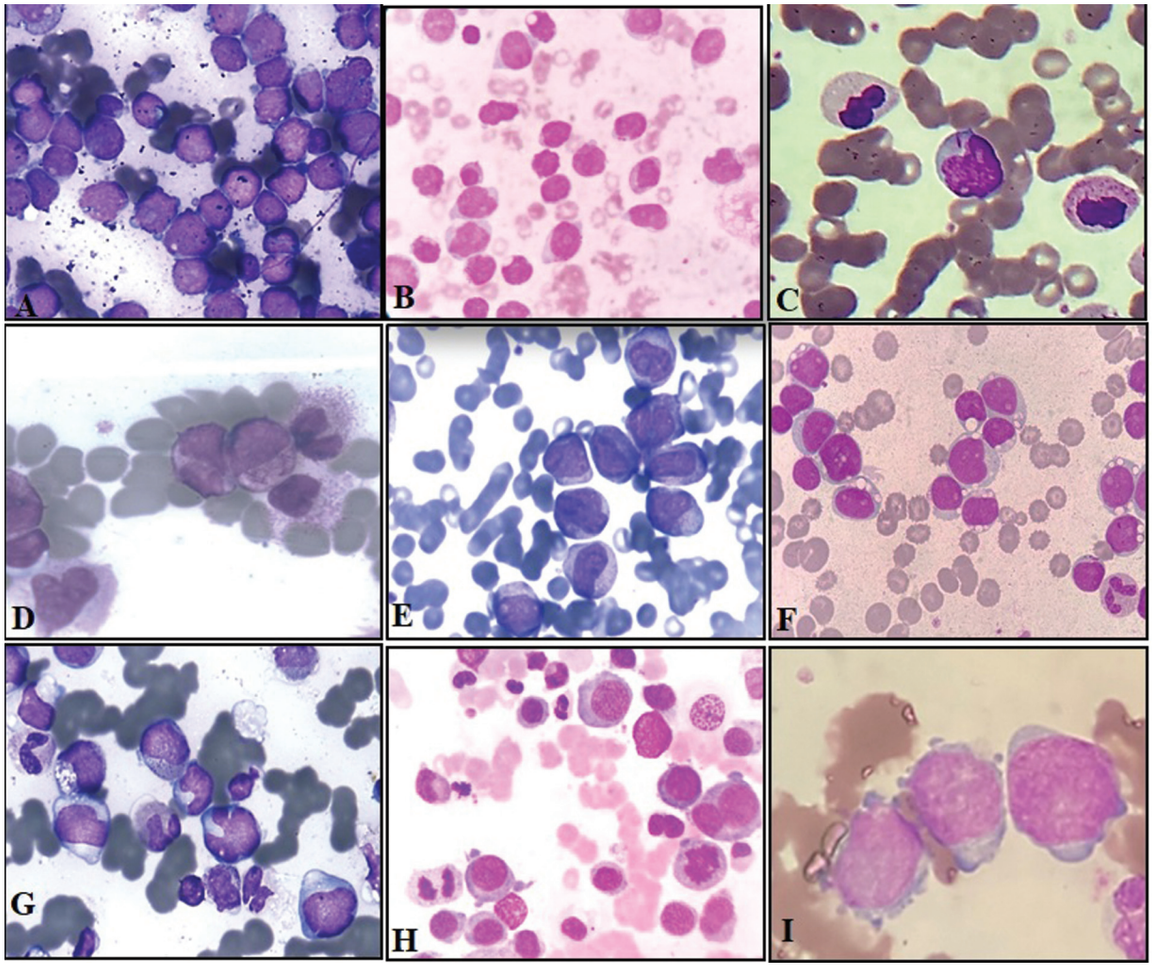

Fig. 1 Morphology of blasts and its equivalents (Giemsa and Leishman stain). (**A**, **B**) Homogenous and heterogeneous lymphoblasts, respectively. (**C**) Myeloblast with Auer rods. (**D**, **E**) Hypergranular and microgranular promyelocytes, respectively. (**F**, **G**) Monoblasts and promonocytes, respectively. (**H**) Dyspoietic erythroid cells with increased proerythroblasts. (**I**) Megakaryoblasts.

Auer rods randomly distributed within the cytoplasm are present in majority of the cases of acute promyelocytic leukemia (APL). These cells are characteristically seen in "hypergranular variant" of APL.[20,21] On the other hand, there is apparent paucity or absence of granules due to the submicroscopic size of the azurophilic granules and shows predominantly bilobed nuclei in abnormal promyelocytes. The leukocyte count is frequently markedly elevated in this variant which is called as "microgranular variant" of APL.[20–22]

Monoblasts are large cells having round nuclei with delicate lacy chromatin and one or more large prominent nucleoli with abundant cytoplasm. Scattered fine azurophilic granules, vacuoles, and hemophagocytosis may be present. Promonocytes have a more irregular and delicately convoluted nucleus, the cytoplasm is usually less basophilic and sometimes more obviously granulated with occasional large azurophilic granules and vacuoles.[19,21]

The megakaryoblasts are usually medium to large sized blasts with a round, slightly irregular, or indented nucleus with fine reticular chromatin and 1 to 3 nucleoli. The cytoplasm is basophilic, often agranular, and may show distinct blebs or pseudopod formation.[3,19,21]

Significance of Cytochemistry in the Resource-Constrained Setting (►Fig. 2)

The use of cytochemistry has greatly helped in the discrimination of ALL from the various subtypes of AML. The single best cytochemical stain which acts as an adjunct to morphological confirmation of myeloblasts in the resource-constrained setting is the MPO stain because of its enzyme specificity. This fact has been endorsed by WHO in assigning the cells as myeloblasts. Even in myelodysplastic syndrome (MDS) with hypogranular myeloid cells, MPO stain could be useful because some amount of its enzymatic activity would still be demonstrated.[13,19]

The other stain used as a surrogate for MPO is Sudan Black B (SBB) stain which is sensitive for myeloblasts help in identifying the cell membrane component. In all AL cases, one should look for at least 3% of the blasts showing positivity to be taken as significant for both MPO and SBB stains. However, it should be noted that approximately 05% of ALL cases do show the positivity for SBB stain.[23,24] Hence, MPO is preferred over the use of SBB for distinguishing these blasts.[18] This SBB positivity in T-ALL cases would point toward myeloid differentiation which helps in identifying early T cell precursor (ETP) ALL cases.[25]

The nonspecific esterase (NSE) with either α-naphthyl acetate or α-naphthyl butyrate as substrate shows diffuse esterase activity in monocytic lineage; however, dot-like positivity can be seen in some cases of T-ALL. Chloroacetate esterase (CAE) stains the maturing granulocytic cells and NSE stains the monocytic series in double esterase stain and useful in diagnosing myelomonocytic leukemias. The significance of NSE stain is strongly recommended as one of the markers for monocytic lineage in WHO classification.[13,19]

The evaluation of periodic acid-Schiff (PAS) activity is based on the pattern of the reaction such as coarse granularity or block positivity against a negative cytoplasmic background in ALL; and diffuse blush positivity is usually appreciated in APL cases.[13] The other stain that is highly correlated with T-ALL is acid-phosphatase (AP) positivity which generally shows polar positivity, however, focal AP positivity can also be found in 15% of the cases of B-ALL.[26]

Perls' stain often used for evaluation of iron stores in the BM and its significance in AL cannot be overemphasized as it helps in identifying the ring sideroblasts. It is useful in cases of pure erythroleukemia and also points toward the origin of AL from the underlying MDS.[19]

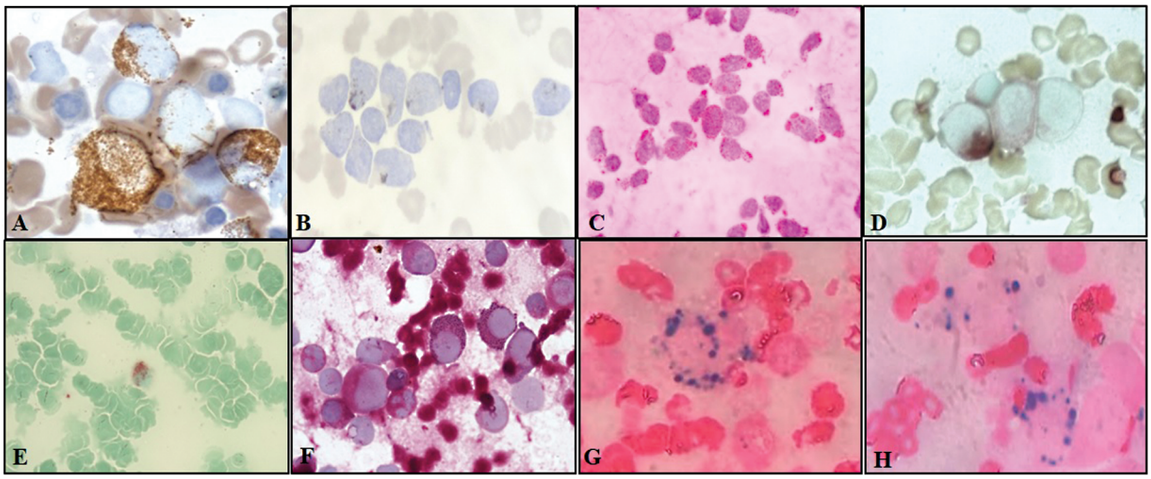

Fig. 2 Various cytochemical stains in acute leukemias. (**A**) Myeloperoxidase stain showing myeloblast with Auer rod. (**B**) Early T cell precursor acute lymphoblastic leukemia showing Sudan Black B positivity. (**C**) Lymphoblasts with typical block positivity in periodic acid-Schiff (PAS) stain. (**D**) Monoblasts showing nonspecific esterase positivity. (**E**) T-lymphoblast showing polar acid phosphatase activity. (**F**) Dyserythropoiesis with granular PAS positivity. (**G** and **H**) Perls' stain showing ring sideroblasts.

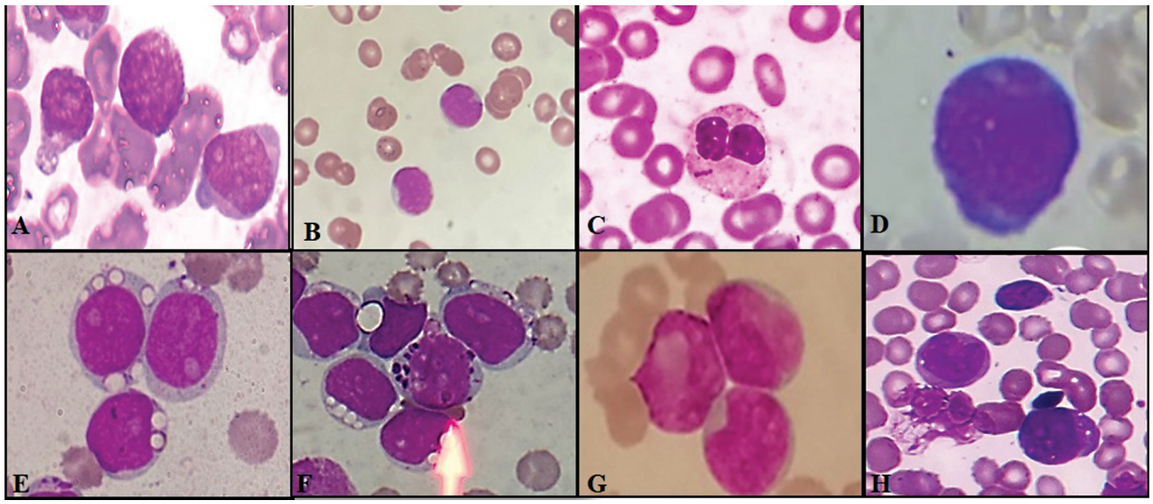

Fig. 3 Specific morphological findings in acute leukemias (Giemsa and Leishman stain). (**A**) Lymphoblast with "hand-mirror" morphology. (**B**) T-lymphoblasts with nuclear cleavage. (**C**) Pseudo-Pelger-Huet neutrophil with Auer rod. (**D**) Myeloblast with Phi body. (**E**, **F**) Monoblasts with prominent cytoplasmic vacuoles and pseudo-Chediak-Higashi granules, respectively. (**G**) Myeloblasts with cup-like nucleus. (**H**) Mixed phenotype acute leukemia showing smaller lymphoblast and larger myeloblast.

Morphological Clues Associated with Recurring Genetic Abnormalities in AL (►Fig. 3)

With the advances in the molecular techniques, the diagnosis and classification for both ALL and AML have undergone remarkable changes over the past two decades. However, WHO classification still gives importance to the morphology and integrates these findings with the clinical features, IP, and cytogenetics/molecular genetics. This indicates that morphology cannot be completely replaced by the advanced tests and these techniques cannot be used as surrogate for morphology.[3]

It is not possible to differentiate morphologically the lymphoblasts seen in B-ALL/LBL from T-ALL/LBL. However, prominent nuclear cleavage along with mediastinal involvement is quite common with T-ALL/LBL. Occasionally, the lymphoblasts may resemble the mature lymphocytes in T-ALL and only IP would be helpful in confirming this finding.[25]

The classical lymphoblasts along with no specific morphological or cytochemical findings are seen in B-ALL associated with t(9:22)(q34.1;q11.2); BCR-ABL1, t(v;11q23.3); KMT2A- rearranged, t(12:21)(p13.2;q22.1); ETV6-RUNX1, hyperdiploidy, hypodiploidy, t(1;19)(q23;p13.3); TCF3-PBX1, BCR-ABL1 like, and iAMP21. In some case of B-ALL, especially associated with t(5;14)(q31.1;q32.1), IGH/IL3 shows typical morphology of lymphoblasts along with prominent eosinophilia which is a reactive population and not a part of the leukemic clone.[27] B-ALL with KMT2A rearrangement might show both lymphoblasts and monoblasts, such cases should be better considered as mixed phenotype ALs (MPAL) with t(v;11q23.3); KMT2A-rearranged. T-ALL with a mediastinal mass are associated with immature myeloid precursors, eosinophils, and also with recurring chromosomal abnormality, t(8,13)(p11;q12). No specific morphological findings are noted in ETP-ALL and near ETP-ALL.[25]

The presence of myeloblasts having an indented nucleus, representing the Golgi zone, and often there is a long thin or fusiform Auer rod sometimes within the nuclear "hof."[21] Few blasts show very large granules suggesting an abnormal fusion called as pseudo-Chediak-Higashi granules. Neutrophils often show dysplastic features and Auer rods in their cytoplasm which are characteristically seen in AML with t(8;21)(q22;q22.1); RUNX1-RUNX1T1.[20,21]

APL with PML-RARA (promyelocytic leukemia/retinoic acid receptor alpha) often presents with severe thrombocytopenia, leucopenia with presence of abnormal hypergranular promyelocytes with Faggot cells which shows strong positivity for MPO stain and red cells show polychromatophils, nucleated red blood cells, and schistocytes suggestive of disseminated intravascular coagulation. Its morphological variant includes microgranular type which usually presents with leukocytosis with bilobed abnormal promyelocytes poses difficulty in identifying the presence of Auer rods. The submicroscopic nature of these granules is very well highlighted by MPO stain.[20]

AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11 has features which are those of acute myelomonocytic leukemia with at least 5% of cells showing dysplastic eosinophils at various stages of maturation (M4Eo variant). On the other hand, AML with t(9;11)(p21.3;q23.3); KMT2A-MLLT3 show features of either AML-M4 or M5. Some cases of AML often show dysplastic features with basophilic differentiation along with either M2 or M4 phenotype in AML with t(6;9)(p23;q34.1); DEK-NUP214.[19–21]

There are certain cases of AML which presents with normal or even an increased platelet count with dysplastic megakaryocytes often giving morphological clue toward AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2);

Table 1 Acute myeloid leukemia with specific gene mutations

S. no.	Gene mutations	Morphology	Other findings
1	Acute myeloid leukemia with mutated NPM1	Blast can resemble M1, M2, M4, or M5, but the distinctive morphological feature is the presence of cup-shaped nuclei[28,29]	CD34 and HLA-DR are negative and shows a good response to induction therapy[30]
2	Acute myeloid leukemia with CEBPA mutation	No distinguishing morphology and requires at least 20% blasts, often associated with erythroid dysplasia	CD34, CD117, and HLA-DR are positive; strong cMPO and asynchronous expression of CD15 and CD65; additionally, CD7 and CD56 are often expressed[31]

GATA2-MECOM. The other entity typically occurs in infants and shows basophilic blast cells with cytoplasmic blebs giving strong clue for AML (megakaryoblastic) with t(1;22)(p13.3;q13.1); RBM15-MKL1.[20,21] AML with cup-like nuclei is strongly associated with mutations involving NPM1 and FLT3[28–30]; and CEBPA-associated AML also show unique phenotypic profile.[31]AML with specific gene mutations are described in ►**Table 1**.[20,21]

Other Morphological Features in AL (►Fig. 3)

"Hand-mirror" morphology, is nothing but cytoplasmic pseudopods are seen in some cases of ALL but it has also been described in rare instances of AML.[18] FAB classification used in ALL comprised of L1 (homogenous), L2 (heterogeneous), and L3 blasts has now been completely obsolete. Sometimes, the cytoplasmic vacuolations are more commonly noted in the lymphoblasts because of artifactual reasons and it should not be confused with Burkitt lymphoma cases previously described as L3 blasts in FAB classification.[13,32] The crushing artifact is more commonly noticed in ALL cases in BMB. Rarely, spontaneous tumor lysis could be picked up from the morphology that most of the blasts show fragmentation of their nuclei resembling "apoptotic bodies" either in PB or BM.[13,16]

One of the defining features of AML with MDS-related changes (ARC) includes the distinct morphological dysplasia in at least $\geq$ 50% of the cells in minimum of two lineages. The dysplastic features in erythroid lineage include megaloblastosis, nuclear irregularity, karyorrhexis, fragmentation, or multinuclearity. The cytochemical stain such as Perls' stain would demonstrate the ring sideroblasts; vacuoles and positivity are noted in PAS stain.[33] Dyspoiesis in the myeloid lineage are appreciated by hypogranularity, Auer rods, and hyposegmented nuclei in the neutrophils called as pseudo-Pelger-Huet anomaly. Monolobated or hypolobated nucleus or multinucleation in large megakaryocytes indicates the dysmegakaryopoiesis.[21,33]

Therapy-related myeloid neoplasms (t-AML/MDS) show AML with findings of myelodysplasia in the morphology and only the history will differentiate from the above entity. This includes therapy-related AML, therapy-related MDS, or therapy-related MDS/myeloproliferative neoplasms (MPNs).[21,34]

The maturing cells of the granulocytic lineage constitute $<$ 10% of the nucleated BM cells comprising of cells ranging from promyelocytes to mature neutrophils and usually 80 to 90% blasts are seen showing azurophilic granules and/or Auer rods. MPO positivity in $\geq$ 03% of blasts would differentiate AML without maturation (AML-M1) from minimal differentiation (AML-M0).[19] If there are $\geq$ 10% of maturing cells of granulocytic lineage and $>$ 20% blasts; with monocytic lineage constitute $<$ 20% in the BM along with frequent presence of Auer rods are classically seen in AML with maturation (AML-M2).[19]

In acute myelomonocytic leukemia (AML-M4), neutrophils and their precursors and monocytes and their precursors each should constitute $\geq$ 20% of BM cells. Myeloblast shows MPO, SBB, and CAE positivity. Monoblasts, promonocytes, and monocytes are positive for NSE. Double esterase may reveal dual positive cells. In AML-M5a, $\geq$ 80% of the monocytic cells are monoblasts, whereas in M5b, most of the monocytic cells are either promonocytes or monocytes. Monocytes and its precursors are NSE positive and MPO negative.[13,19]

If $>$ 80% of the BM cells are erythroid, with $>$ 30% of them are proerythroblasts showing block-like positivity in PAS and NSE; MPO and SBB are negative in pure erythroleukemia (AML-M6). On the other hand, AL with $\geq$ 20% blasts, of which $>$ 50% should be megakaryoblasts showing punctate and focal positivity in PAS and NSE; MPO and SBB are classically negative in AML-M7. Acute basophilic leukemia is rare and characterized by $>$ 20% blasts having moderately basophilic cytoplasm containing coarse basophilic granules and vacuolation showing positivity for toluidine blue, AP (diffuse), and PAS (large blocks). Mature basophils are usually sparse. CAE negativity helps in distinguishing these blasts from mast cells leukemia.[35]

Morphologically at times, it is possible to come across two different types of blasts with two lineage-specific markers which was previously described as "bilineage" MPAL.[21] If they express antigens of more than one lineage in the same blasts, it was called as "biphenotypic" MPAL and acute undifferentiated leukemias, which exhibit no lineage-specific antigens. The characteristic IP profile for each of the lineage are described in ►**Table 2**.[36,37]

Morphology in Predicting the Progression to AL from Other underlying Diseases

Transient abnormal myelopoiesis is classically encountered in 10% of newborn with Down syndrome where there is a

Table 2 Immunophenotyping using immunohistochemistry or flow cytometry

Lineage cells	Immunophenotype marker(s)
All leucocytes	CD45
Immaturity markers	HLA-DR, CD34, nTdT, CD117, CD99
B cells	CD19, cCD79a, CD10, CD20, cCD22
T cells	cCD3, sCD3, CD4, CD8, CD7, CD5, CD2, CD1a
NK cells	CD16, CD56
Erythroid cells	E-cadherin, CD36, CD71
Myeloid cells	MPO, CD13, CD33, CD117
Monocytes	CD64, CD14, CD11c
Megakaryocytes	CD61, CD41
Plasma cells	CD38, CD138, kappa, and lambda

Abbreviation: NK, natural killer.

brief period of appearance of blasts mostly of megakaryocytic lineage at the age of 3 to 7 days and are completely asymptomatic clinically. Often it undergoes spontaneous remission within first 3 months of life and 20 to 30% of these cases show progression after 1 to 3 years of life.[21,38]

ARC and t-AML/MDS are already described. Myeloid sarcoma is described as extramedullary proliferation of blasts mostly of myeloid or monocytic lineage associated with tissue destruction. It may show progression or might coexist with AML.[21,39] Similarly, the underlying MPN or MPN/MDS overlap category might show progression to blast crisis which are mostly of myeloid lineage and 10 to 20% might show lymphoid lineage.[40] Inherited BM failure such as Fanconi anemia and acquired aplastic anemia with or without paroxysmal nocturnal hemoglobinuria show progression to AL in 5 to 10% of cases.[41]

Role of BMB in AL (►Fig. 4)

It is generally accepted fact that BMB is complimentary to BMA/imprint smear; however, BMB is better in accessing the overall cellularity, focal marrow involvement, stromal changes, and useful adjunct for IHC and molecular studies.[15]

Hypoplastic AL/MDS generally shows hypocellularity in BMB and generally associated with suppressed trilineage hematopoiesis. It is characterized by interstitial increase in immature cells and BMA might be hemodiluted. The use of immaturity and lineage-specific markers would be helpful in arriving at the final diagnosis.[17]

The stromal changes such as myelonecrosis may be extensive, and in such cases IHC would be useful as the antigens are better preserved even in the necrotic cells.[42,43] Other changes include gelatinous transformation of marrow and myelofibrosis can be seen in AL in 1 to 2% of cases.[44] Another rare condition called as acute panmyelosis with myelofibrosis in which PB shows pancytopenia with no or minimal anisopoikilocytosis, variable macrocytosis, and no teardrop

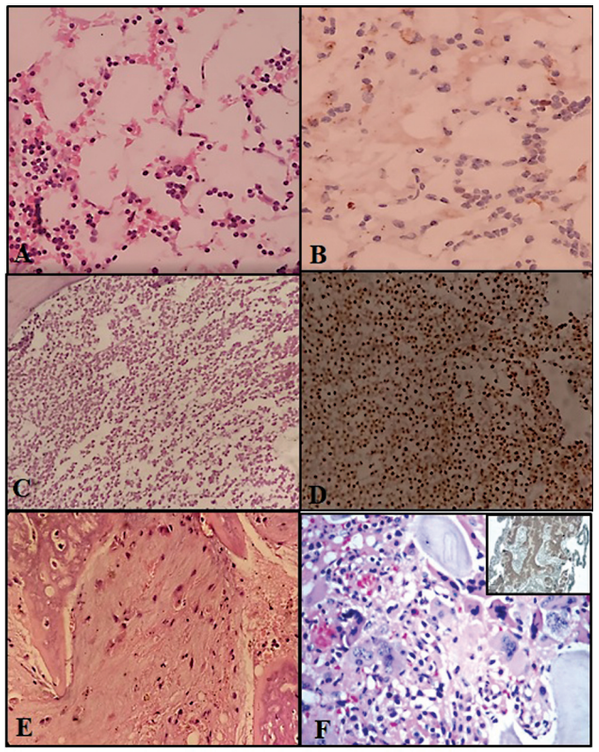

Fig. 4 Bone marrow biopsy in acute leukemias (hematoxylin and eosin [H&E] stain). (**A**, **B**) Hypoplastic B-acute lymphoblastic leukemia (B-ALL) with CD34 positive immunohistochemistry (IHC), respectively. (**C**, **D**) Myelonecrosis in B-ALL with TdT positive IHC. (**E**) Gelatinous marrow transformation. (**F**) Primary myelofibrosis with inset showing increased reticulin fibrosis with progression to acute leukemia.

cells. BMB is hypercellular and shows diffuse fibrosis in reticulin and Masson trichrome stain along with the proliferation of all lineage elements (panmyelosis) and foci of increased blasts with dysplastic megakaryocytes are the characteristic findings. Immunohistochemistry with all the three lineage markers would be very helpful in diagnosing this condition.[19]

Morphology Helps in Predicting the Prognosis in AL

Generally good prognosis is expected if the morphological clues to the underlying genetic abnormalities are present such as t(8:21), t(15:17), and t(16:16) in AML with eosinophilia.[21] Myeloid sarcoma, ARC, t-AML/MDS, and MPAL are associated with poor prognosis.[19]

Morphological Mimics of AL (►Fig. 5)

Hematogones, which are normal B cell progenitors, can resemble lymphoblasts but typically have even greater nuclear-to-cytoplasmic ratio, more uniform chromatin, and discrete nucleoli. Blastoid variants of lymphoma like blastoid mantle cell lymphoma, high grade B cell lymphoma with blastoid morphology, Burkitt leukemia, prolymphocytes in prolymphocytic leukemia, and hairy cell leukemia variant

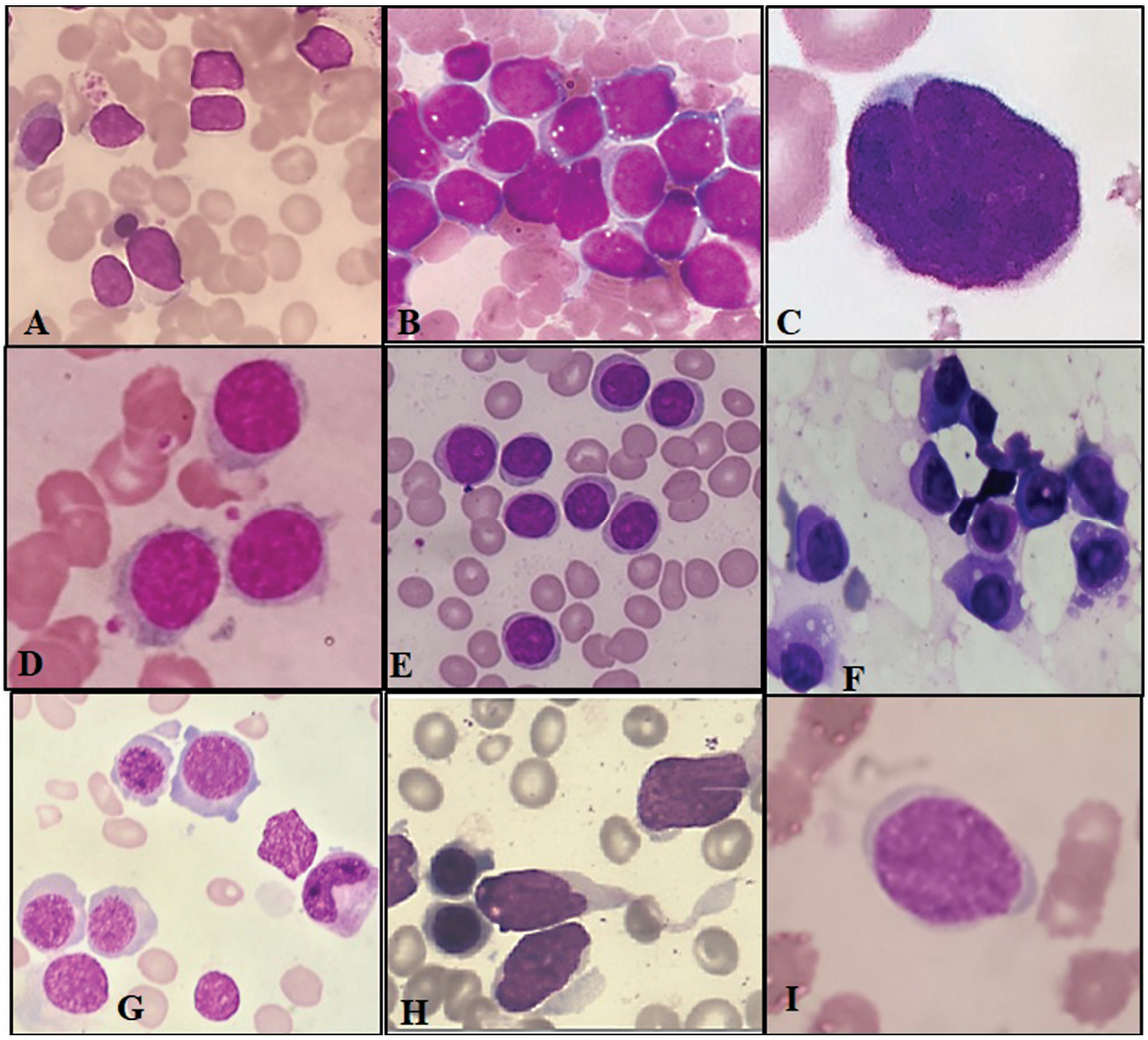

Fig. 5 Morphological mimics of acute leukemia (Giemsa and Leishman stain). (**A**) Hematogones, (**B**) Burkitt leukemia, (**C**) blastoid mantle cell lymphoma, (**D**) hairy cell leukemia variant, (**E**) T cell prolymphocytic leukemia, (**F**) plasmablasts, (**G**) megaloblastosis, (**H**) blastic plasmacytoid dendritic cell neoplasm, and (**I**) carcinocythemia from metastatic neuroblastoma.

can mimic AL and IP is useful in these situations. The plasmablasts especially in plasmablastic leukemia/plasma cell leukemia also mimic AL.[17]

MDS with excess blasts (EB2) and MDS/MPN overlap category such as chronic myelomonocytic leukemia in elderly and juvenile myelomonocytic leukemia in pediatric population are the most confusing mimicker of AML-M4/M5 and distinction must be made based on accurate blast and promonocyte counts.[44] Megaloblasts with open sieve-like nuclear chromatin could be confused with AML-M6; blastic plasmacytoid dendritic cell and circulating tumor cells called as "carcinocythemia" occurs in neuroblastoma and other adenocarcinoma would be easily confused with undifferentiated leukemia.[17] The advanced molecular research in the future will generate huge data that cannot be given by morphology; however, it should serve as complementary to each other.

Conclusion

The morphology as such can give clues for specific genetic abnormalities and mutations in AL even with the gaining importance to IP and molecular genetics. This endorsement by WHO classification gives boost to the morphologist/hematopathologist and should be considered as a kind of "gold-standard" starting point for the analysis of AL cases. Morphological examination cannot be replaced and advanced tests cannot be used as surrogate for morphology.

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None declared.

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Flow Cytometry in the Diagnostic Laboratory Workup of Acute Lymphoblastic Leukemias

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Abstract

Acute lymphoblastic leukemias (ALLs) are hematological neoplasms characterized by clonal proliferation of lymphoid blasts, which can be B- or T-cell type. Flow cytometric immunophenotyping is an integral component in establishing blast lineage during the diagnostic workup of ALLs, aiding in appropriate therapy, prognostication, and monitoring of the disease. The current review focuses on the utility of flow cytometry in the workup of ALLs, including the usefulness of various antibodies and pitfalls in diagnosis.

Keywords

- acute lymphoblastic leukemia
- flow cytometry
- B-ALL
- T-ALL

Introduction

Multiparametric flow cytometry is an indispensable tool for the diagnosis and subclassification of acute lymphoblastic leukemia (ALL). Accurate classification of ALLs into B- or T-cell types is crucial for the optimal choice of therapeutic regimens that varies based on the ALL subtype. The antigenic expression profile, particularly the immunophenotypic aberrancies by the blasts deviating from those encountered during normal hematopoiesis, aids in the differentiation of the blasts from their normal benign counterparts. The panel of antigens for clinical testing has evolved from 4 to 13 colors or more thanks to the substantial development in antibody clones, the fluorochrome conjugate options, and a wide variety of lasers that have dramatically increased the number of antigens that can be simultaneously studied. In this article, we attempt to discuss the strategy and approach to the classification of ALL into B- or T-cell subtypes and the evolution of consensus groups for antigen/antibody/fluorochrome selection, choice of reagents, sample processing methodology for appropriate diagnosis, and classification.

B-Acute Lymphoblastic Leukemias

B-lymphoblastic leukemia/lymphoma or B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is the most common malignancy seen in childhood. Approximately 75% of BCP-ALL cases occur in children under 6 years of age.[1] However, it shows a bimodal age distribution, with a small peak occurring during the fifth decade of life.[2] BCP-ALL is diagnosed by morphology combined with immunophenotyping, typically done by multiparametric flow cytometer (MFC). Immunophenotyping is essential for differentiating BCP-ALL from acute leukemias of other lineages, like T-lymphoblastic leukemia (T-ALL), acute myeloid leukemia (AML), mixed phenotype acute leukemia (MPAL), etc.

The flow cytometric diagnosis of BCP-ALL is based on identifying an expanded population of immature B cells showing immunophenotypic aberrancies that help distinguish the leukemic blasts from normal B-cell precursors, or hematogones.[3] These aberrancies are in the form of increased or decreased intensity of expression of an antigen on the leukemic blasts compared to the normal counterparts or gain of antigen expression, which are not specific to B-lineage.[4] Knowledge of the spectrum of antigenic expression on the B-progenitor cells during development is essential to differentiate them from B-leukemic blasts.[5] The normal B-progenitors show the expression of certain antigens in a sequential, tightly regulated manner.[3] The B-progenitor cells are derived from common lymphoid progenitor cells in the bone marrow, and they undergo three stages of maturation to become mature B-lymphoid cells. These three stages of

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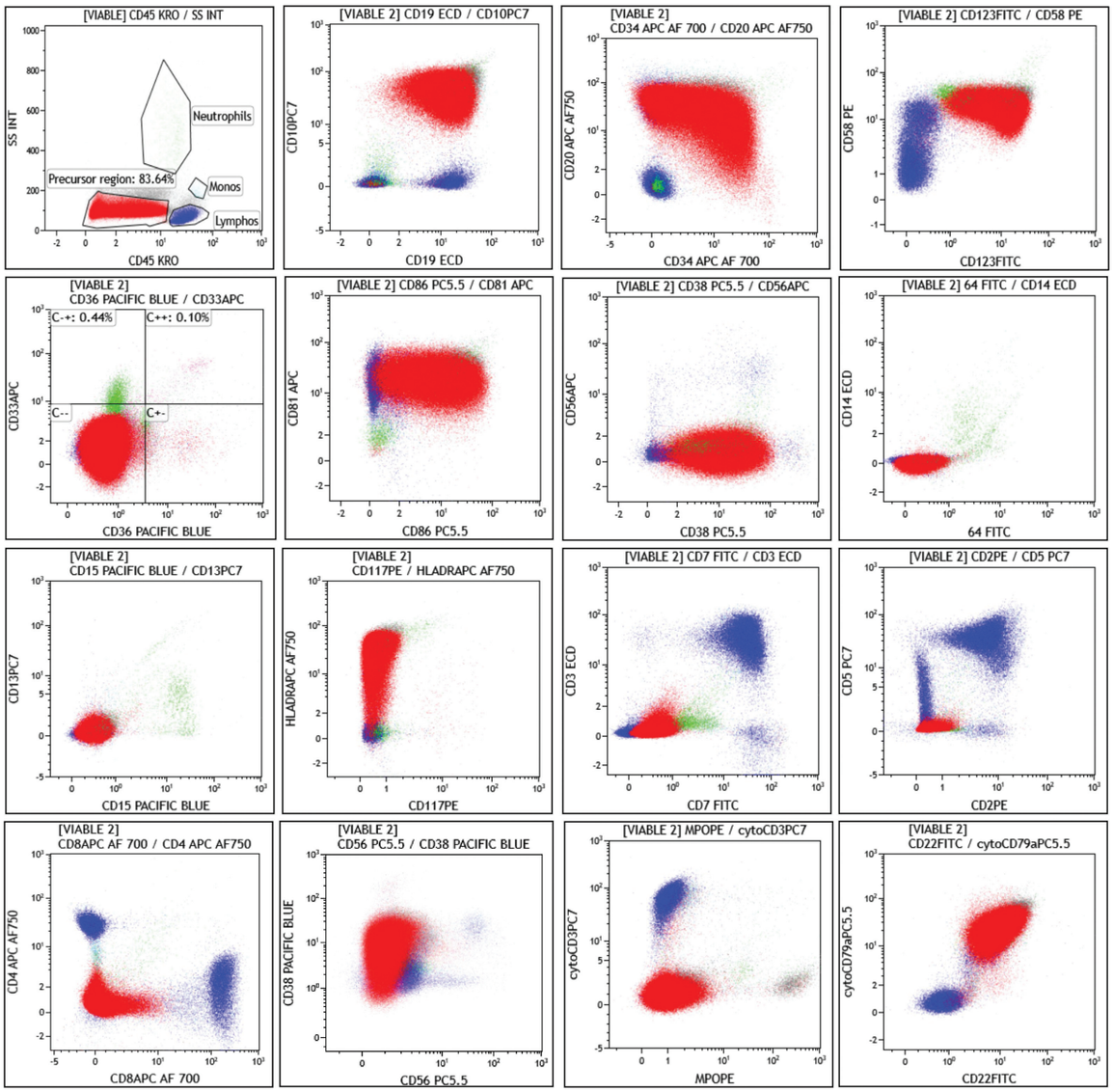

Fig. 1 Flow cytometric dot plots of a case of BCP-ALL. The blasts (*red population*) are SSC low, CD45 dim to negative, positive for CD19, CD10, CD34, CD20, CD58, CD123, CD81, CD86, CD38, HLA-DR, CD22, and CytoCD79a. T-cell markers like CD3, CD7, CD5, CD2, CD4, CD8 and NK cell marker like CD56, and, myeloid markers like CD13, CD15, CD33, CD26, CD117, CD14, CD64 and MPO are negative.

cells are called hematogones type I (early/pre-B-I), hematogones type II (intermediate/pre-B-II), and hematogones type III (late/transitional B-cells).[6] The three stages of hematogones show a stepwise increase in the intensity of expression of CD45 and CD20 while showing a stepwise decrease in intensity of CD34 and CD10, which are finally lost at the late stage. CD19 expression is the lowest in stage I, and increases significantly in stage II, with an eventual mild reduction in mature B cells. Mature B cells show loss of CD34, CD10, and TdT, bright expression of CD20 and CD45, and a surface expression of polytypic immunoglobulins.[5] ►**Fig. 1** shows flow cytometric dot plots of a BCP-ALL patient.

The B-leukemic blasts show maturation arrest at any stage and deviation from the normal sequential immunophenotypic maturation kinetics seen in hematogones.[6–8] Other than this, over- or underexpression of markers like CD123, CD58, CD81, CD86, CD73, CD200, CD9, and CD304 can be seen in the B-leukemic blasts, as shown in ►**Table 1**. Additionally, they may express aberrant lineage markers like CD13, CD33, CD66c, CD15, CD56, and CD7 that aid in differentiating the B-leukemic blasts from hematogones.[4,6,7,9–17]

In 1995, the European Group for the Immunological Characterization of Leukemia (EGIL) proposed guidelines for diagnosing and subclassifying BCP-ALL based on immunophenotyping by flow cytometry.[18] According to the EGIL guidelines, B-lineage markers (CD19, CD20, CD22, and CD79a) and immaturity markers (CD34 and TdT) are used to diagnose BCP-ALL. Expression of CD10 and cytoplasmic and surface immunoglobulin M (IgM) are used to subclassify BCP-ALL into four subgroups based on their level of maturation (►**Table 2**).

Table 1 Aberrant antigen expression in BCP-ALL compared to hematogones[a]

Antigen	Aberrancy
TdT	Negative/uniform expression
CD34	Negative/uniform expression
CD45	Negative/uniform expression
CD10	Negative/overexpression (uniform bright)
CD20	Uniform expression or negative
CD22	Negative/under expression
CD38	Under expression
CD19	Under/overexpression
CD73	Overexpression
CD58	Overexpression
CD86	Overexpression
CD123	Overexpression
CD200	Overexpression
CD81	Under expression
CD304	Aberrant expression
CD9	Overexpression
CD44	Overexpression
CD13, CD33, CD66c, CD15, CD7, and CD56	Lineage aberrant markers

Abbreviations: BCP-ALL, B-cell precursor acute lymphoblastic leukemia; TdT, terminal deoxynucleotidyl transferase.
[a]Please note that all cases of BCP-ALL may not have these aberrancies, and in many cases individual antigens may have expression profile similar to hematogones.

In 2001, the World Health Organization (WHO) introduced a drastic shift in the classification of acute leukemia, including associated genetic abnormalities in the workup for subclassification of BCP-ALL. With an improved understanding of cytogenetic and molecular abnormalities associated with BCP-ALL, and due to their prognostic and therapeutic implications, a complete subtyping and risk stratification of BCP-ALL now require a plethora of molecular tests.

However, since certain specific genetic abnormalities are associated with the expression of particular antigens on the leukemic cells, MFC-based immunophenotyping may provide clues about the genotype[12] and thus help decide which molecular tests to do first.

In 2012, the EuroFlow Consortium put forward guidelines for optimal eight-color antibody panels to diagnose various hematological neoplasms, including BCP-ALL.[8] They initially suggested using an acute leukemia orientation tube (ALOT) for lineage identification (►**Table 3**). After confirmation of B-lineage by ALOT tube, further markers for additional characterization are studied in four eight-color tubes. All four tubes contain three backbone markers (CD45, CD19, and CD34) to identify and gate the B-leukemic blasts. The rest of the five antibodies are used to distinguish hematogones from B-leukemic blasts to subclassify the blasts based on their level of maturation, and to identify leukemia-associated immunophenotype (LAIP), which is especially useful in minimal residual disease (MRD) assessment during follow-up. The four-tube eight-color panel recommended by the EuroFlow Consortium for the characterization of B-ALL is shown in ►**Table 3**. With technological advancements in flow cytometry, 10-colour (and higher) panels are now commonly used for diagnostic purposes. The obvious advantage is that fewer tubes (e.g., single-tube assays) can now be utilized by incorporating a sufficient number of LAIP markers.

Specific markers have been found to be associated with the BCP-ALL genotype in various studies (highlighted in ►**Table 4**). The expression of CD66c is positively associated with *BCR-ABL1* rearrangement and a few cases of hyperdiploidy. It is rarely seen associated with *KMT2A* and *PBX1* rearrangements.[12,19,20] CD13 and CD33 (myeloid markers) are found to be aberrantly expressed in *BCR-ABL1* translocated BCP-ALL.[21] The NG2 expression on the surface of B-leukemic blasts is strongly associated with *KMT2A* rearrangement.[22,23] Also, *KMT2A* rearrangement shows loss of CD10 and aberrant expression of CD15.[12,24]

CD9 is found to be positively associated with the *TCF3::PBX1* rearrangement. The *TCF3::PBX1* rearranged BCP-ALL also shows a pre-B phenotype with complete loss of CD34, and is positive for cytoplasmic IgM.[23,25] CD123 is found to be aberrantly overexpressed in BCP-ALL with hyperdiploid karyotype. At the same time, its expression is suppressed in association with the *ETV6::RUNX1* rearrangement.[12,26] Overexpression of the CRLF2 (also known as TSLPR) antigen on the surface of B-leukemic blasts is associated with Ph-like B-ALL, and it is found to be associated with *IKZF1* deletion.[27,28]

Another increasingly important role of flow cytometry in the diagnostic workup of BCP-ALL is the analysis of the ploidy

Table 2 EGIL immunophenotypic subclassification of B-ALL

Subclassification	CD10	Cytoplasmic IgM	Surface IgM
B I (pro-B) ALL	Negative	Negative	Negative
B II (pre-pre-B or common B) ALL	Positive	Negative	Negative
B III (pre-B) ALL	Positive	Positive	Negative
B IV (mature B) ALL	Negative/positive	Negative/positive	Positive

Abbreviation: ALL, acute lymphoblastic leukemia; EGIL, European Group for the Immunological Characterization of Leukemia; IgM, immunoglobulin M.

Table 3 EuroFlow consortium recommended antibody panel for B-ALL

Tubes	Fluorochromes							
	PacB	AmCyan	FITC	PE	PerCPCy5.5	PECy7	APC	AF700
ALOT Tube	CyCD3	CD45	CyMPO	CyCD79a	CD34	CD19	CD7	SmCD3
BCP-ALL Tube 1	CD20	CD45	CD58	CD66c	CD34	CD19	CD10	CD38
BCP-ALL Tube 2	SmIgk	CD45	CyIgμ	CD33	CD34	CD19	SmIgM and CD117	SmIgλ
BCP-ALL Tube 3	CD9	CD45	NuTdT	CD13	CD34	CD19	CD22	CD24
BCP-ALL Tube 4	CD21	CD45	CD15 and CD65	NG2	CD34	CD19	CD12 3	CD81

Abbreviations: A-LOT, acute leukemia orientation tube; BCP-ALL, B cell precursor acute lymphoblastic leukemia.

Table 4 Genotypic associations of a few BCP-ALL antigens detected by MFC

Marker	Genotype association
CD66c	Positively associated with *BCR-ABL1* translocation Rarely seen in *KMT2A* and *PBX1* rearrangement
CD13 and CD33	Positively associated with *BCR-ABL1* translocation
CD15, NG2, and loss of CD10	Strongly associated with *KMT2A* rearrangement
CD9	Positively associated with *TCF3::PBX1* rearrangement
CD123	Overexpressed in BCP-ALL with hyperdiploidy Underexpression associated with *ETV6::RUNX1*
CRLF2	Positively associated with Ph-like BCP-ALL

Abbreviations: BCP-ALL, B-cell precursor acute lymphoblastic leukemia; MFC, multiparametric flow cytometer.

status of the blasts. It is well established that BCP-ALL cases with high hyperdiploidy have better outcomes, while low-hypodiploid and near-haploid patients have poorer outcomes. Although conventional cytogenetics (CC) is the gold standard for ploidy analysis, many studies have shown that flow cytometry–based DNA ploidy analysis correlates well with CC. Various nucleic acid binding dyes (propidium iodide, DAPI, acridine orange, etc.) have been used for this purpose. Recent studies have found that FxCycle Violet is an excellent DNA selective dye excited by the violet laser, which allows simultaneous six- to seven-color immunophenotyping (using blue and red lasers) to separate the blasts from the background normal population and thus accurately assess the ploidy status of the abnormal B-lineage blasts in BCP-ALL.[29,30]

In recent years, immunotherapy has emerged as another important tool in the treatment of BCP-ALL, and it is often guided by flow cytometric immunophenotyping. A study from MD Anderson Cancer Center showed higher 3-year overall survival with the addition of rituximab (monoclonal antibody [moAb] against CD20) in patients younger than 60 years.[31] Another study showed that the addition of rituximab led to a longer event-free survival and lower frequency of relapses in adult Ph-negative BCP-ALL cases.[32] Both these studies used rituximab in patients whose blasts showed at least 20% CD20 expression. Ofatumumab, another anti-CD20 moAb, has been used with good results in patients in whom CD20 expression was as low as 1%.[33]

CD19 is expressed ubiquitously in BCP-ALL. Immunotherapy targeting CD19, in the form of bispecific T-cell engager (BiTE) called band anti-CD19 chimeric antigen receptor T cells (CARTs), has emerged as a promising agent in relapsed refractory BCP-ALL. CD19-negative blasts have been described in relapses following treatment with Blinatumomab and CART, and pose a challenge to their detection using MFC. CD22 is also expressed in a vast majority of BCP-ALL, and Inotuzumab ozogamicin (directed against CD22) has also shown good results in refractory BCP-ALL.[34]

T-Acute Lymphoblastic Leukemia

T-acute lymphoblastic leukemias (T-ALLs) account for approximately 15% of the pediatric ALLs and 25% of the adult ALL groups.[35] T-ALLs are defined by surface or cytoplasmic CD3 (CyCD3) expression. The markers of the T-cell lineage are CD1a, CD2, CD3, CD4, CD5, CD7, and CD8. CD7 expression is usually bright and uniform in patients with T-ALL. CD7 is highly sensitive for T-ALL; however, it is nonspecific as it is also aberrantly frequently expressed in blasts of AML.[36] The expression of other markers like CD1a, CD2, CD3 (surface

Table 5 EGIL classification of T-ALL

Markers	Pro-T-ALL	Pre-T-ALL	Cortical T-ALL	Medullary T-ALL
CD1	–	–	++	–
CD2	+	++	++	++
SmCD3	–	–	– (except in SmCD3+ subtypes, ++)	++
CyCD3	++	++	++	++
$CD4^-/CD8^-$	++	+	–	–
$CD4^-/CD8^+$	–	±	±	±
$CD4^+/CD8^-$	–	±	±	+
$CD4^+/CD8^+$	–	–	+	±
CD5	–	++	++	++
CD7	++	++	++	++

Abbreviations: EGIL, European Group for the Immunological Characterization of Leukemia; T-ALL, T acute lymphoblastic leukemia.
Note: –, positive in <10% of the T-ALLs; ±, positive in 10–25% of the T-ALLs; +, 25–75% of the T-ALLs are positive; ++, positive in >75% of the T-ALLs.

membrane [Sm]), CD4, CD5, and CD8 is variable depending on the maturational stage of the blasts.[37] Markers like CD2, CD5, and CD7 correspond to most immature T cells; however, they lack lineage specificity. CyCD3 is a marker constantly expressed by T-ALLs and is lineage specific with no cross-reactivity, appearing at the early stages of maturation. A combination of CyCD3 and SmCD3 is helpful as it identifies a $CyCD3^+/SmCD3^{-/dim}$ population, most frequently observed in patients of T-ALL.[8] Using anti-CD3 epsilon chain antibody is important and as per available literature, the CD3 clone S4.1 gives increased autofluorescence, whereas UCHT-1-PE gives the highest specific fluorescence intensity for intracellular staining.[38,39]

Maturation abnormalities observed in T-ALLs serve to differentiate abnormal T-lymphoblasts from normal thymic cells. According to the degree of thymic differentiation, the EGIL classified T-ALLs into four major subgroups.[18] These subgroups included pro-T-ALL (T-1), pre-T-ALL (T-2), cortical T-ALL (T-3), and medullary T-ALL (T-4; ►**Table 5**). Immature T-ALLs, $CD1^-/CD7^+$, constitute the pro-T-ALLs, and those expressing CD2 and CD5 represent the pre-T-ALL subtype. Cases positive for CD1 and frequently dual positive for CD4/CD8 form the cortical type, and the ones with $SmCD3^+/CD1^-$ form the mature T-ALL subgroup. Flow cytometry data from a patient with T-ALL are shown in ►**Fig. 2**. Further, based on the expression of SmCD3 and the type of T-cell receptor, T-ALLs can be classified as $SmCD3^+/TCR\text{-}ab^+$ and $SmCD3^+/TCR\text{-}gd^+$ subgroups.

CD45, the pan-leukocyte gating marker, is often expressed brighter in T-ALLs than B-ALLs, and the expression level might significantly overlap with mature lymphocytes.[37] Other markers indicating an immature phenotype include CD34 and terminal deoxynucleotidyl transferase (TdT), which are positive in a proportion of T-ALL patients. CD10 (common acute lymphoblastic leukemia antigen [CALLA]) is positive in some patients (10% of all T-ALLs), but it seldom carries any diagnostic significance in classifying T-ALLs. CyCD79a, a marker associated with B-cell lineage, can be expressed at low levels in a fraction of T-ALLs, particularly the $TCRgd^+$ subgroup.[40] Expression of myeloid-associated markers such as CD13, CD33, and CD117 is found in some cases of T-ALL, especially in the recently described category of early T-cell precursor T-ALL (ETPALL).

ETPALL is a recently described category of immature T-ALLs (~15% of the cases) with low response rates to chemotherapy and dismal prognosis.[41] It is characterized by the absence of CD1a and CD8. CD5 is found weakly expressed (negative or dim, in <75% of the blast population) along with the expression of myeloid/stem cell–associated antigens (in at least >25% of the blast population) such as CD34, HLA-DR, CD13, CD33, CD11b, CD65, and CD117. Scoring systems incorporating a 6- or an 11-marker panel are used to identify patients of ETPALL. The expression of CD5 in patients with ETPALL is heterogeneous, and cases exhibiting a strong positivity for CD5 were further classified as near-ETPALL.[42]

The EuroFlow Consortium, in 2012,[8] designed an antibody panel for the diagnosis and immunophenotypic characterization of T-ALLs. This antibody panel was run when the ALOT tube suggested T-lineage blast proliferation. The panel was set with a combination of markers aimed at the identification of the blasts (cyCD3, CD45, and smCD3), markers for the differential diagnosis of T-ALL, and to specify the maturational stage of the blasts for subclassification of T-ALL with markers such as CD1a, CD2, CD3, CD4, CD5, CD7, CD8, CD10, TCR-ab, and TCR-gd. CD1a, CD34, CD99, and nuTdT were included to indicate the precursor nature of the blasts. Additional markers, including CD44, CD45RA, and CD123, were also used, which contribute to maturational staging.[8,43–45] Myeloid antigens such as CD13, CD33, and CD117 were added to the panel to identify cases with early precursor T-cell immunophenotype. ►**Table 6** shows the T-ALL panel designed by the EuroFlow Consortium.

T-ALL, especially the ETPALL, can be confused with MPAL (T/myeloid type). A combination of markers like CD3, CD7, and myeloperoxidase must be used for accurate lineage identification. The recent update on the classification of myeloid and histiocytic/dendritic cell neoplasm has redefined the lineage assignment criteria for the diagnosis of MPAL.[46] CD3

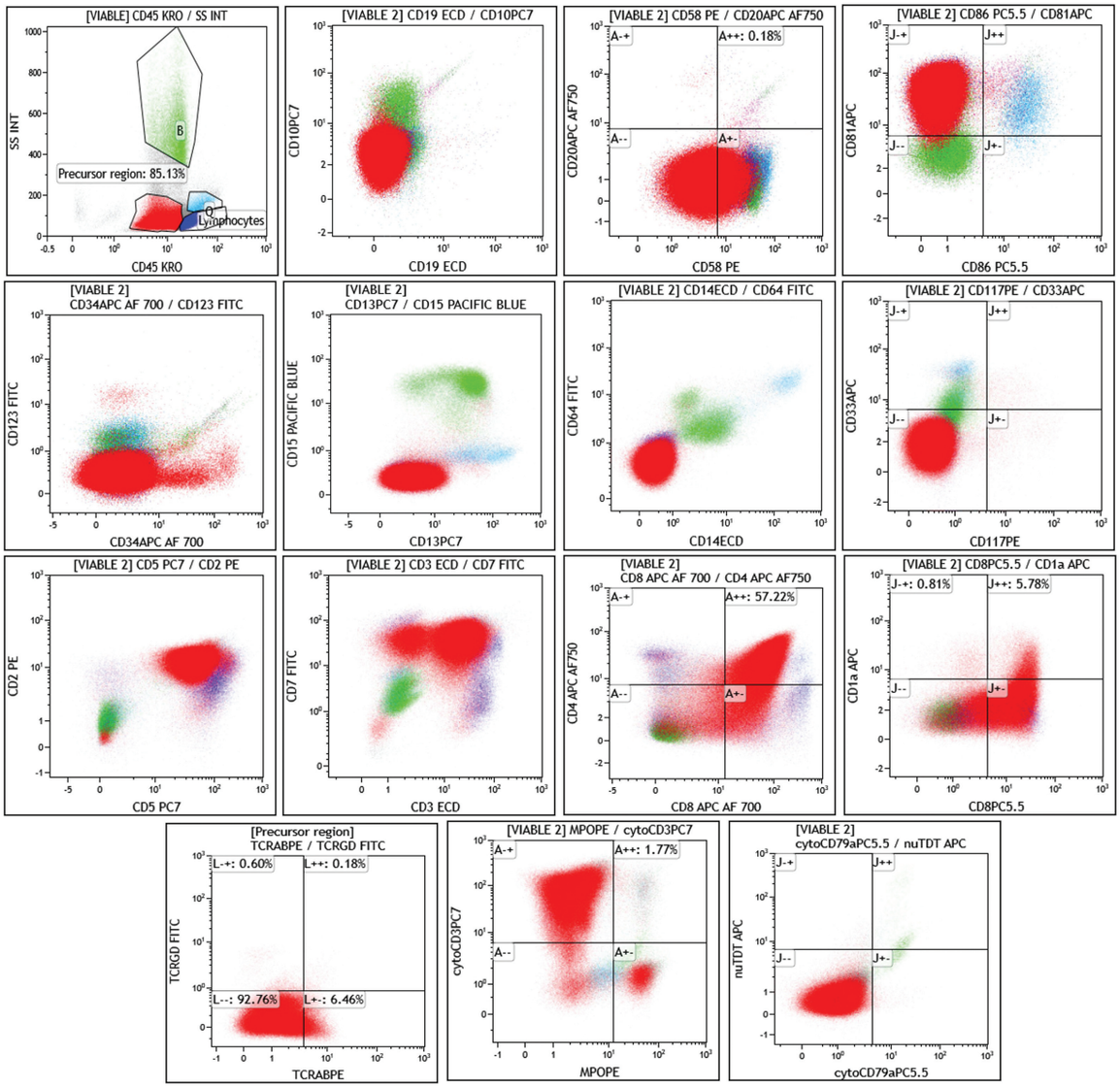

Fig. 2 Flow cytometric dot plots of a case of T-ALL. The blasts (*red population*) are SSC low and CD45 dim, positive forcytoCD3, CD2, CD5, smCD3, CD7, CD4, CD8, and negative for CD1a, TCRab, TCRgd, and nuTdT. The blasts are negative for B-cell and myeloid markers.

expression (cytoplasmic or surface), with an expression intensity exceeding 50% of the mature T-lymphocytes on flow cytometry, is recommended for the assignment of T-lineage. Similarly, for myeloid lineage, myeloperoxidase expression intensity in part exceeds greater than 50% of the neutrophil level or expression of two or more WHO-defined markers of monocytic differentiation.[46]

Finally, information on the certain antigens expressed by the leukemic blasts can guide for targeted immunotherapies in T-ALL.[47] A lot of clinical trials have proved the efficiency of

Table 6 EuroFlow consortium recommended eight color antibody panels for T-ALL

T-ALL tubes	Fluorochromes							
	PacB	AmCyan	FITC	PE	PerCPCy5.5	PECy7	APC	AF700
Tube 1	CyCD3	CD45	NuTdT	CD99	CD5	CD10	CD1a	SmCD3
Tube 2	CyCD3	CD45	CD2	CD117	CD4	CD8	CD7	SmCD3
Tube 3	CyCD3	CD45	TCRgd	TCRab	CD33	CD56	CyTCRb	SmCD3
Tube 4	CyCD3	CD45	CD44	CD13	HLADR	CD45RA	CD123	SmCD3

Abbreviation: T-ALL, T-acute lymphoblastic leukemia.

moAbs against specific antigens, particularly in relapsed/refractory T-ALLs. MoAbs to CD38 (daratumumab) and CD52 (alemtuzumab) have been tried in T-ALL patients and several new antigens such as CXCR4, IL7R, CD30, CD43, CD44, CD99, and CD194 are investigated as potential new targets for immunotherapy in T-ALL patients.[47,48] These antibodies can be utilized in the diagnostic panel of antibodies if clinical settings for immunotherapies are available.

Conclusion

To summarize, immunophenotyping by flow cytometry is an essential component in the diagnosis of ALL and distinguishing it as B- or T-ALL and from other leukemias such as AML and MPAL. A suitable panel of antigens needs to be analyzed for improved sensitivity and specificity of the test. Flow cytometric immunophenotype at baseline is essential to identify the antigenic aberrancy in the leukemic blasts, which aids in minimal/measurable disease (MRD) assessment at follow-up and to differentiate residual leukemic blasts from their normal counterparts.

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Conflict of Interest
None declared.

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Role of Cytogenetics and FISH in Laboratory Workup of B Cell Precursor Acute Lymphoblastic Leukemia

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Abstract

Modern therapeutic protocols in acute leukemias risk stratify disease based on genetic characterization of the neoplastic cells and their response to treatment. Genetic characterization is routinely performed by cytogenetic testing of leukemic cells and is a standard component of modern risk-adapted therapy in acute lymphoblastic leukemia (ALL). High-throughput technologies like RNA sequencing have identified multiple novel subtypes in recent years. The cytogenetic strategy using GTG and fluorescent in-situ hybridization (FISH) has to be adapted to identify not only the primary principal chromosomal abnormalities but also the novel subtypes. In the review, we describe a systematic comprehensive cytogenetic strategy that integrates information from immunophenotyping, flow-based DNA ploidy, and karyotyping complemented by targeted FISH studies to identify more than 70% of genetic abnormalities described in B cell precursor ALL. The simplified strategy includes a four-probe FISH and flow ploidy strategy, ± karyotyping that identifies high risk (*KMT2A, BCR::ABL1*, hypodiploidy, iAMP21) and standard risk (*ETV6::RUNX1* and high hyperdiploid) cytogenetic groups. The extended FISH panel includes probes targeting *MEF2D, ZNF384*, and *CRLF2* rearrangements that are used intuitively on integrating the immunophenotyping features that characterize these entities. The strategy also includes a systematic approach to identify masked hypodiploidy integrating targeted FISH analysis directed toward identifying monosomies of chromosomes 7, 15, and 17 and flow cytometry-based DNA ploidy analysis. The recently described PH-like ALL is characterized by *ABL* class fusions and rearrangements of *CRLF2 and JAK2* genes. FISH analysis using break-apart probes can be used to identify these aberrations. The cytogenetic approach also includes FISH analysis to identify intragenic and whole gene deletions of the *IKZF1* genes that identify a subset of patients associated with high risk of treatment failure.

Keywords

- acute lymphoblastic leukemia
- *ETV6::RUNX1* fusion
- hyperdiploidy
- *BCR::ABL1* fusion
- *KMT2A* gene rearrangement
- iAMP21
- FISH in ALL
- karyotyping

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Introduction

Modern treatment protocols in acute lymphoblastic leukemia (ALL) risk stratify patients based on genetic characteristics of leukemic cells and response to treatment.[1] Genetic characterization is routinely performed by cytogenetic testing of leukemic cells and is a standard component of modern risk-adapted therapy in ALLs.[2,3] Arising from the precursors of the lymphoid lineage, ALLs are either B cell precursor (BCP-ALL) or T cell type (T-ALL). BCP-ALL accounts for 75% of ALLs in children and 80% of ALLs in adults and comprises multiple genetic subtypes defined by their sentinel finding chromosomal abnormalities that are essential in risk stratification. T-ALLs, on other hand, are treated as a high-risk disease upfront and hence this review will focus on cytogenetic characterization of BCP-ALL.

Recurring Chromosomal Alterations in BCP-ALL

BCP-ALLs are characterized by a spectrum of finding chromosomal abnormalities that occur early in the course of the disease and are prognostic and predictive of outcomes.[4] Copy number abnormalities and sequence mutations are cooperating secondary lesions in leukemogenesis and may either be acquired or enriched at disease progression (►**Table 1**).[5,6] The clinically significant chromosomal abnormalities include aneuploidies and chromosomal translocations/gene rearrangements. High hyperdiploidy (HH) and *ETV6::RUNX1* fusions have more than 90% cure rates and are categorized as standard risk[7,8] and *TCF3::PBX1* fusions are categorized as intermediate risk. Hypodiploidy, intrachromosomal amplification of chromosome 21 (iAMP21), *BCR::ABL1*, *TCF3::HLF1* fusions, complex karyotypes, and *KMT2A* rearrangements are categorized as high risk.[9]

The distribution of the cytogenetic subtypes is variable in different age groups with good risk cytogenetic subtypes, that is, HH and *ETV6::RUNX1* fusions being more frequent in pediatric patients and *BCR::ABL1* being more frequent in adults. *KMT2A* rearrangements are characteristic of infant ALL with prevalence rising in adults (10–15%).[9,10]

Cytogenetic and Molecular Methods to Characterize BCP-ALL

Karyotyping

Chromosome banding technique is a morphological assessment of whole genome of a single cell and requires fresh samples and skilled manpower. The turnaround time varies from 5 to 10 days. The chromosomes are best studied at the metaphase stage of the cell cycle when the chromatin is condensed and chromosome morphology is well defined. The reliability of karyotyping analysis depends on obtaining good quality analyzable metaphases from neoplastic cells. Blasts with low proliferative potential and a proportion of HH cases may not yield analyzable metaphases, the results being interpreted erroneously as normal karyotype in such cases. Correlating FISH and flow ploidy results in these cases aids in accurately identifying the cytogenetic subtype.

Fluorescent in Situ Hybridization

Fluorescent in situ hybridization (FISH) is based on the principle of hybridization of single-stranded DNA probes labeled with fluorophores to their complementary genomic sequences. FISH does not require live cells, is relatively inexpensive, and allows transport of samples for testing at referral laboratories. FISH microscopy images can also be reviewed centrally to ensure standardization and diagnostic accuracy across treatment centers. Sensitivity varies from 1 to 5%.

Preanalytical Variables and Quality Control in Karyotyping and FISH

The first pull bone marrow aspirate sample is the preferred sample for cytogenetic studies that should be transported as soon as possible to the laboratory and processed with minimum delay. However, peripheral blood samples with high blast counts can also be utilized for FISH studies. Samples should be collected in heparin vials.

A minimum of 20 metaphases obtained from two independent cultures should be analyzed. In the presence of a clonal cytogenetic abnormality, evaluation of fewer than 20 metaphases is acceptable.

In FISH analysis, each probe should be validated for various thresholds, aberrant signal patterns to establish false positive/negative ranges. A minimum of 100 interphase nuclei by two analysts for diagnostic samples and 200 interphase nuclei for follow-up samples are recommended. The cutoff value for fusion probes is 1% and a higher cutoff for break apart probes is recommended. Cutoff values for each probe can be calculated by either using coefficient of variation with standard deviation and β inverse function or by using the Excel (Microsoft, Redmond, Washington, United States) statistical function CRITBINOM (n, p, α) with a confidence level of 95%. When results are just above the cutoff value, the report should mention that the clinical significance is unclear.[11]

In patients with lymphoblastic lymphoma without bone marrow involvement the cytogenetic characterization can be performed on either touch preparations, or on formalin fixed paraffin embedded sections.

Single-Nucleotide Polymorphism Array

Single-nucleotide polymorphism (SNP) arrays is a useful tool to study copy number abnormalities, polymorphisms, and copy neutral loss of heterozygosity at a whole genome level. The technology is based on the principle of hybridizing fragmented nucleic acid sequences derived from patient's DNA that are labeled with fluorescent dyes on the allele-specific oligonucleotide probes immobilized on array chip. The hybridization signal is recorded using a detection system and results are interpreted. SNP arrays can identify aneuploidies, chromosomal duplications, deletions, and copy neutral loss of heterozygosity. High-density SNP array in ALLs identifies hyperdiploidy, hypodiploidy, hidden hypodiploidy, *IKZF1* deletions, and *IKZF1* Plus patients (deletions in *PAX5*, *CDKN2A*, *CDKN2B*, *PAR1* deletions with absence of ERG deletions).[12,13] The utility of SNP array in routine

Table 1 Primary cytogenetic and secondary genetic abnormalities in BCP-ALL

Primary Cytogenetic Abnormalities					Secondary Genetic Abnormalities			
Cytogenetic Subtype	**Characteristics**	**Frequency In Children**	**Frequency in Adults**	**Prognosis**	**Genes involved**	**Change**	**Frequency in children**	**Frequency in adults**
High Hyperdiploidy	>50 chromosomes	25–40%	2%	Good	**DEVELOPMENT**			
Hypodiploidy	<46 chromosomes	2–3%	9–11%	Poor	*PAX 5*	Deletion/ Translocation/ Mutations	32%	30–35%
Near Haploidy	24–29 chromosomes				*IKZF1*	Deletions/ Mutations	15%	40–50%
Low hypodiploidy	30–39 chromosomes							
High Hypodiploidy	40–45 chromosomes				**CELL CYCLE REGULATION**			
Near Triploidy	66–79 chromosomes	1%	1%	Intermediate				
Near Tetraploidy	84–100 chromosomes	1%	1%	Intermediate				
					CDKN2A	Deletions	20–25%	
iAMP 21	Amplification	3–5%	1%	Poor	*TP53*	Deletions/ Mutations	<2% (Increased at relapse)	<2% (Increased at relapse)
					SIGNALLING			
FUSIONS					*JAK1/2*			
t(12;21)	*ETV6::RUNX1*	15–25%	<1%	Good	*CRLF2*	Rearrangements	5–16%	4–6%
t(9;22) **t(1;19)**	*BCR::ABL1* *TCF3::PBX1*	2–5% 2–6%	35% 3%	Poor Intermediate	*RAS*	Mutations	Increased at relapse	Increased at relapse
KMT2A (MLL)	Multiple partners	1–2%	10–15%	Poor				
(Ph like)	*ABL1/CRLF2*	10%	20–25%	Poor	**OTHERS**			
ZNF384	Multiple partners	1–5%	2–7%		*CREBBP*	Deletions/ Mutations	Increased at relapse	Increased at relapse
MEF2D	Multiple partners	3%	7%	Poor	*NT5C2*	Mutations	Increased at relapse	Increased at relapse
DUX4/ERG		7%	4–5%	Good	*NR3C1*	Deletions	Increased at relapse	Increased at relapse

IKZF1 Plus profile (deletions in IKZF1 deletions plus *PAX5*, *CDKN2A*, *CDKN2B*, *PAR1* deletions with absence of ERG deletions) seen in 6% of paediatric ALL[74] and 21% of adult ALL[9]

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diagnostics laboratories is limited by high cost per test and inability to identify fusions resulting from balanced translocations.

Transcriptome Analysis

Transcriptome sequencing and analysis identifies fusions and unique gene expression signatures identifying novel genetic subtypes like *BCR::ABL1* like, *ETV6::RUNX1* like, *KMT2A* like, and *PAX5* altered ALLs.[14–16] High costs, lack of expertise in analysis of data, and limited access to the technology have restricted its wider use in routine diagnostic laboratories.

Cytogenetic Strategy in BCP-ALL

The cytogenetic strategy is based on karyotyping, FISH analysis using a panel of four probes and DNA index using flow cytometry. The cytogenetic strategy is designed to identify aneuploidies (hyperdiploidy and hypodiploidy), rearrangements (*ETV6::RUNX1*,*BCR::ABL1*, *KMT2A* rearrangements, *TCF3::PBX1*, *TCF3::HLF1*), and amplifications (iAMP21).

While karyotyping identifies aneuploidies (hyperdiploidy, hypodiploidy and near haploidy), balanced chromosomal rearrangements (*BCR::ABL1*, *TCF3::PBX1* and *KMT2A* rearrangements), and structural abnormalities (duplications and deletions larger than 5MB), the resolution of karyotyping is low (<5MB) and it fails to identify cryptic translocations like t (12;21)/*ETV6::RUNX1* and some of the *KMT2A* rearrangements. Karyotyping also serves as a discovery tool identifying multiple structural and numerical abnormalities and identifies complex karyotype (5 abnormalities) that is associated with very high risk in adult ALL.[9]

FISH-Based Strategy

We developed a cytogenetic strategy based on three probe FISH testing and flow ploidy to identify principal genetic subtypes of BCP-ALL (►**Fig. 1**).[17] The three-probe FISH strategy includes dual-color fusion probe targeting *ETV6::RUNX1* fusion, dual color fusion probe targeting *BCR::ABL1* fusion, and *KMT2A* break-apart probe. The three-probe strategy has evolved into four-probe strategy by adding the *TCF3* triple color probe to identify *TCF3::PBX1* and *TCF3::HLF1* fusions.

FISH analysis is performed in a stepwise manner, the first step involves testing using *ETV6::RUNX1* probe in pediatric ALL patients and setting up sample for karyotyping and flow based detection of DNA index/ploidy. Samples that test positive for *ETV6::RUNX1* fusion are not tested using additional probes. If the sample tests negative, reflex testing is performed using *BCR::ABL1* dual-color fusion probe, *KMT2A* break-apart probe, and *TCF3* break-apart probe. Adult ALL patients are tested for *BCR::ABL1* fusion in the first step.

Additional Findings Using ETV6::RUNX1 Dual-Color Fusion Probe

FISH analysis using *ETV6::RUNX1* dual color probe not only identifies the fusion but also identifies iAMP21 and is a screening tool to identify HH.[3,17]

HH: Presence of three to four discrete additional *RUNX1* signals is suggestive of HH as these patients universally gain chromosome 21. Almost 25 to 30% of HH patients show normal karyotypes. In these patients (hidden HH) presence of additional *RUNX1* signals is suggestive of HH and can be confirmed by using centromeric probes targeting chromosomes 4, 10, and 17 and correlating with flow ploidy.

iAMP21: iAMP21 is defined by the presence of five or more total copies of *RUNX1* in interphase cells or three or more extra *RUNX1* signals on a single abnormal chromosome 21 in a tandem step ladder arrangement.[18]

Additional Findings Using BCR::ABL1 Dual-Color Fusion Probe

FISH analysis using the dual-color fusion probe identifies *BCR::ABL1* fusions and is also a screening tool to identify other *ABL1* translocations. Presence of additional *ABL1* signal

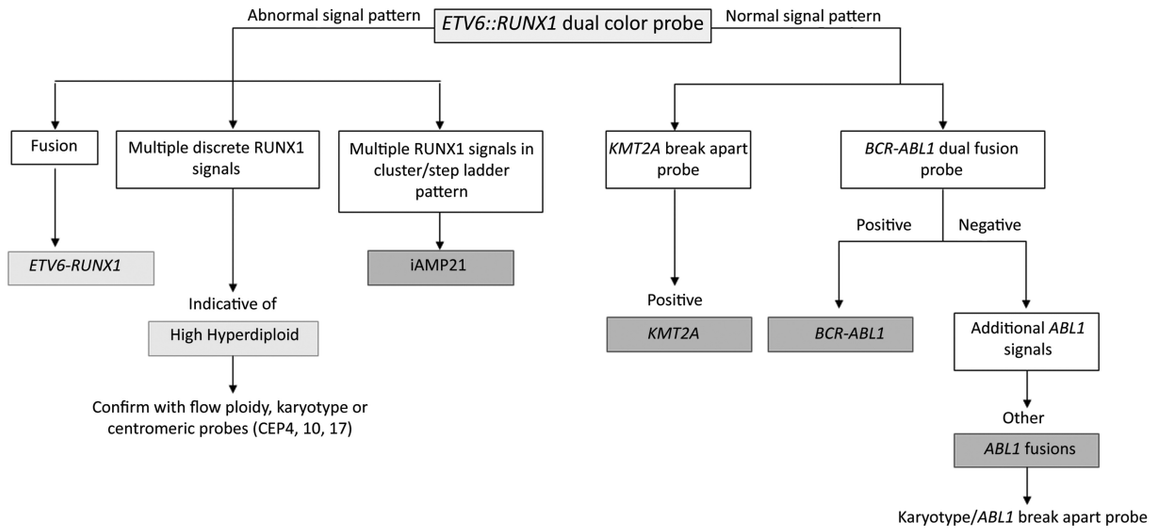

Fig. 1 Fluorescent in-situ hybridization (FISH) screening strategy to identify principal cytogenetic subtypes.

indicates the presence of *ABL1* rearrangements involving other partner genes. The *ABL1* rearrangement in these patients needs to be confirmed using *ABL1* break-apart probe.

Aneuploidies

The clinically relevant aneuploid entities in ALL comprises of HH, near triploidy, near-tetraploidy, near haploidy, and low hypodiploidy. HH and low hyperidiploidy are associated with intermediate prognosis.

High Hyperdiploidy

HH ALL forms one of the largest subgroups accounting for 30 to 40% of pediatric B cell ALL making it one of the most common malignancy in the pediatric population. HH ALL is characterized by non-random gains of chromosomes X,4,6,10,17,18, and 21 more frequently as trisomies followed by tetrasomies, with the modal chromosome number ranging from 51 to 67 chromosomes.[19] HH ALL is generally associated with favorable clinical features like low white cell counts, age 2 to 7 years and a very low incidence of extramedullary disease.[4] The outcome is superior with more than 90% overall survival in modern treatment protocols. Gains of specific chromosomes in HH ALL have been implicated as a significant factor affecting outcomes. Analyzing the cytogenetic data +6, +4/+ 10, +10/+ 17, and +4/+ 18 has been reported as good risk indicators in various studies.[4,20,21] The Children's Oncology group uses the presence of triple trisomies (trisomies of chromosomes 4,10 and 17) as a good risk indicator.[22] Lower modal numbers and the presence of trisomy 5 have been implicated as poor risk indicators in some studies.[20] However, these associations were not found to be consistent between the various clinical trials. Nearly 50% of HH patients show structural changes duplication of long arm of chromosome 1 being most common. Fusions are rare in HH patients. Patients with *BCR::ABL1*, *ETV6::RUNX1* or *KMT2A* rearrangements may be seen along with chromosomal gains and a hyperdiploid karyotype. These gains are usually secondary and the patients are assigned to the risk subtype based on the fusions present.

Diagnosis of HH ALL: Identified by karyotyping, flow cytometric-based DNA index, and SNP array. About 25 to 30% of HH patients show normal karyotype and diagnosis is based on flow cytometric DNA index and additional *RUNX1* signals on *ETV6::RUNX1* FISH analysis and CEP probes targeting chromosome 4,10, and 17.[23,24] In patients with failed karyotype result, gain of *RUNX1* signals on *ETV6::RUNX1* FISH analysis suggests presence of HH, and the diagnosis is to be confirmed by FISH analysis using CEP probes targeting chromosomes 4,10, and 17.

Hypodiploidy and Near-Haploidy

Hypodiploidy is characterized by less than 46 chromosomes and is further subtyped into HH (40–43 chromosomes), low hypodiploidy (30–39 chromosomes), and near haploidy (25–29 chromosomes), the latter two being associated with extremely poor prognosis.[25] It is seen in 1% of childhood and 3 to 4% of adult BCP-ALL. Near haploid and low hypodiploid karyotypes show monosomies of chromosomes 3,4, 7,13,15,16, and 17, while chromosomes X,14,18, and 21 show two copies.[26]

Masked Hypodiploidy: In a proportion of patients with hypodiploidy, the hypodiploid clone can be masked through a process called endoreduplication. In these patients, the hypodiploid clone duplicates the number of chromosomes and the karyotype can reveal only metaphases derived from the endoreduplicated clone with 50 to 79 chromosomes.[27] The patients are erroneously categorized as HH ALL. Masked hypodiploidy can be identified by SNP arrays, where the chromosomes with two copies showing uniparental isodisomy and chromosomes with four copies showing 2:2 allelic ratios.[28] Flow-based ploidy analysis may show two peaks corresponding to the hypodiploid clone and the endoreduplicated hyperdiploid clone. Presence of characteristic pattern of gains of chromosomes also aids in identifying a masked hypodiploidy warranting further investigation. We devised a systematic cytogenetic strategy to identify masked hypodiploidy using a combination of flow ploidy, pattern of gains of chromosome on karyotype, and targeted FISH analysis directed to identify monosomies of chromosomes 3,7,15, and 17.[29]

Diagnosis of Low Hypodiploidy/Near Haploidy

Diagnosis can be established by karyotyping, FISH, and SNP arrays. *TP53* mutations are seen in 90% of low hypodiploid patients.[30,31]

Near Triploidy (66–79 Chromosomes)

There is evidence that near triploidy represents a hidden low hyperdiploid clone.[26] Once masked hypodiploidy has been ruled out, near triploidy is to be considered as an intermediate risk factor. Diagnosis rests on karyoytping, FISH, and SNP arrays.

Near Tetraploidy (84–100 Chromosomes)

Near tetraploidy is seen in 1% of childhood ALL and is more frequent in T ALL. Near tetraploidy does not carry any prognostic impact by itself.[32] Once the presence of *ETV6::RUNX1*, *BCR::ABL1* fusions and *KMT2A* rearrangements have been ruled by FISH, near tetraploidy is to be considered as an intermediate risk factor.

Amplifications: Intrachromosomal Amplification of 21

iAMP21 is seen in 2% of pediatric ALL and is rare in adults.[33] It is characterized by gains and losses along the long arm of chromosome 21 with *RUNX1* gene being present in the common area of amplification.[34] The common regions of deletions involve subtelomeric region of chromosome 21.[18,35] SNP array and whole genome studies have inferred that the formation of iAMP21 involves breakage-fusion-bridge cycle, resulting in amplification and formation of dicentric chromosomes and chromothripsis.[34] Associated with high relapse risk, intensive therapies have improved outcomes in recent years.[36]

Diagnosis of iAMP21: On karyotype iAMP21 is seen as a grossly abnormal chromosome 21, the diagnosis being supported by FISH analysis showing more than or equal to three

copies of *RUNX1* gene on the arm of the chromosome 21.[35] On interphase FISH iAMP21 is defined as more than or equal three five copies of *RUNX1* gene per cell. On interphase FISH care should be taken to differentiate from HH as more than or equal to five copies of *RUNX1* may be seen in HH patients as well. In doubtful cases FISH targeting the subtelomeric region of chromosome 21 may help in diagnosis. SNP array analysis identifies a typical pattern of gains and losses associated with iAMP21 and is diagnostic in rare atypical cases where additional copies of *RUNX1* gene may be present on other chromosomes.[37]

Rearrangements

The chromosomal translocations in BCP-ALL commonly involve transcription factors, epigenetic modifiers, cytokine receptors, and tyrosine kinases. The translocations deregulate gene expression either by forming a chimeric transcript or overexpression of a gene by juxtaposition of enhancers to the partner gene.

t(12;21)(p13:q22)/*ETV6::RUNX1*

ETV6::RUNX1 fusions are more frequent in childhood ALL and account for 25% of pediatric ALL in Western data and around 18% as per our data.[2,17,38,39] It is associated with favorable outcomes. The *ETV6::RUNX1* fusion is known to occur prenatally where it gives to rise to pre-leukemia state followed by secondary mutations that trigger overt leukemia.[40]

Diagnosis: The t(12;21) is cryptic and is best identified by FISH analysis using dual-color fusion probes. The FISH approach has more advantages than the molecular RTPCR approach as the transcript expression can be low and also the FISH analysis using *ETV6::RUNX1* probe additionally identifies HH, iAMP21, and other *ETV6* fusions as well.

t(9;22)/*BCR::ABL1*

BCR::ABL1 fusion accounts for 25% of adult ALL and is seen in 2 to 5% of childhood ALL. Associated with high risk,[41,42] the outcomes have improved in recent years with the use of tyrosine kinase inhibitors in both children and adults.[43–47] Ikaros deletions are common in *BCR::ABL1* positive patients and are associated with treatment failure and relapse.[48]

Diagnosis: Identified by karyotyping, FISH and molecular methods.

TCF3 Rearrangements: t(1;19)(q23;p13)/*TCF3::PBX1* and t (17;19)(q22;p13) /*TCF3::HLF1*

TCF3::PBX1 fusions occurs in approximately 6% of BCP-ALL and associated with intermediate risk.[49,50] The fusion protein is formed by joining the homeobox (*HOX*) gene *PBX1* (for pre-B cell homeobox 1) on chromosome 1 with the two activation domains of the basic helix-loop-helix transcription factor *TCF3* on chromosome 19 leading to transcriptional activation of *PBX1*.

TCF3::HLF fusion is a rare genetic subtype seen in less than 1% of BCP ALL.[51] Patients frequently present with disseminated intravascular coagulation, hypercalcemia,[52] low WBC counts, and absence of CD34 expression on immunophenotyping. It is associated with a dismal prognosis, despite treatment intensification and allogenic stem cell transplantation.[53]

Diagnosis: Identified by karyotyping, FISH, and molecular methods. The translocation t(1;19) may occur either as a balanced form or an unbalanced form (only derivative 19 present), the unbalanced form being more common.[50,54] Not all patients with t(1;19) identified on karyotype carry the *TCF3::PBX1* transcript especially those associated with HH.[55] FISH analysis using a break-apart probe identifies these translocations accurately. The translocation t(17;19) can be identified by karyotyping and FISH. The *TCF3* break-apart probe is included in our FISH panel. Patients testing positive for *TCF3* rearrangement using break-apart probe are investigated using tricolor fusion probe that identifies the specific partners, that is, *PBX1* and *HLF1*.

KMT2A Rearrangements

KMT2A rearrangements are hallmark of infant ALL with an increased prevalence in adolescents and young adults (4%) and peaking in adults (15%). *KMT2A* (*MLL*) gene can rearrange with more than 80 partners and more than 100 different translocations have been described. They are strong drivers of leukemogenesis with very few secondary alterations and are associated with poor prognosis.[56]

Diagnosis: Best identified by FISH analysis using break-apart probes. Karyotyping identifies partners and in patients with poor chromosome morphology match metaphase FISH analysis helps in identifying partner. *KMT2A* rearrangements involving genes located close to the *KMT2A* gene on chromosome 11 (*ATP5L* and *USP2*) show a normal FISH pattern and these rearrangements are best identified using molecular techniques or RNA sequencing.[57]

B-Other ALL

B-Other ALL is a heterogenous group defined by absence of all routinely assessed classifying cytogenetic abnormalities described above and are classified as intermediate risk cytogenetics. In the preceding decade, over 18 different genetic subtypes in the B-Other group have been identified, with differing treatment outcomes (►**Fig. 2**).[14] Subtypes with good outcomes are *DUX4* fusions (associated with *ERG* deletions). Poor outcome subtypes include Ph-like ALL which may benefit from intensive therapy along with tyrosine kinase inhibitors.[43,44] *MEF2D* and *ZNF384* fusions have been reported to be associated with intermediate prognosis.[58] Independent of these cytogenetic groups, somatic copy number alterations and gene mutations are also of prognostic significance. Among these, are deletions in *IKZF1* (*IKZF1*^{del}) especially when accompanied by deletions in *CDKN2A/B*, *PAX5* and the *PAR1* locus; deletions in *RB1*, *NR3C1*, *BTG1*; and mutations in *TP53*, and *PAX5*.[8,59]

Role of Karyotyping and FISH in Identifying Different Subtypes of B-other BCP-ALL

Karyotype is a discovery tool and serves as a screening tool to identify structural abnormalities and rearrangements which

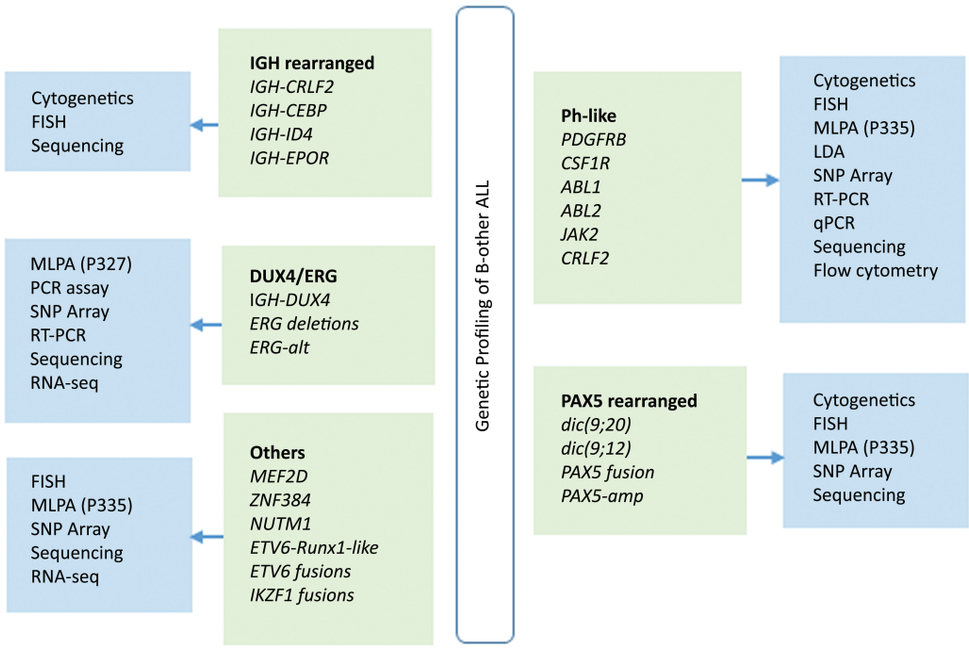

Fig. 2 Techniques available for genetic profiling of B-Other ALL.

can be confirmed through FISH analysis using specific probes.

MEF2D Rearranged BCP ALL

MEF2D (myocyte enhancer factor 2D)-rearranged ALL accounts for 4% of pediatric and 10% of adult ALL with a higher incidence in adolescents. It is characterized by a distinct immunophenotype (CD10 − , CD38 +) and is associated with a poor prognosis.[58,60,61] The common fusion partner genes include *BCL9* (1q21), *HNRPULN1* (19q13.2), *DAZAP1* (19p13.3), *CSF1R* (5q32),*SS18*(18q11.2), *STAT6* (12q13.3), and *FOXJ2* (12p13.3). The fusions result in increased cell growth, resistance to dexamethasone, and increase of *HDAC9* expression. The most common fusion is the *MEF2D::BCL9* fusion which is the result of an interstitial deletion between 1q21 and 1q22. The fusion is cryptic and cannot be identified by karyotyping. *MEF2D* rearrangements lead to increased *HDAC9* expression, therefore amenable to histone deacetylase inhibitor treatment.[58]

Diagnosis: The diagnosis is based on characteristic immunophenotype confirmed by FISH analysis using a *MEF2D* break-apart probe. The tricolor *MEF2D* break-apart probe differentiates between *MEF2D::BCL9* fusions and other partners

B- ALL with Zinc Finger Protein 384 (*ZNF384*) Rearrangements

ZNF384 rearrangements show a peak incidence in adolescents and young adults and account for 1 to 5% of pediatric and 2 to 7% of adult ALL.[62–64] The *ZNF384* gene functions as a transcription factor and multiple partners have been described. The *ZNF384* rearranged ALL have a distinct transcriptomic signature and are associated with aberrant expression of myeloid markers (CD13 and CD33). With a stem cell signature, the *ZNF384* rearrangements have been reported in biphenotypic, mixed phenotype, and acute undifferentiated leukemia.[65]

Diagnosis: The aberrant expression of myeloid markers provides a clue. The rearrangements are undetectable by karyotyping and are identified by FISH analysis using a *ZNF384* break-apart probe.

BCR::ABL1 Like (Ph-Like ALL)

The *BCR::ABL1*-like (Ph-like) is characterized by gene expression profile similar to *BCR::ABL1* fusion positive patients but lacks the *BCR::ABL1* fusions.[15,16] Ph-like ALL is seen in 12% of childhood, 21% of adolescent 27% of young adult, and 20 to 24% in adults more than 40 years of age. It is a heterogeneous group consisting of gene rearrangements, copy number alterations, and mutations that activate tyrosine kinase or cytokine receptor signaling. Similar to *BCR::ABL1* positive ALL, *IKZF1* deletions are found in 70 to 80% of Ph-like ALLs and are associated with poor outcomes.[15]

The definition of Ph-like ALL is based on gene expression profile, while the cytogenetic approach to identify this entity is based on identifying *CRLF2* rearrangements and identifying *ABL* class fusions using break-apart probes (►**Fig. 3**).

CRLF2 Rearranged BCP-ALL

The *CRLF2* gene is located on the pseudoautosomal regions of the sex chromosomes X and Y. The two common genomic alterations resulting in *CRLF2* rearrangement include t(X;14)(p22.3;q32.33) or t(Y:14)(p11.32;q32.33) and *CRLF2::P2RY8* fusion resulting from 320KB interstitial deletion within the PAR1 region bringing the two genes together.[5,66,67] The *P2RY8::CRLF2* fusions have also been identified with other primary cytogenetic abnormalities like HH and iAMP21. *CRLF2* rearrangements result in overexpression of the

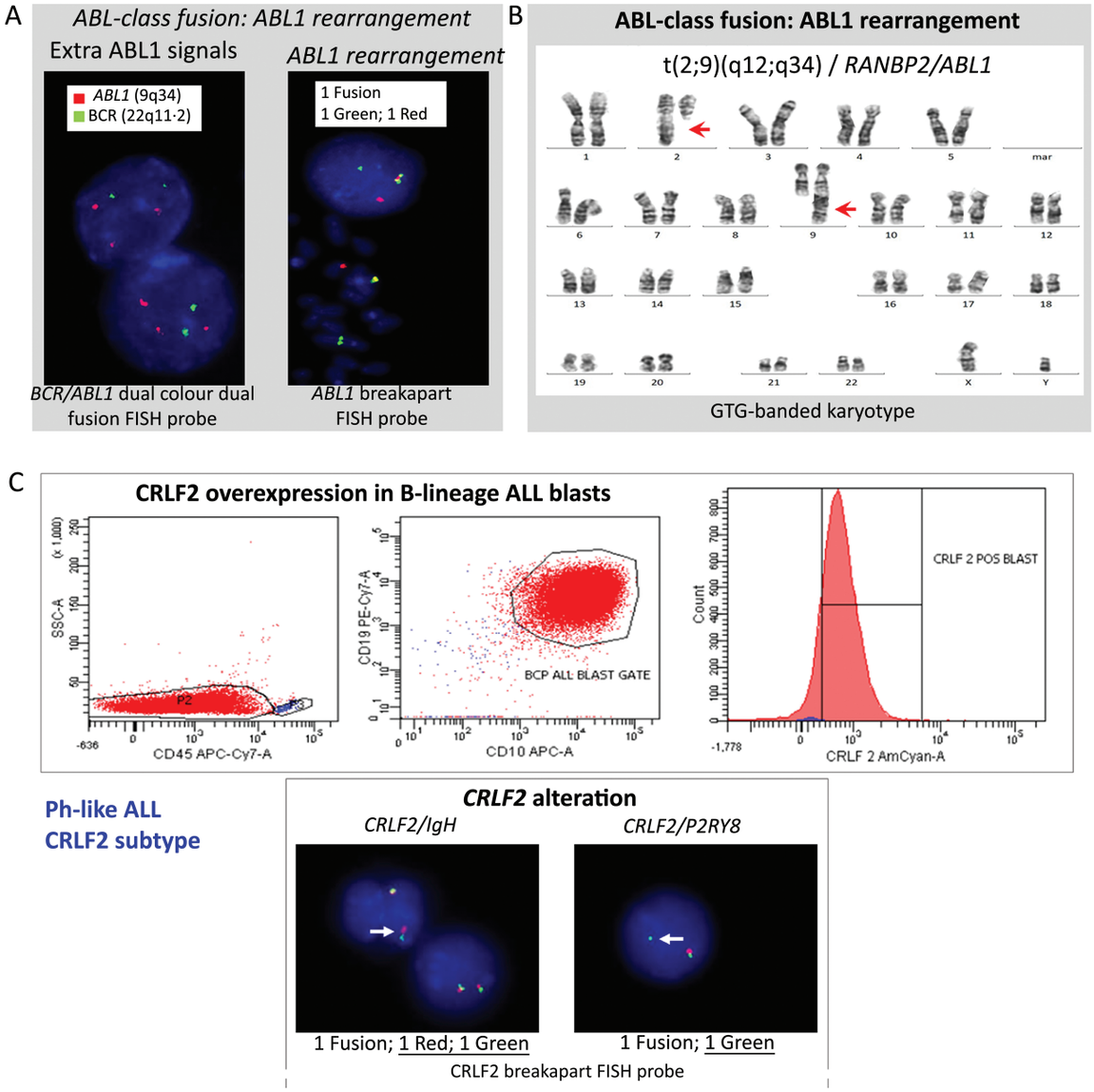

Fig. 3 Cytogenetic approach to identify Ph-like ALL with ABL1 rearrangement (**A** and **B**) and CRLF2 rearrangement (**C**).

CRLF2 protein that can be detected by multiparametric flow cytometry. The *IgH* translocation places the *CRLF2* gene under the impact of IGH enhancer and in *CRLF2::P2RY8* fusion under the influence of promoter sequences of the *P2RY8* gene.

Rearrangement of the *CRLF2* gene is seen in 50% of Ph-like ALL and in half of Down syndrome-associated BCP-ALL.[68] The *CRLF2* rearrangements are associated with poor prognosis in non-Down syndrome pediatric and adult patients.[38,41,69]

Diagnosis: Both the *IgH::CRLF2* and *P2RY8::CRLF2* are cryptic and cannot be detected on karyotype. They can be detected by FISH using a *CRLF2* break-apart probe. *CRLF2::P2RY8* fusions can also be identified by detecting deletion in the *PAR1* region by using multiplex ligation-based probe amplification (MLPA) or chromosomal micro arrays or SNP arrays.

ABL Class Fusions

ABL class fusions although not so frequent in BCP-ALL (3–5% of pediatric ALL, 2–3% of adult ALL) are seen in 10% of Ph-like ALL.[41,42,70] The *ABL* class rearrangements result in fusion of the 5′ partner gene with 3′ of the kinase gene resulting in transcripts that have intact tyrosine kinase domain resulting in activation of the kinase pathway.[70] The frequently involved kinase genes include *ABL1*, *ABL2*, *CSF1R*, *PDGFRB*, and *PDGRFA*.[69]

Diagnosis: The fusions can be identified by RNA sequencing and multiplex PCR assays. Cytogenetically, the presence of an additional ABL1 signal on *BCR::ABL1* FISH is a screening tool and hints toward the presence of the *ABL1* rearrangements. Since the Ph-like ALL is associated with poor response to induction therapy and high MRD, our cytogenetic approach is based on FISH analysis using break-apart probes targeting

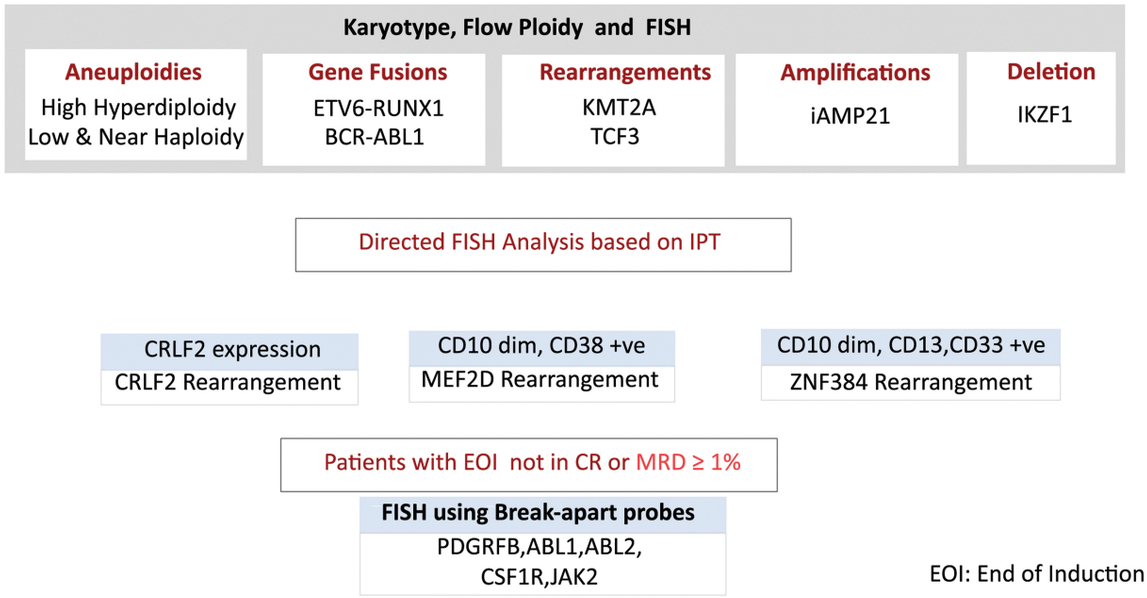

Fig. 4 Cytogenetic strategy to profile B-other ALL including Ph-like ALL.

ABL1, *ABL2*, *CSF1R*, *PDGRFB*, and *PDGRFA* in patients not in remission at end of induction or having a high MRD. Karyotype can also identify translocations involving the kinase genes that are confirmed using specific break-apart probes.

JAK Rearrangements and Mutations

Translocations resulting in rearrangement of *JAK2* gene are seen in 5% of pediatric Ph-like ALL and more frequently in young adults.[69] The *JAK2* rearrangements result in in frame fusion of the 5′ of the partner gene with the 3′ of the *JAK2* kinase gene keeping the tyrosine kinase domain intact. The *JAK2* fusions can be identified by molecular techniques, RNA sequencing, visible chromosomal rearrangements involving the 9p24 loci, and FISH analysis using the break apart probe. The *JAK2* mutations are frequent in *CRLF2* rearranged ALL and result in activation of the JAK-STAT pathway. The mutations can be identified using Sangers sequencing. The other JAK mutations are not so common and involve the *JAK1* gene.

IKZF1 Deletions and IKZF1 plus BCP ALL

IKZF1-deletions are known to have poor outcomes in ALL overall,[71] and are associated with resistance to therapy. *IKZF1* deletions with co-occurring alterations in *CDKN2A/2B*, *PAX5*, and *PAR1* (pseudo-autosomal region) in the absence of *ERG* deletions are defined as *IKZF1* plus are associated with poor outcomes and high rates of treatment failure.[72]

The *IKZF1* deleted and *IKZF1* plus are commonly identified using MLPA or chromosomal microarray analysis.[12] In recent times, FISH probe targeting the exon 4–7 of the *IKZF1* gene has been used to identify the whole gene and intragenic deletions of the *IKZF1* gene.[73] Karyotyping identifies monosomy 7, deletions of short arm of chromosome 7, and dicentric translocations that result in deletion of the entire short arm of chromosome 7. Dicentric translocations between the long arm of chromosomes 7 and 9 result in deletions of the entire short arms of chromosomes 7 and 9 and are consistent with the diagnosis of *IKZF1* plus.

Summary

Cytogenetic study including karyotype and FISH is an efficient tool in identifying the primary chromosomal abnormalities in BCP-ALL. The minimal diagnostic workup suggested is a FISH panel that includes probes targeting *ETV6::RUNX1*, *BCR::ABL1*, *KMT2A* rearrangements and *TCF3* rearrangements along with karyotyping and flow ploidy. In patients with failed karyotype, information from *ETV6::RUNX1* probe, flow ploidy complemented with additional centromeric probes targeting 4,10 and 17 can identify the aneuploidies including the hidden hyperdiploid and a fair proportion of masked hypodiploidy patients. The information from immunophenotyping can be integrated into the cytogenetic analysis and utilized for targeted FISH analysis (►**Fig. 4**). For example, information on dim to negative CD10 expression and CD38 positivity is an indicator to test using *MEF2D* break apart probe. Similarly, dim CD10 expression with aberrant CD13 and CD33 positivity is an indicator to test for *ZNF384* rearrangements. These patients commonly show ETV6 deletions on *ETV6::RUNX1* FISH analysis. Reflex testing for *ABL* kinase and *JAK2* rearrangements in patients is suggested in patients with treatment failure at end of induction or with high MRD. The above strategy can identify genetic aberrations in more than 70% of BCP-ALL patients.

Authors' Contributions

R.I. and M.P. wrote the original draft, K.R., A.D. collated the data and figures; M.P. and R.I. have full access to all data and the final responsibility for publication. All authors reviewed the manuscript draft submitted for publication.

Conflict of Interest
None declared.

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Flow Cytometric MRD Assessment in Acute Lymphoblastic Leukemias

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Abstract

Acute lymphoblastic leukemia (ALL) is one of the very first malignancy where the assessment of early response to therapy by minimal/measurable residual disease (MRD) monitoring has proven to be cardinal tool for guiding therapeutic choices. At present, MRD detection is not only used for the assessment of initial treatment response and subsequent risk stratification but also for monitoring disease burden in the setting of hematopoietic stem cell transplant. Multicolor flow cytometry (FCM) for the assessment of MRD has been in existence for more than two decades. It is presently the most commonly used technique worldwide for MRD assessment in ALL. The technique has evolved from two to three color assays in its early phases to eight and more color assays in present time, which enables detection of one leukemic cell in 10^4 or more cells. The assessment of MRD is based on analysis of expression of lineage-associated markers and either looking at "leukemia associated immunophenotypes" or identify "different from normal" patterns. A rapid turn-around-time and direct quantification of viable residual leukemic cells are advantages of FCM over molecular techniques of MRD assessment. On the other hand, one of the prime limitations of detection of residual cells by FCM is the immunophenotypic shifts that are observed as a result of chemotherapeutic reagents. In addition, introduction of immunotherapy, especially against important gating markers like CD19, has posed significant challenge to FCM-based MRD assays, and requires modification of antibody panels for an alternate gating and analysis strategy. Finally, standardization and validation of MRD assay and use of internal and external quality controls are extremely important aspects for a clinical laboratory providing MRD reports for patient care.

Keywords
- hematology
- acute lymphoblastic leukemia
- flow cytometry
- MRD

Introduction

Acute lymphoblastic leukemia (ALL) is a malignant neoplasm arising from the precursor lineage hematopoietic cells. The proliferation of these cells can be seen in bone marrow (BM), peripheral blood (PB), and extramedullary tissues. It represents the most common leukemia in childhood and can also be seen in substantial cases in adulthood.[1] Conventionally these are classified as either arising from B lineage (B-ALL) or T lineage (T-ALL) precursor cells. Recently, there has been considerable research to subclassify these leukemias based on their molecular pathogenesis and this has become necessary in recent times due to their prognostic and therapeutic relevance.[2] In majority of the cases, the etiology remains unknown and ALL develops as a de novo neoplasm. The remaining includes either the genetically predisposing syndromes like Down syndrome, Fanconi anemia, few rarer entities like Bloom syndrome, ataxia telangiectasia, and

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Nijmegen breakdown syndrome or exposure to ionizing radiation, pesticides or solvents exposure, or infections like Epstein-Barr virus and human immunodeficiency virus.[3] Although chromosomal aberrations represent the hallmark of ALL, these, however, are not the sole pathogenetic etiology. Characteristic translocations especially in B-ALL include t (12;21) [*ETV6::RUNX1*], t(1;19) [*TCF3::PBX1*], t(9;22) [*BCR::ABL1*] and *KMT2A* gene rearrangements.[4] Accurate diagnosis of ALL by definition requires more than 20% blasts either in PB or BM[4] that is confirmed further for lineage assignment by multiparametric flow cytometry (MFC). Fluorescent in situ hybridization (FISH) and/or molecular tests like reverse-transcriptase polymerase chain reaction (RT-PCR) are required to subcategorize these cases for further prognostication and implications in therapy.

For the management of ALL, a prognostic evaluation is crucial. The clinical team can accurately determine the best course of treatment and the decision to consider the patient for an allogeneic stem cell transplant by an optimal risk stratification.[5] ALL is one of the first cancers where monitoring for minimal/measurable residual disease (MRD) has established itself as a crucial tool for determining the best course of treatment. Currently, MRD detection is used for initial treatment response evaluation, disease burden monitoring, and risk stratification in the context of hematopoietic stem cell transplant.[6,7]

MRD in ALL

By definition, MRD refers to the presence of measurable tumor cells that are persistently present in a sample after the cancer treatment. While the assessment of morphological remission in ALL is still impeccable, the blasts are not always easy to distinguish especially when there is proliferation of hematogones, in the presence of regenerating normal myeloid precursors and after granulocyte colony stimulating factor therapy. Moreover, this morphological assessment is highly subjective and only a few cells are assessed at a given time.[8–10]

Techniques for MRD

The most widely used techniques for analyzing MRD in ALL at the moment are MFC and PCR amplification based techniques, including the use of molecular markers specific to leukemia (fusion gene transcripts) or to the patient (rearrangements of the immunoglobulin/T cell receptor [TCR] gene). In addition, FISH cytogenetics, cell culture systems, Southern blotting, Sanger sequencing, and next-generation sequencing (NGS) are other modalities to assess the residual disease. PCR-based techniques have an advantage of high sensitivity that reaches $1:10^4$-$1:10^5$ (0.5–1.0 log higher than FCM). Furthermore, requirement of lesser amount of tissue compared with FCM is another important advantage of PCR techniques. However, this is a time-consuming methodology and requires highly specialized personnel for technical work, trouble shooting, and interpretation. Moreover, applicability of MRD analysis by real-time quantitative PCR (RQ-PCR) for fusion-genes is limited to only those cases that have a typical translocation at diagnosis and is usually not helpful in ALL with a normal karyotype.[11–15]

MRD by Flow Cytometry

MFC assesses the expression of differently expressed antigens by leukemic cells. Signals emitted by fluorochrome-conjugated monoclonal antibodies against these antigens are studied for MRD analysis. Two broad approaches have traditionally been in place to explain MRD assessment strategy, that is, leukemia-associated immunophenotype (LAIP) where the expression of antigens at diagnosis is tracked at subsequent time points on the residual leukemic cells; another method uses immunophenotypic shift in comparison to a normal counterpart population (hematopoietic progenitors of similar lineage and maturational stage) to identify leukemic blasts. This so-called "different from normal" analysis has the advantage of studying MRD without the requirement of knowledge of the diagnostic immunophenotype.[16] In real-world scenario, the boundaries between both these approaches are blurred, and a typical MRD-analysis is a combination of both of the concepts. FCM has the advantage of lesser turnaround time, its applicable in almost all ALL cases (up to 95%), and its readily available in routine hematology laboratories compared with other advanced molecular testing. In addition, a simultaneous assessment of cell qualities required for targeted therapies can be done. However, this technique also comes with its fair share of challenges. Immunophenotypic shifts are a known phenomenon postchemotherapy, which hinders the exact characterization of the leukemic cells on follow-up, most importantly when there is downregulation of the gating markers, like CD19 for B-lineage ALL. Steroids that are used extensively in all treatment protocols have been implicated in modulating the gene expression and hence alterations in immunophenotypic profile of the cells. Recent trends to utilize targeted therapy in ALL have also posed a challenge with respect to MRD assessment. Especially with B-ALL, there becomes a need to standardize a different antibody panel.[17,18]

MRD in B-ALL

Time Points for Assessment

For B-ALL, day 15 is considered as the first check point for the assessment of fast and early elimination of leukemic cells. Studies reported importance of MRD analysis at this nascent stage with patients having MRD negativity at this time point exhibiting an excellent prognosis.[19,20] The main advantage of day 15 MRD assessment is less interference from the proliferating hematogones as compared with the end of induction time point which needs a wider panel of antibodies to delineate these cells from leukemic B cells. Absence of hematogones increases the confidence of reporting with minimum set of markers and hence is more cost-effective specially in resource constraint laboratories.[21,22]

Nevertheless, most of the laboratories nowadays assess residual disease at postinduction, postconsolidation, and end of maintenance phases of treatment protocol. Each of

these time points exhibit prognostic relevance in risk stratification of patients. However, any suspicion of relapse or plan of change of therapy including decision for transplant warrants a more frequent assessment.[23–26]

Technical Considerations

Heparin or ethylenediaminetetraacetic acid-anticoagulated PB or BM samples can be used. The BM sample has been shown to have more sensitivity to detect MRD cells in B lineage ALL, where the level tends to be 1 to 3 logs lower in PB than in BM. However, PB sample does have its advantage of being minimally invasive and hence more frequent monitoring is less cumbersome. Moreover, existence of PB-MRD positivity indicates high risk of relapse than isolated BM-MRD positivity. In fact, some studies have shown that a day 15 PB-MRD can provide an early, minimally invasive and easily accessible MRD screening option.[27] However, BM remains the sample of choice for B lineage ALL due to an overall higher detection rates than PB. This may not be true for T lineage ALL, where studies have shown a similar sensitivity for the detection of residual leukemic cells from PB and BM samples.[23,28] Minimal residual disease levels in BM and PB are comparable in children with T-ALL, but not in precursor-B-ALL.[29]

The optimal way to assess MRD is via an initial pull of not more than 3 mL of BM aspirate to avoid hemodilution of the specimen. Study by Helgestad et al showed 2.5 mL of first pull BM aspirate from one puncture site was appropriate for MRD analysis.[30] Samples should be processed within 24 to 48 hours and delivered ideally at room temperature. The EuroFlow group recommends "erythrocyte bulk lysis" procedure that seems to yield a higher number of leukocytes for acquisition of flow cytometer and hence likely to increase the sensitivity of the assay. Incubation with a prestandardized set of antibody cocktail(s) in dark, followed by thorough washing for removal of excess antibodies, should be done. The antibody cocktail should preferably of eight or higher colors for achieving a high sensitivity assay. A tube containing sheath fluid should be run prior to each sample run to avoid carry-over. To obtain a good sensitivity MRD data, acquisition of large numbers of cells becomes important. Acquisition of at least 1.5 million clean cells is recommended. With acquisition of more than or equal to 4×10^6 the sensitivity of detection of MRD can be comparable to PCR-MRD data.[31] However, analysis of the data generated by acquisition of millions of cells, in a multicolor-high sensitivity assay settings, requires high-end computers with state-of-art processors, graphic cards, and advance data-analysis software. The high-sensitivity flow data analysis also requires special considerations. Careful removal of cell-aggregates, debris, and nonspecific events is necessary. Certain mimickers and artifacts can hinder MRD detection especially in high-sensitivity settings. Cells with overlapping immunophenotype like plasmacytoid dendritic cells, CD10+ transitional B cells, CD19+ natural killer (NK) cells, mesenchymal stromal/stem cells, as well as endothelial cells could well interfere with the rare true residual leukemic cells and should be kept in mind.[32] There is no clear recommendation yet on the number of cells in a cluster required to define residual disease; however, most of the studies in B-ALL indicate a minimum number between 10 and 50 events.[33] Majority of the treatment protocols agree upon 0.01% clinically defined threshold to define MRD positivity. During the early phases of studies on clinical relevance of MRD in ALL, researchers from the International Berlin-Frankfurt-Munster (I-BFM) Study Group discovered that patients with MRD levels of 0.1% or above on both days 33 and 78 of therapy experienced a relapse rate of 75%, which led to the treatment of this particular patient population being intensified.[34] The Dana-Farber Cancer Institute ALL Consortium also noted an MRD threshold of 0.1% best predicted relapse hazard. The subsequent results with higher sensitivity assays documented the clinical relevance of 0.01% MRD cutoff, especially at end of induction time point.[35] The Children's Oncology Group noted that the presence of MRD of 0.01% or greater, on day 29 was the strongest prognostic indication and predicted a worse outcome.[20] The UKALL2003 study group also showed benefits in event-free survival of augmented postremission therapy in children with presence 0.01% or greater MRD at the end of induction.[36] For the Indian Collaborative Childhood Leukaemia Group (ICiCLe), developed in 2013, the cutoff for categorization into high risk group based on MRD levels at end of induction time point is 0.01%.[37] However, it may be relevant to note that depending on the protocol and on the time point at which MRD is examined, other threshold levels can also be informative.

Strategy for MRD Analysis

FCM MRD in B-ALL was conceived in two or three-color flow cytometers that utilized patient-specific immunophenotyping to assess response compared with the baseline expression of the concerned markers. To enable pattern recognition in FCM plots and to detect aberrant regions showing MRD cellular phenotypes, various combinations of B-lineage maturation markers (CD10, CD20, CD22, CD19, CD34, CD38, CD45, and CD58) were utilized.[38,39] With further enhancements of the labeling capacities of cells, four- to six-color flow cytometers came into picture, thereby increasing MRD profiling capabilities and improving the sensitivity of MRD detection.[40] Recent efforts in standardization of MRD have shifted the capabilities of laboratories to an eight-color panel and beyond. For backbone markers, CD19, CD10, CD20, CD34, and CD45 are considered optimal. CD38 is also added and considered to be of high diagnostic relevance.[41–43] Other markers considered to be relevant for enhancing sensitivity of the assay include CD73, CD86, CD66c, CD123, CD81, CD44, CD58, CD9, and CD304.[44–47] CD15/NG2 might also be relevant in ALL with *KMT2A* gene rearrangements that is predominantly seen in infants. In addition, these cases are usually CD10 negative.[48]

The core approach of MRD analysis is to distinguish residual leukemic blasts from regenerative normal B cell precursors (BCP) in postchemotherapy setup.[49] The BCPs are known to follow a well-conserved immunophenotypic maturation sequence for the backbone markers viz CD10, CD19, CD20, CD34, CD38, and CD45. The premise for identifying

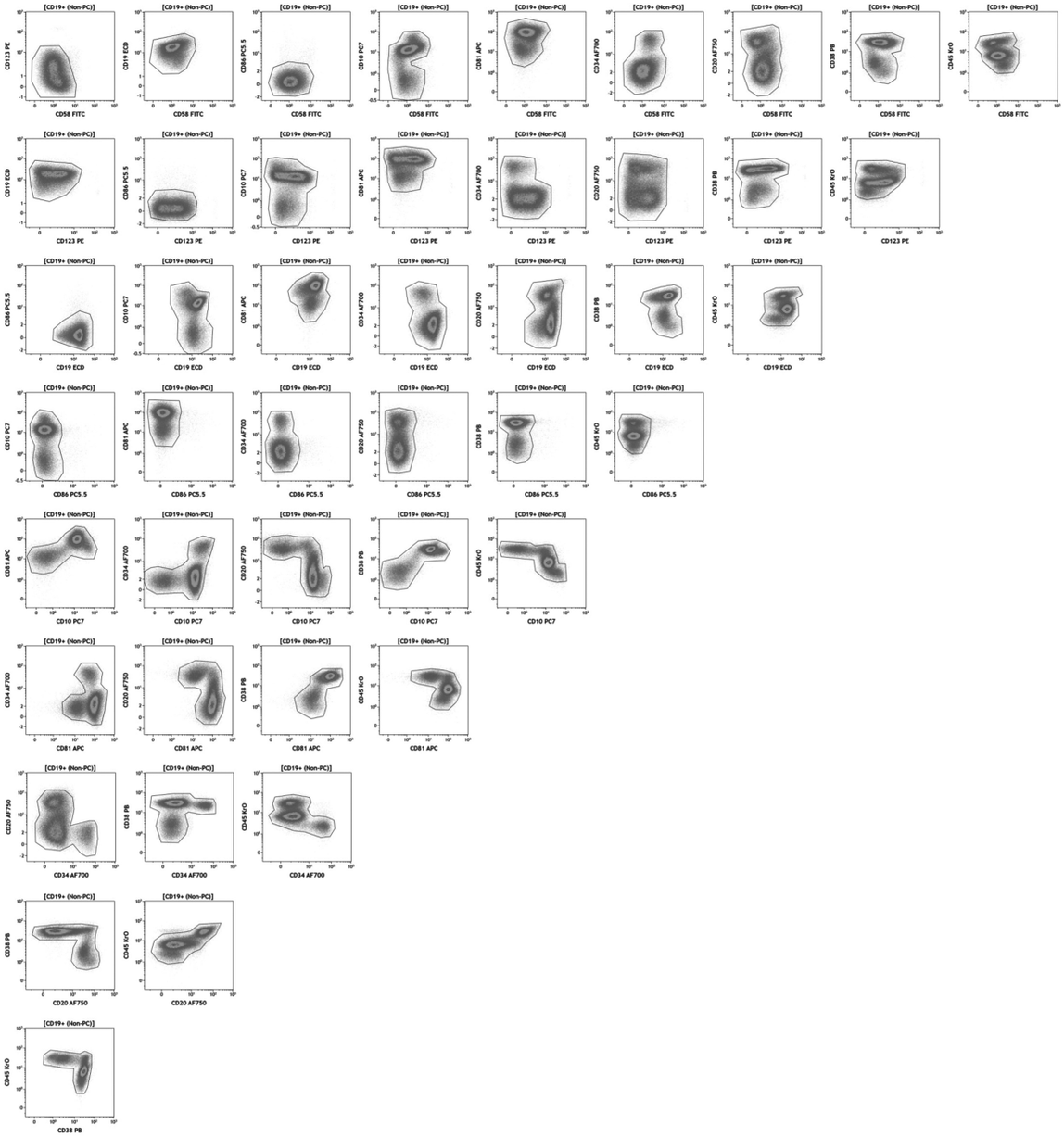

Fig. 1 Panel of biaxial plots showing hematogone maturation pattern using a 10-color antibody cocktail comprising of CD58 FITC, CD123 PE, CD19 ECD, CD86 PC5.5, CD10 PC7, CD81 APC, CD34 APCAF700, CD20 APCAF750, CD38 PB, and CD45 KrO. The plots depict CD19 gated population of cells after removal of plasma cells to create a template for assessment of B-ALL MRD.

remaining leukemic cells is any variation from these normal expression or patterns brought on by overexpression, underexpression, or asynchronous expression.[50] Among these CD19 positive cells, plasma cells should be delineated based upon their $CD38^{high}$, $CD10^{neg}$, $CD20^{neg}$ expression. Hematogones proliferation usually occurs after consolidation therapy and is recognized by their specific patterns of maturation (►**Fig. 1**). Hematogones are usually $CD81^{bright\text{-}pos}$ and $CD58^{moderate\text{-}pos}$. The early hematogones are $CD123^{neg}$ and $CD86^{neg}$; however, late hematogones can show a dim expression of CD123 and CD86. Any deviation from these recognized patterns or maturation arrest hints toward a possible MRD (►**Fig. 2**).[51] Although CD34 is a marker for immaturity, its expression is noted only in approximately 70% of cases of B-ALL.[52–54] Also, treatment may cause reduced levels of this marker, sometimes complete absence.[55] In addition, aberrant expression of myeloid markers, such as CD13, CD15, CD33, and CD66c, make these LAIPs useful for MRD evaluation.[56,57]

Newer parameters have been increasingly utilized to differentiate abnormal blasts from normal BCPs. Another effective marker for MRD diagnosis is CD73, which has great specificity and equal sensitivity to other common LAIPs. However, because of the bimodal nature of its expression in mature B cells, its interpretation must be done in the context of the expression of other markers. In hematogones,

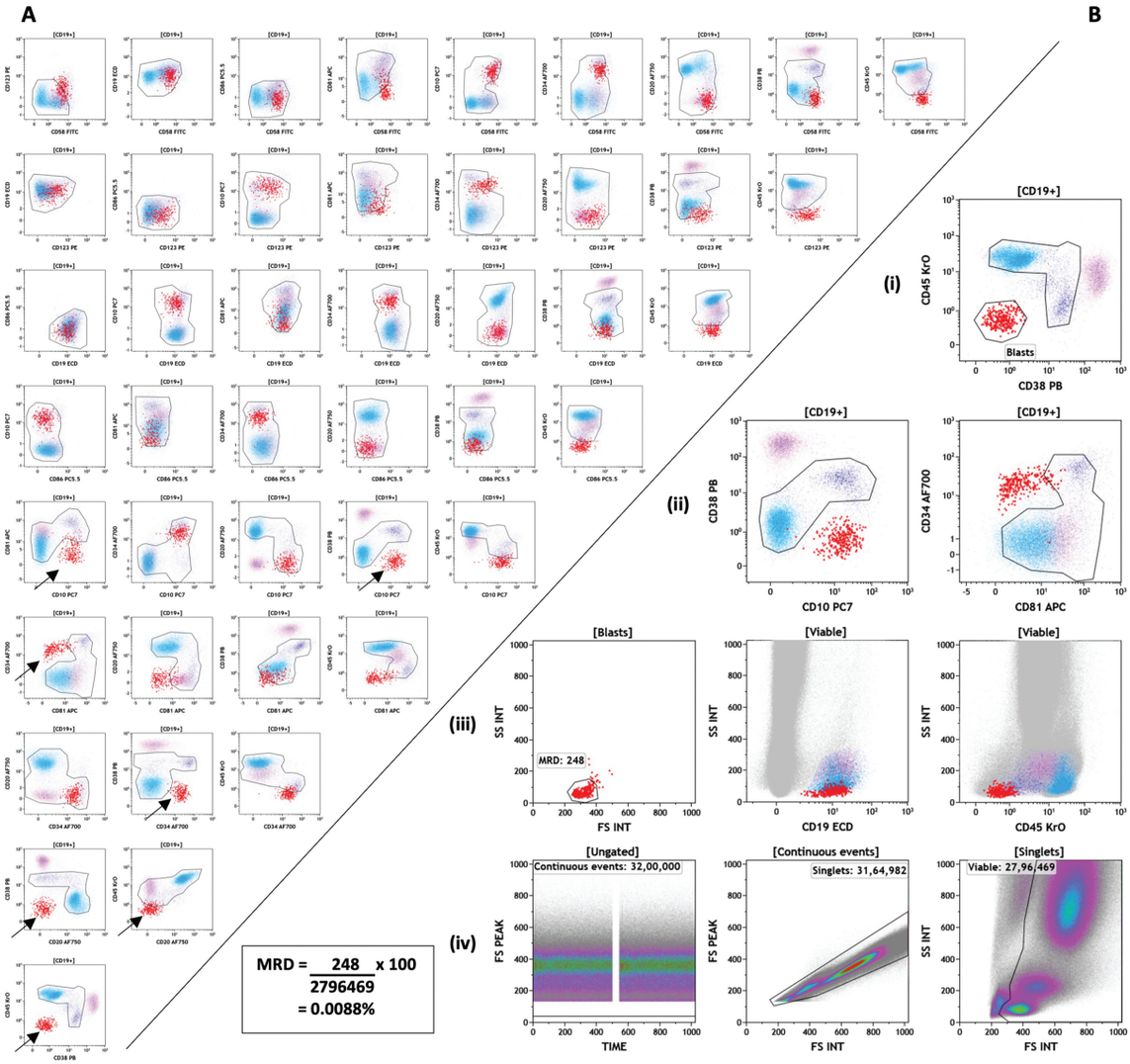

Fig. 2 (**A**) Panel of plots showing assessment of MRD on bone marrow aspirate sample from a patient of B-ALL at end of induction time point. The mature B cells are shown in light blue color, hematogones in dark blue, and plasma cells are in pink color. The events representing MRD are colored red and depicted in bolder dots than rest of the events. The MRD events are distinctly separate and lying outside the "hematogone-maturation pattern" in some of the plots (indicated by arrows), mostly in lower part of the panel. (**B**) Representative plots to show calculation of percentage of residual leukemic cells; (i) the residual blasts are gated on CD38 versus CD45 plot (red dots) where these show distinctly different expression for the two markers when compared with normal B cells; (ii) the gated residual blasts are shown in two other plots, that is, CD10 versus CD38 and CD81 versus CD34, highlighting their deviation from normal maturation pattern; (iii) forward scatter (FS) versus side scatter (SS) plot showing gating of tight cluster of cells to refine residual blast population which provides the numerator for calculating MRD, and a retrospective depiction of this MRD-cluster on CD19 versus SS and CD45 versus SS plots; (iv) the denominator for calculation of MRD is provided by gating viable cells on FS INT versus SS INT plot after gating continuous events on time versus FS PEAK plot and removing cell-aggregates on FS INT versus FS PEAK plot. The "text box" shows calculation of percentage of residual leukemic cells.

CD304 expression is weak and heterogenous. As B cells matures, its expression declines until it is completely missing. However, CD304 exhibits a robust expression when present on blasts.[58]

A common strategy is to create templates using the fixed scatter plots and predefined gates. These gates are designed after evaluating the normal BCPs on the treatment-naïve samples. End induction T-ALL, un-involved neuroblastoma/retinoblastoma, or non-Hodgkin lymphoma staging BM aspirate samples and BM samples from patients of immune thrombocytopenia are some of the scenarios where the template for B-ALL can be made. Any "deviation- from-normal" is readily identified.[59]

Effects of Immunotherapy

Recent enhancements in our understanding of disease biology have led to the development of targeted therapies. With the introduction of CD19 directed therapies (chimeric antigen receptor [CAR-T] and BiTE [bispecific T cell engaging small molecules], blinatumomab), the MRD assessment by FCM has become more challenging. Studies have shown that these therapies can lead to downregulation of CD19 on

leukemic cells by different mechanisms, which minimize its utility to be used as gating marker.[60,61] In this regard, alternate markers have to be used to delineate the abnormal cells. A study by Cherian et al had shown that CD22 and CD24 may be used to detect MRD B-ALL after anti-CD19 therapy. The B cells are identified by the expression of CD22 or CD24 without CD66b and include cells that may or may not have CD19 positive B cells, but express either CD22 or CD24. Such populations may include some dendritic cell populations and basophils, and as well include CD34 positive progenitor populations. Other considerations are relevant while using these two markers for MRD analysis. With regard to hematogones, there will always be a maturation pattern for CD22 and CD24. However, the expression of CD22 is dim in the early hematogones and gradually increases till mature B cell stage. CD24, on the other hand, shows bright expression in hematogones and diminishes as the cell mature. Therefore, these markers have to be used in addition to CD10 and CD34 among others to avoid false negative or false positive results.[62] Inotuzumab ozogamicin is an anti-CD22 monoclonal antibody conjugated to calicheamicin, a cytotoxic agent and causes double-stranded DNA breaks initiating apoptosis.[63] Anti-CD22 therapy leads to decreased expression of CD22 on B cells. In these cases, gating using CD79a along with use of additional markers CD24/CD66b among others is done.[64]

FCM-based analysis in patients receiving CAR-T therapy needs to be done in CAR-T infusion bag and in PB samples. The assessment has to be done at various time points following the infusion with the knowledge that there is known variations in CAR-T cell percentage over time. Usually, a peak in CAR-T cell percentage occurs 12 days after the infusion. Although exact time point is still undetermined, analysis at day 1, day 7, day 14, and day 30 ($\pm$2 days for each time point) following the CAR-T infusion is done. In terms of CD4+ and CD8+ balance within CAR-T cell subset, CD4+ cells are the main lymphocytes shortly after the infusion, CD8+ lymphocyte subset increased later on to reach first an equilibrium with CD4+ subset and then become the most significant lymphocyte population within CAR-T cells.[65] Having said that, it is extremely important for a clinician to mention the targeted therapy used in a given patient while sending out the requisition forms to avoid false negative results.

MRD in T-ALL

MRD is one of the key predictors of prognosis in T-ALL. MRD-based categorization of patients into low-risk allows them to be exposed to less intensive therapy and thus a lower risk of chemotherapy-related morbidity and mortality. MRD assessment is also useful in the setting of stem cell transplantation and for early recognition of impending relapse.[66–68]

Time Points for MRD Detection

The time points of measurement of MRD detection in T-ALL also depend upon the protocol used in therapy: As with B-ALL, in modified BFM, it is done 4 weeks postinduction; 22 week at the end of reinduction and end of therapy. An alternate protocol prefers at 6 weeks postinduction and at the end of therapy. In India, many centers use the ICiCLe protocol in children which involves MRD assessment only postconsolidation (week 11).[37,69,70]

Flow Cytometric MRD Analysis

Standard techniques for T-MRD include multiparametric FCM and PCR to detect clone-specific TCR gene and leukemia-specific fusion transcripts.[14] In contrast to precursor B-ALL, in T-ALL, PB sampling is equally useful as compared with BM sample and recent studies indicate that they have equal sensitivity in MRD detection. Precursor-B-ALL is of BM origin, which probably explains the higher MRD levels in BM than in the PB samples. T-ALL, on the other hand, is of thymic origin and shows a highly disseminating character, resulting in comparable MRD levels in BM and PB samples. PB blood MRD levels in T-ALL patients are comparable or up to 1 log lower than in BM.[23,71]

To find residual leukemic cells, it was initially suggested to look for immature T cells (cells co-expressing cCD3/TdT or CD7/TdT) outside of the thymus. However, it has been demonstrated that after treatment, the expression of immature antigens such TdT and CD99 is downregulated. A more effective strategy takes use of the difference in surface CD3 expression between mature and immature T cells, as well as leukemic T cells (►**Fig. 3**). For T lineage ALL, the MRD assay should preferably comprise of CD2, CD3, CD4, CD5, CD7, CD8, CD16, CD34, CD38, CD45, CD48, and CD56. Additional markers that may be used include CD1a, TdT, CD13, CD33, and CD117.[72] Mature T cells express a high level of surface CD3 and leukemic T cells often lack the surface expression but show a cytoplasmic CD3 expression. To achieve lineage specificity, cytoplasmic CD3 is combined with surface CD3, but to exclude the typically tiny subset of NK cells that may express cytoplasmic CD3, NK cell-associated antigens like CD56 and CD16 must also be included. Notably, immature NK cells are also CD16/56 negative. CD38, in combination of other T cell markers, helps in their exclusion. Aberrant T cells may show a lower antigen expression or complete absence of markers, such as CD5, sCD3, CD2, and dual negative or dual positivity of CD4 & CD8. Another frequent observation is the bright expression of CD7, and the expression of CD13/CD33 (lineage infidelity) can frequently aid in the identification of MRD.[73–75] Recently, a few new parameters have been studied for assessing the MRD in T-ALL. Median fluorescence intensity of CD48 was compared between normal T and NK cells and leukemic blasts that showed consistently decreased expression of CD48 on leukemic blasts which persisted even at postinduction phase of chemotherapy.[76]

Measures of Analytical Sensitivity of MFC Assays

Establishing the sensitivity of an assay designed for rare event analysis, such as MRD detection, is of paramount importance. The introduction of concepts of limit of blank (LOB), limit of detection (LOD) and lower limit of

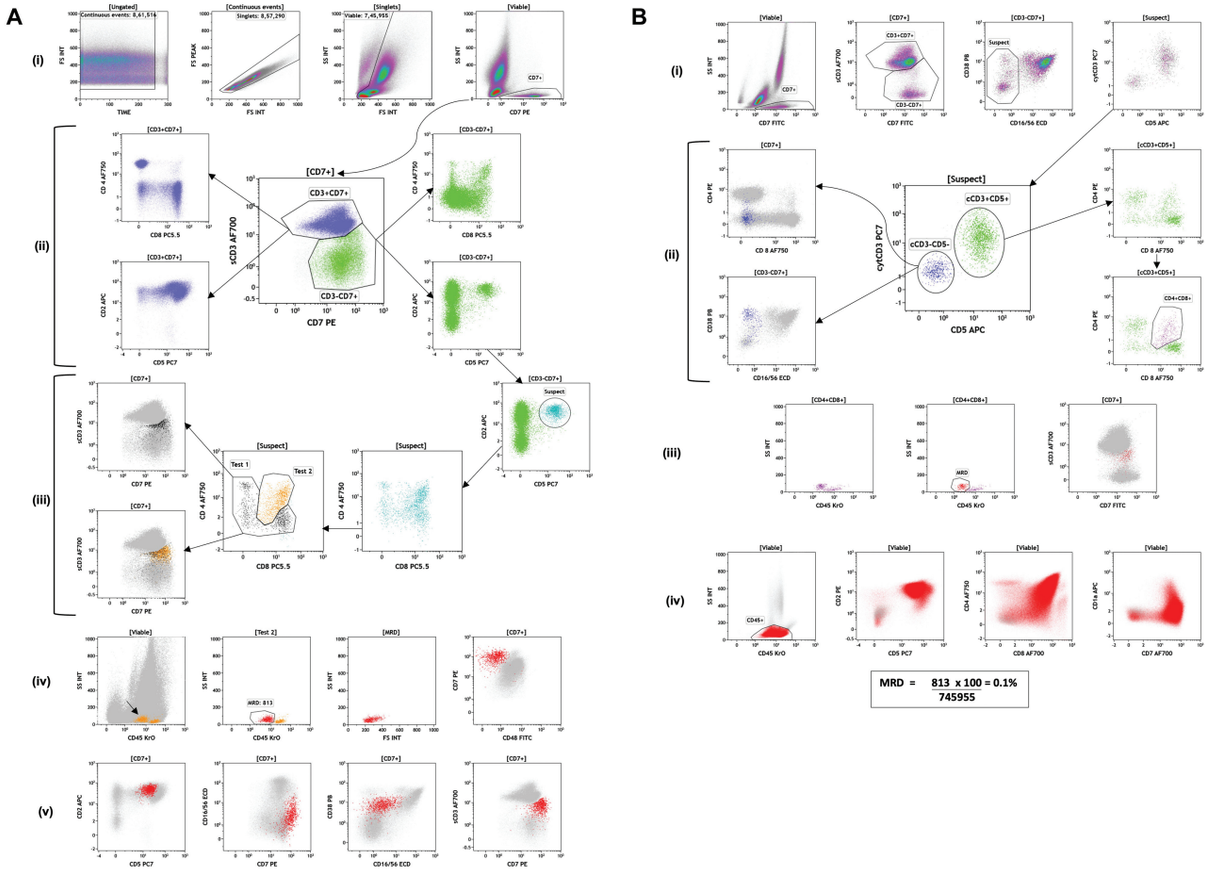

Fig. 3 (**A**) MRD analysis from bone marrow aspirate of a case of T-ALL on therapy, using a 10-color and two-tube assay. These representative plots are from analysis of first tube comprising of CD48 FITC, CD7 PE, CD16/CD56 ECD, CD8 PC5.5, CD5 PC7, CD2 APC, CD3 APCAF700 (Surface), CD4 APCAF750, CD38 PB, and CD45 KrO. (i) The four biaxial density plots show gating of CD7 variably positive and side scatter (SS) low events (labeled as "CD7 + "), after sequentially gating the continuous, nondoublet (single cell) and viable events. (ii) Panel of plots where CD7+ events have been shown in the central biaxial dot plot of CD7 versus CD3 (surface), which reveals two major populations, one of which is surface-CD3 positive and CD7 variably positive, labeled as CD3 + CD7+ (blue dots), and the other population is surface-CD3 negative and CD7 positive, labeled as CD3-CD7+ (green dots). The CD3 + CD7+ population represents normal T lymphocytes and show normal profile of CD4, CD8, CD2, and CD5, as noted in CD4 versus CD8 and CD2 versus CD5 plots (left side plots). The CD3-CD7+ population predominantly revealed immunophenotypic profile consistent with NK cells, that is, CD4 and CD8 negative, CD5 negative and CD2 variably positive (right side plots). However, a small discrete subset showed dual CD2 and CD5 positivity. (iii) The CD2 and CD5 dual positive cells were gated (labeled Suspect). These cells showed heterogeneity in CD4 and CD8 expression and were further gated as test 1 (black dotes) and test 2 (Orange dotes). Test 1 population was distributed among CD4 and CD8 and was normal T cell with slight downregulation of surface CD3 expression. Test 2 population had a dual CD4 and CD8 positivity and showed a dim surface CD3 expression. (iv) Retrospective look in CD45 versus SS plot showed a subset of test 2 population with dim CD45 expression (arrow) which was gated as the residual leukemic cell population, labeled MRD (red dots). This population showed a tight cluster on FS versus SS plot. It also showed loss of CD48 expression a with brighter CD7 expression than normal T cells on CD48 versus CD7 plot. (v) Retrospective analysis of residual leukemic cells with normal T/NK cells in the background. (**B**) Representative plots from analysis of second tube comprising of CD7 FITC, CD4 PE, CD16/CD56 ECD, CD34 PC5.5, CD3 PC7 (Cytoplasmic), CD5 APC, CD3 APCAF700 (Surface), CD8 APCAF750, CD38 PB, and CD45 KrO. (i) Biaxial density plots show gating of clean CD7+ events and further gating of two major subpopulations on CD7 versus surface CD3 plot. The CD3-CD7+ population is shown in CD16/CD56 versus CD38 plot with gating of CD16/CD56 negative and CD38 variable events (labeled as Suspect). This population has been shown in cytoplasmic-CD3 and CD5 plot that reveals two discrete subpopulations. (ii) Panel of plots where the "suspect" events have been shown in the central biaxial dot plot of CD5 versus cytoplasmic-CD3. The events labeled as cCD3-CD5- (blue dots) represent immature NK cells, noted further as CD4 and CD8 dual negative, and CD38 bright positive with absence of CD16/CD56. The cCD3 + CD5+ population (green dots) shows a cluster of CD4 and CD8 dual positive population in addition to normally distributed T cells. This dual-positive population is gated and labeled as CD4 + CD8+ (purple dots). (iii) The CD4 + CD8+ events reveal a CD45 dim cluster on CD45 versus SS plot which is further gated and labeled as MRD (red dots), noted to show a dimmer surface CD3 and a brighter CD7 expression than normal T cells, similar to that noted in first tube. (iv) Representative plots from the diagnostic sample showing CD45 dim blasts with dual positivity for CD2 and CD5 and for CD4 and CD8. The blasts also show a bright CD7 expression with only a minor population with CD1a expression. The text box shows percentage of MRD with numerator (MRD events) and denominator (viable events) calculated from the first tube.

quantification (LLOQ) in rare event analysis has been a remarkable advancement to ensure robust and reliable measurements of rare events. However, the LOB is the highest *apparent* analyte concentration which is expected to be found when multiple replicates of a blank sample containing no analyte are tested and are calculated as mean blank + 1.645 SD (standard deviation [blank]). LOD, on the other hand, is the lowest analyte concentration likely to be reliably distinguished from the LOB and is commonly defined as LOB + 1.645 SD (low positive samples). LOD is the level where 95% of low levels of events measure and are detected above LOB. LLOQ is the lowest level of measure and that can be reliably detected and the total error (bias and imprecision) meets a desired criterion for accuracy, defined on basis of clinical utility. The LLOQ is usually higher or at times equal to the LOD, but is never lower than it.[77] For rare event analysis like MRD assay, a strategy of replicate assay of negative samples as well as samples with low target population frequency can be used for estimating analytical sensitivity. For example, LOB can be established by acquiring five list mode files each from five replicates of five separate samples, which do not have target population of interest. LOB is confirmed when no more than 5% of the blank replicates exceed the low positive target. Similarly, LOD can be established by repeated assay of samples having low levels of the target population to determine SD and calculated imprecision. For clinical samples, 20 to 30 "relevant" events, with acquisition of one million or more clean events (devoid of debris and cell aggregates), are considered enough to classify rare population as "detectable," and at least 50 events can make such population "quantifiable." Increasing number of relevant events decreases CV, which is considered acceptable if around 10%, and increasing the total number of clean events increases the sensitivity of assay. The functional sensitivity or LLOQ may be determined by an approach in which serial dilutions of cells from a positive sample are spiked into a negative sample. It is then appropriate to verify a desired LLOQ by assaying at least five replicates near the LLOQ, each replicate analyzed and confirming that an acceptable level of imprecision is achieved.[78,79]

Limitations of Flow Cytometry-Based MRD

One of the prime limitations of detection of residual cells by FCM is the immunophenotypic shifts that are observed as a result of chemotherapeutic reagents. While the targeted therapy mentioned above results in overall reduced expression of the antigens concerned, other conventional agents (alkylating drugs and steroids) cause subtle shifts in the antigenic profile in the normal BCPs, accurate interpretation of which poses a great amount of challenge.[80] Some of the notable shifts include brighter expression of CD10 on early hematogones and proliferation of type 1 hematogones in end of induction samples compared with postconsolidation and subsequent samples.[81,82] Leukemic cells also show some changes in the antigenic profile most importantly downregulation of CD34 and CD10 and upregulation of CD19, CD20, and CD45.[55,74]

With respect to T-ALL, studies have reported decline in TdT and CD99, variable expression of CD10 and CD34 on leukemic blasts during therapy, while the lineage specific markers including CD2, CD3, CD4, CD5, CD7, and CD8 were relatively stable.[74]

Another important consideration includes the diluted sample that stresses upon the need to provide first pull of BM sample for achieving a high sensitivity MRD analysis.

The challenges mentioned above along with differences in antibody panel design and fluorochrome combination complicate interlaboratory standardization. Improved participation in external quality programs, like those provided by United Kingdom National External Quality Assessment Scheme (UKNEQAS) and by the College of American Pathologists (CAP), can help to improve and sustain quality of the assay. In India, an interlaboratory comparison program (ILCP) is being run by the Department of Hematopathology, ACTREC, Tata Memorial Centre, Navi Mumbai.

Future Considerations

Past decade has witnessed a plethora of advancements in methodologies for the detection of MRD that aim at higher sensitivity and broader applicability. EuroFlow Consortium has developed a high throughput "next-generation flow cytometry" which analyzes the data on multivariate plots wherein a test sample is compared with that of the standard one. A revolutionary piece of software, like Infinicyt, gauges how far cells in the test sample have deviated from the standard in terms of cell maturation and differentiation. Regular QA evaluations have completely standardized this method.[83,84]

Another breakthrough in MRD quantification is the NGS. EuroClonality-NGS Consortium has made guidelines/workflow as well as standardization for NGS-based Ig/TCR assays in hemato-oncology. This method entails identifying clonal Ig/TCR gene rearrangements that are unique to leukemia in the diagnostic sample. The identical set of primers as for RQ-PCR is used. These sequences are then employed for the detection of MRD in the subsequent follow-up samples. Compared with Sanger sequencing-based assays, NGS has a quicker turnaround time and greater sensitivity (down to even 10^{-7}), although this depends heavily on the capability of the laboratory. However, the amount of input DNA necessary to attain this high level of sensitivity should be substantial (~65 µg), which is difficult in postinduction marrow samples. Although becoming increasingly popular for acute myeloid leukemia, this technique is in its nascent stage to be implicated as a routine investigation in ALL patients.[85–87]

Conclusions

To conclude, detection of MRD using FCM has been in practice since 1990s and has superseded the light microscopy with much enhanced sensitivity. As the protocols have been standardized, there have been significant advancements in risk assessment and prognosis resulting in even more improvement in childhood and adult ALL cure rates.

Lymphocyte profiling and myriad of immunophenotypic markers allow quick and reliable assessment of MRD in ALL. In the light of recent development of novel and targeted therapies in ALL, the FCM-based MRD analysis has garnered even broader applicability. The EuroFlow consortium has developed new high-throughput concepts for flow MRD of ALL employing an eight-color single tube panel based on multivariate analysis, with the use of newer fluorochrome combinations projected to rise in the future decade. This would help to increase the applicability and specificity of measurements of flow MRD.

Conflict of Interest
None declared.

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Role of Conventional Cytogenetics and FISH in the Laboratory Work Up of Plasma Cell Dyscrasias

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Abstract

Plasma cell dyscrasias are a heterogeneous group of neoplasms characterized by abnormal proliferation of plasma cells with or without over production of monoclonal immunoglobulins. Chromosomal abnormalities are acquired either early in the course of the disease or during disease progression. Plasma cell dyscrasias are categorized into multiple cytogenetic subtypes that form an integral component of risk-stratified treatment protocols. The primary genetic events are *IgH* gene translocations and non-random gains of chromosomes 3/5/7/9/11/15/19 and or 21. The secondary genetic events consist of chromosome 1 abnormalities (1p deletion and 1q gain or amplification), deletion 17p/TP53, deletion 13q, and *MYC* gene rearrangements. Plasma cells being at the end of differentiation spectrum of B cells, have low proliferative potential precluding the use of karyotyping in identification of chromosomal abnormalities. Analysis of enriched plasma cells using interphase fluorescent in situ hybridization (FISH) is the technique of choice for identifying these abnormalities. It is essential to enrich plasma cells before the FISH analysis, and numerous plasma cell enrichment techniques have been described. In the paper, we review the cytogenetic approach to identify clinically significant genetic aberrations including the effective use of FISH panels and plasma cell enrichment techniques.

Keywords
- plasma cell dyscrasia
- multiple myeloma
- FISH
- MGUS
- SMM
- plasma cell leukemia
- cytogenetics in myeloma

Introduction

Plasma cell dyscrasias are a group of heterogeneous neoplasms characterized by clonal proliferation of plasma cells. Based on the percentage of plasma cell infiltration in the bone marrow, the type of monoclonal protein, association of CRAB (hypercalcemia, renal impairment, anemia, or lytic bone lesions) plasma cell dyscrasias are classified as[1,2]:

IgM monoclonal gammopathy of undetermined significance
Non IgM monoclonal gammopathy of undetermined significance
Smoldering (asymptomatic) plasma cell myeloma
Multiple myeloma (plasma cell myeloma):

- Multiple myeloma NOS
- Multiple myeloma with recurrent genetic abnormality
- Multiple myeloma with *CCND* family translocation
- Multiple myeloma with *MAF* family translocation
- Multiple myeloma with *NSD2* family translocation
- Multiple myeloma with hyperdiploidy

Solitary plasmacytoma of bone
Extraosseous plasmacytoma
Monoclonal immunoglobulin deposition disease
Immunoglobulin light chain amyloidosis (AL)
Localized AL amyloidosis
Light chain and heavy chain deposition disease

The plasma cells undergo several rounds of differentiation in the bone marrow and secondary lymphoid organs and

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involve V(D)J rearrangements and somatic hypermutation with class switch recombination.[3] Sentinel chromosomal abnormalities acquired in the process of maturation and differentiation result in neoplastic transformation of the plasma cells.[3] These cytogenetic abnormalities are integral to risk stratified treatment protocols. The founding or primary chromosomal abnormalities that occur early in the course of the disease are *IgH* gene rearrangements and aneuploidy.[3,4] Secondary chromosomal abnormalities are either acquired or enriched at disease progression and include deletion of short arm of chromosome 17 (deletion 17p/*TP53*), deletion of short arm of chromosome 1 (1p deletion), gain or amplification of long arm of chromosome 1 (1q gain/amp), deletion of long arm of chromosome 13 (13q deletion) and *MYC* gene rearrangements.[4]

In *IgH* gene rearrangements, the promoter sequences of the *IgH* enhancers cause overexpression of the partner genes. The recurrent *IgH* translocations in multiple myeloma (MM), involve *CCND1* at 11q13, *CCND3* at 6p21, *FGFR3*/*MMSET*/*CCND2* at 4p16 resulting in increased expression of cyclin D family of genes that promote cell cycle progression and increased proliferation. *IgH* translocations involving the *MAF* family of genes include *MAF* at 16q23 and *MAFB* at 20q11, resulting in the upregulation of *MAF*-associated transcription process.[5]

Aneuploidies include hyperdiploid MM and the non-hyperdiploid MM. Hyperdiploidy in plasma cells neoplasms is characterized by nonrandom gains of chromosomes 3, 5, 7, 9, 11, 15, 19, and 21. The gains of chromosomes result in gene dosage effects, altering gene expression. Gains of chromosome 11 is associated with the overexpression of the *CCND1* gene. Nonhyperdiploid MM includes hypodiploid (<45 chromosomes) and pseudodiploid (45–46 chromosomes).[5,6]

Secondary cytogenetic abnormalities can be present either at diagnosis or may be enriched or acquired during progression of the disease. The molecular mechanisms that promote progression include the activation of the *RAS* pathway and *MYC* overexpression accompanied by DNA hypomethylation leading to genomic instability (►**Fig. 1**).

In this review, we will focus on the role of cytogenetics for the work up of plasma cell dyscrasias.

Cytogenetic Risk Stratification

MM is a heterogeneous disease characterized by multiple genetic subtypes that have varied response to treatment and are an integral component of risk stratification of the disease. The impact of cytogenetic abnormalities on time to progression (TTP) varies according to the type of plasma cell dyscrasia. In MM, the standard risk abnormalities include t(11;14), t(6;14), and hyperdiploidy (gains of 3/5/7/9/11/15/19 and or

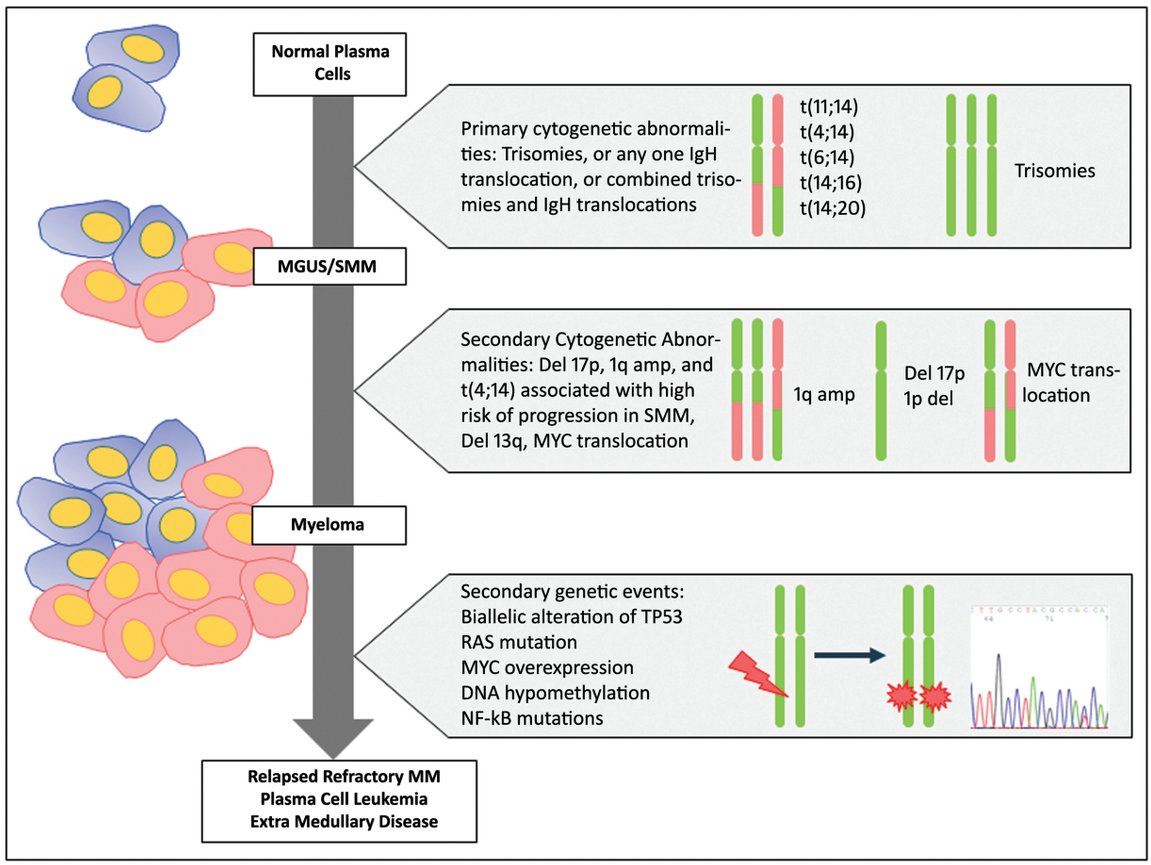

Fig. 1 Cytogenetic and genetic abnormalities occurring in the evolution of plasma cell dyscrasias.

21) and high-risk abnormalities include t(4;14), t(14;16), t (14;20), deletion of short arm of chromosome 17 (Del 17p), gain or amplification of long arm of chromosome 1 (1q gain/amp), deletion of short arm of chromosome 1 (1p deletion), *MYC* gene rearrangements, and deletion of long arm of chromosome 13 (13q deletion).[1,2,7,8]

The revised international staging system for myeloma established by the International Myeloma Working Group includes high-risk cytogenetic abnormalities such as t(4;14), t(14;16) deletion 17p/*TP53* along with serum albumin, serum β-2 microglobulin, and serum lactate dehydrogenase levels. The second revision of the RISS excluded t(14;16) and included 1q amplification in the scoring system.[9]

The Mayo Clinic includes both the standard risk and high-risk cytogenetic abnormalities in the Stratification for Myeloma and Risk Adapted Therapy (mSMART) protocol (►**Table 1**).[10–13]

Double-Hit and Triple-Hit Myeloma

The mSMART 3.0 proposed by the Mayo Clinic has proposed a concept of double-hit myeloma and triple-hit myeloma similar to the concept seen in lymphomas.[14] These are defined as follows:

High-risk abnormalities include deletion 17p/*TP53*, 1q gain/amp, t(4;14), t(14;16), t(14;20)

Double-hit MM (DH-MM) includes any two high-risk abnormalities

Triple-hit MM (TH-MM) includes three or more high-risk abnormalities

These are considered as the 'ultra-high risk' abnormalities showing rapid disease progression and shorter overall survival as compared to MM having a single or no high-risk abnormality. The most common high-risk abnormalities seen were 1q gain/amp, t(4;14), deletion 17p/*TP53* in several studies.[15,16]

Table 1 mSMART 3.0 criteria for risk stratification of active multiple myeloma

mSMART 3.0 (risk stratification of active MM)	
Standard risk	**High risk** [a,b]
Trisomies of 3/5/7/9/ 11/15/19 and or 21*	t(4;14)
t(11;14) [d]	t(14;16)
t(6;14)	t(14;20)
	Del 17p
	TP53 mutation
	1q gain
	• R-ISS stage 3 • High-plasma cell S-phase[c] • GEP: High-risk signature
Double-hit myeloma: any 2 high-risk abnormalities	
Triple-hit myeloma: ≥3 high-risk abnormalities	

[a]Trisomies may ameliorate.
[b]By FISH or equivalent method.
[c]Cutoffs vary.
[d]t(11;14) may be associated with plasma cell leukemia.

Cytogenetic Lab Approach in MM

The cytogenetic strategy in plasma cell dyscrasia is based predominantly on FISH analysis of neoplastic plasma cells. The use of karyotyping has decreased in recent years due to reasons explained below. Although single nucleotide polymorphism (SNP) array has been used to identify copy number abnormalities, it does not identify fusions and the results have to be integrated with FISH analysis.[17,18]

Transcriptomic analysis has been used to identify high-risk gene expression signatures.[19,20] This review will focus on FISH-based approach to identify the genetic subtypes.

Karyotyping (Conventional Cytogenetics)

Karyotyping relies on the ability of plasma cells to divide, which is limited, making it difficult to acquire metaphases for study. Stimulants such as lipopolysaccharide (LPS), 12-O-tetradecanoylphorbol-13-acetate (TPA or phorbol 12-myristate 13-acetate), and cytokines (interleukin-6 and granulocyte–macrophage colony-stimulating are used to increase the yield of metaphases, their role in IgM-negative B cells being limited.[21,22] Oligonucleotides containing CpG motif, such as synthetic DSP30, can stimulate cells of the immune system in vitro and hence can be used to increase the yield of metaphases in vitro.[23] Various cytokines such as IL-10, IL-2, and TNF-alpha can also be used as mitogens for increasing the yield of metaphases.[23] Though G-banded analysis has the advantage of whole genome analysis at a low resolution (►**Fig. 2A**), low or no yield of metaphases from the plasma cells limits its utility in identifying the subtypes. Karyotyping fails to identify cryptic translocations involving the *IgH* locus such as t(4;14) and t(14;16) and a subset of cryptic 17p deletions. Hence, FISH is a superior tool with better sensitivity and specificity in identifying the clinically relevant genetic subtypes.[17,24]

Fluorescent in-situ Hybridization

FISH does not require live cells and can be performed on interphase cells. Plasma cell infiltration of the bone marrow can be patchy in MM, and the percentage of plasma cells in the marrow may vary. It is essential to enrich plasma cells before application of probes and performing the FISH analysis. Currently, FISH is the preferred tool to identify the genetic subtypes for risk stratification in MM patients.[24–27]

Various Plasma Cell Enrichment Techniques

1) Magnetic cell sorting (MACS)
2) Fluorescence activating cell sorting (FACS)
3) Targeted manual sorting
4) Customized automated image analysis
5) Cytoplasmic immunoglobulin FISH (cIg-FISH)
6) Target FISH[28]

Magnetic Cell Sorting

The adhesion of the plasma cells to an antibody cocktail serves as a basic principle for sorting the cells. Fresh heparin bone marrow samples are treated with an anti-CD138 and

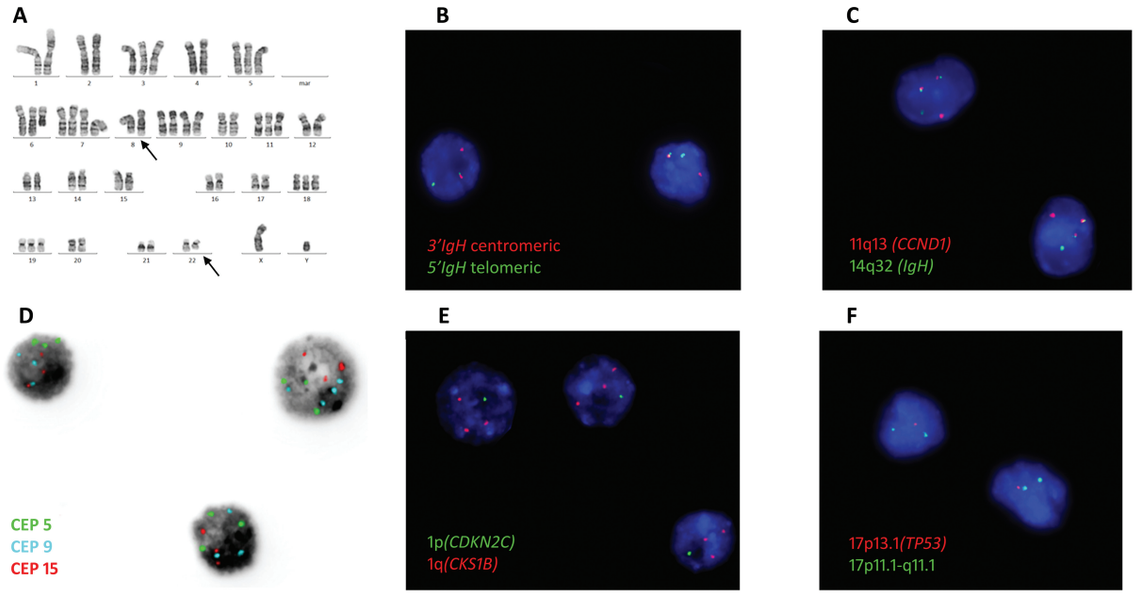

Fig. 2 (**A**) GTG banded karyotype image showing hyperdiploidy with deletion of long arm of chromosome 6 and a balanced translocation between the long arms of chromosome of 8 and 22. (**B**) Interphase FISH with *IgH* break-apart probe positive for *IgH* rearrangement. (**C**) Interphase FISH with *CCND1::IgH d*ual color dual fusion probe positive for t(11;14). (**D**) Interphase FISH with LSI D5S23, D5S721/CEP 9/CEP 15 tricolor probe depicting trisomies for chromosomes 5,9,15. (**E**) Interphase FISH with 1p(*CDKN2C*)/1q(*CKS1B*) LSI probe depicting deletion of 1p(*CDKN2C*) and gain of 1q(*CKS1B*). (**F**) Interphase FISH targeting*TP53* gene depicting *TP53* deletion.

anti-dextran antibody complex. Magnetic beads are added that bind to the dextran complex and this is subjected to a magnetic chamber for separation. The unbound particles are washed off and the mixture obtained contains the sorted plasma cells (►**Fig. 3**). The technique is cost-effective and requires fresh samples, as the yield decreases with the age of the sample.[28]

Fluorescence Activating Cell Sorting

Flow cytometric sorting of neoplastic plasma cells is performed using a cocktail of various antibodies (e.g., anti-CD45, anti-CD56, and anti-CD38) along with assessment of light scattering ability of plasma cells. FACS is more efficient than MACS as it uses multiple antibodies and parameters to identify and sort neoplastic plasma cells, thereby increasing the yield of plasma cells available for characterization. Higher capital and maintenance costs and restricted access coupled with cost of monoclonal antibodies have limited the use of sorter in most of the routine diagnostic laboratories.[28,29]

Targeted Manual Sorting

Targeted manual sorting relies on morphological identification of large mononuclear cells. One of the limitations of Target FISH is the intensity of signals that is weak and not uniform across the slide. The technique is subjective, requires skilled manpower, is time consuming with an increase possibility of false-negative results.[28]

Customized Automated Image Analysis

FISH slides are subjected to automated slide scanning where the mono nuclear cells are identified using a software classifier. The ability to count more cells increases the sensitivity of this technique as compared to manual analysis. The major drawback is the capital costs and infrastructure required to store and analyze the images.[28]

Cytoplasmic Immunoglobulin

Plasma cells are differentiated from other cells in the marrow by staining them with anti-kappa or anti-lambda antibodies (cytoplasmic immunoglobulin FISH [clg-FISH]). The method is tedious, requires additional time for careful meticulous examination of the slides. In cases with aggregation of small plasma cells, the analysis is difficult. The modified cIg FISH has refined the identification of plasma cells by fixing the plasma cells to avoid clumping or aggregation by replacing 96% ethanol wash with 100% methanol. The method is the preferred technique of FISH on plasma cells in a large number of laboratories.[30–32]

Target FISH

Plasma cells are sorted by centrifugation using Ficoll and are then stained with May–Grünwald Giemsa (MGG) stain. Plasma cells are identified based on morphology and are captured using an automated system. These slides are then de-stained and FISH is performed on the same slides.

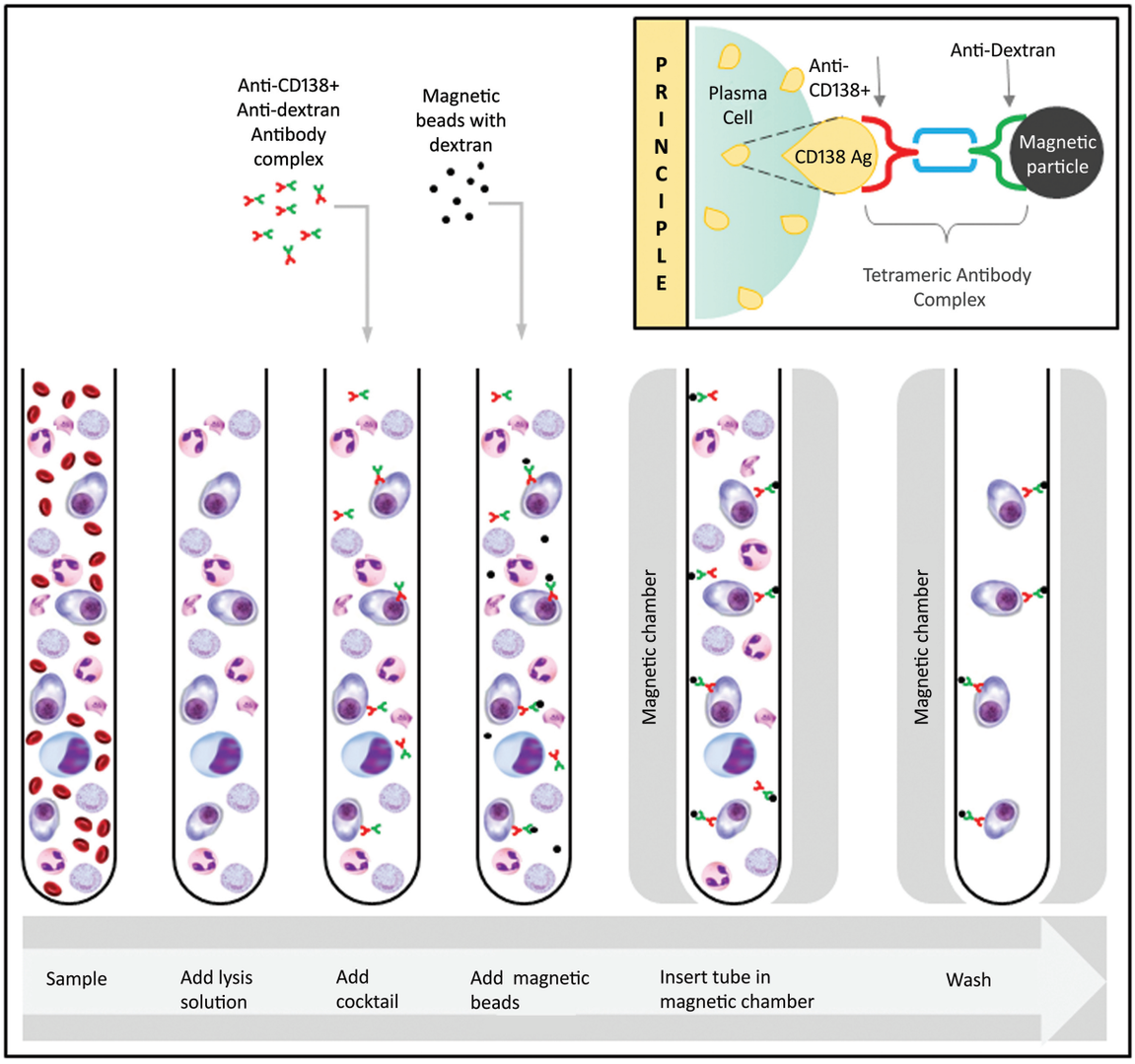

Fig. 3 Magnetic cell sorting (MACS) of CD138+ plasma cells using anti-CD138 and anti-dextran complex through positive selection of plasma cells in a magnetic chamber.

Plasma cells are identified based on the analysis of previously captured and analyzed images and FISH signals are recorded on the same cell. The disadvantage is that thousands of images are captured and there are reports of discordant results between the images captured.[33]

Quantitative Multigene Fluorescent in-situ Hybridization

Quantitative multigene fluorescent in-situ hybridization (QM-FISH) is a cytological high-resolution technique used to identify heterogeneity and clonal evolution in cases of MM. Single cell analysis could provide new insights into the clonal evolution in MM. Because MM is a heterogenous disease, clonal evolution is a continuous process and QM-FISH can only identify selective abnormalities. QM-FISH could prove to be an important tool to analyze the new emerging clones with its sensitivity being similar to conventional FISH.[34]

Pre-analytical Variables and Quality Control

The first pull bone marrow aspirate sample is the preferred sample for cytogenetic studies in MM. The sample should be transported as soon as possible to the laboratory and processed with minimum delay. A delay in the transport and processing of samples results in the depletion of the plasma cells impacting the results of the study. Hemolyzed and clotted samples adversely impact the plasma cell enrichment process and a repeat sample should be requested. While standardizing the plasma cell enrichment process using MACS, flow cytometry-based analysis should be performed to evaluate the efficiency of the enrichment process to positively select the CD138-positive plasma cells. The laboratory should perform analysis on known positive and

negative samples to calculate the cut-off for each probe. A recommendation of 10% for fusion or break-apart probes and 20% for numerical abnormalities has been mentioned in some studies.[35]

These recommendations are not universally accepted and ideally the laboratories should define their own cut-off values for each probe. It is expected that in samples with plasma cell enrichment the primary abnormalities will be present in the majority of the cells as compared to the secondary abnormalities. Ideally, 100 cells should be evaluated by at least two analysts.[35] Cut-off values for each probe can be calculated by either using CV with standard deviation and beta inverse function or using the Excel (Microsoft, Redmond, WA) statistical function CRITBINOM (n, p, α) with a confidence level of 95%.[36]

FISH studies on bone marrow biopsies are technically challenging with high frequency of failure due to the use of acids in decalcification process. Fixation in 10% neutral buffered formalin for at least 24 hours and EDTA-based decalcification are recommended for optimal FISH analysis on bone marrow biopsy specimens. In patients, showing patchy interstitial infiltration by plasma cells in the bone marrow biopsy, identifying the plasma cells is challenging and essential expertise is required to avoid false-negative results.

Table 2 Stepwise FISH strategy in multiple myeloma

At diagnosis
IgH gene rearrangement
Deletion 17p
1q gain/amplification
1p deletion
8q24.1 rearrangement, *MYC* break apart
Deletion 13q
CEP 5/9/15 (By FISH or flow ploidy)
If positive for IGH gene rearrangement
t(11;14)(q13;q32), *CCND1::IgH* fusion
t(6;14)(p21;q32) *CCND3::IgH* fusion
t(4;14)(p16.3;q32) *FGFR3::IgH* fusion
t(14;16)(q32;q23) *IgH::MAF* fusion
t(14;20)(q32;q12) *IgH::MAFB* fusion
At follow-up
Deletion 17p
1q gain/amplification
8q24.1 rearrangement, *MYC* break apart

Probe Selection

To identify deletions/amplifications dual-color locus specific probes with an internal control is recommended. A break-apart probe is used for identifying *IgH* and *MYC* rearrangements. Dual color fusion probes are used to identify specific *IgH* partners.[35,37]

Step Wise FISH Strategy

FISH analysis to identify the clinically relevant genetic subtypes is performed in a stepwise manner to judiciously utilize the enriched plasma cells and save costs (►**Table 2**). The first step involves testing for 17p,13q deletion, 1q gain/1p deletion, *IgH* and *MYC* rearrangement using respective break-apart probes and centromeric probes targeting 5,9,15 to identify trisomies. Based on the results of the initial panel, patient samples positive for *IgH* rearrangement (►**Fig. 2B**) are reflex tested using specific fusion probes to identify the partner. Follow-up samples are tested for deletion 17p, *MYC* rearrangements, and 1q gain/amp.

Subtypes Based on Genetic Abnormalities

IgH Rearrangements

IgH rearrangements occur early in the course of the disease. The rearrangements result in over expression of the partner gene due to the impact of enhancer elements in the promoter regions of the *IgH* gene.[38] The *IgH* rearrangements based on the partner gene can be sub grouped as

IgH Rearrangements with CCND Family: t(11;14) and t(6;14)

Translocations involving the *CCND* family of genes are associated with standard risk as per the mSMART criteria. t(11;14)(q13;q32) involves the *CCND1* gene (►**Fig. 2C**) and is seen in 15-20% of patients, while t(6;14) (p21;q32) involves the *CCND3* gene and is seen in < 5% of patients. The translocations cause dysregulation in cyclin D, resulting in the *RB1* gene inactivation leading to cell cycle progression.[5,6,39,40] Patients with t(11;14) also show overexpression of *BCL2* and are known to benefit from therapy with Venetoclax (*BCL2* inhibitor).[41,42] Though t(11;14) is associated with standard risk it has been reported in cases of primary plasma cell leukemias and in association with *MYC* rearrangements.[43,44]

IgH Rearrangements with MAF Family Translocation

Translocations t(14;16)(q32;q23) and t(14;20)(q32;q11) are seen in < 3% and < 1%, respectively, are associated with high risk. These upregulate the *MAF* family of genes, thereby activating the transcription by overexpression of *CCND2*.[5,6,40]

IgH Rearrangements with NSD2 Translocation

Translocation t(4;14)(p16;q32) is seen in 15% of the cases and is associated with high risk. It causes the overexpression of *FGFR3* (fibroblast growth factor receptor 3) and *NSD2* (nuclear receptor binding SET domain protein 2), the latter also known as *MMSET* (multiple myeloma SET domain protein). *FGFR3* plays an essential role in regulating cell proliferation, differentiation, and apoptosis. The majority cases of t(4;14) show the overexpression of *CCND2*, a cell cycle regulator, though the exact mechanism is not understood.[5,6]

Aneuploidy

Aneuploidies are recurrent cytogenetic subtypes in MM and can be categorized into hyperdiploid and nonhyperdiploid groups.

Hyperdiploidy

Hyperdiploidy (>46 up to 75 chromosomes) is associated with standard risk and is seen in ~55% of patients. It is characterized by non-random gain of chromosomes 3/5/7/9/11/15/19 and or 21 (►**Fig. 2D**). Studies have shown that trisomy 3 and trisomy 5 are associated with a better overall survival (OS), whereas trisomy 21 is associated with a poor OS.[45,46] The diagnosis of hyperdiploid MM is based on the presence of at least gains of two or more chromosomes in the absence of any monosomies on FISH analysis.[47]

The non-hyperdiploid MM group includes hypodiploidy (<45 chromosomes) and pseudodiploidy (45–46 chromosomes). The most common monosomies seen are for chromosomes 13, 14, 16, and 22. This group is associated with a poor prognosis.[6,48]

Chromosome 1 Abnormalities

1q Gain/amplification

Gain/amplification of long arm of chromosome 1 (1q21) is seen in 28 to 44% cases of MM. It is a secondary abnormality acquired during progression of disease. The copy number gain of 1q21 (+1q21) is subclassified into gains (3 copies) and amplification (≥4 copies). Prognostically, 1q21 amplification is associated with a worse overall survival as compared to gain of 1q21[49] (►**Fig. 2E**). Over expression of *CKS1B* was initially thought as the driver event, but later multiple genes such as *MUC1*, *MCL1*, *ANP32E*, *BCL9*, *PSMD4*, *PDZK1*, *NEK2*, *ARNT*, *ILF2*, and *ADAR1* have been known to play a role in progression.[49–54] Jumping translocations involving 1q is another known mechanism that results in gains of 1q.[53] The gain of 1q21 is considered as high risk as per the mSMART v 3.0 criteria and has been incorporated into the second revision of RISS.[9]

1p Deletion

Deletion of short arm of chromosome 1 is seen in 18 to 38% of patients and is associated with poor outcomes. The recurrent deletions are seen at loci 1p22 and 1p32 (►**Fig. 2E**). While deletions involving the locus 1p22 have not shown significant impact on survival, deletions involving the locus 1p32 (houses genes *CDKN2C* and *FAF1*) are associated with t(4;14), deletion 17p/*TP53* and 13q deletion. Some studies have suggested deletion of 1p32 as an independent prognostic risk factor with a high risk for relapse and early death.[55–57]

Deletion 17p/*TP53*

Deletion 17p/*TP53* is associated with high-risk disease and can be acquired or enriched on disease progression[58] (►**Fig. 2F**). The bi-allelic inactivation of the *TP53* gene is a marker of 'ultra-high risk disease.'[59] Recently, this entity has also been included as a criteria for double/triple-hit myeloma.[13]

Chromosome 13

Monosomy 13 and large deletions on long arm of chromosome 13 are established recurrent cytogenetic abnormalities in MM, resulting in deletion of the *RB1* gene. The size of the deletions varies and a commonly deleted region of pathogenic significance has not been clearly defined. Previous studies have inferred that poor prognosis in 13q deletions is associated with large deletions/monosomy 13 identified on karyotype rather than smaller deletions identified on FISH only. [60] However, the observation was not consistent in subsequent studies and it is now known that adverse prognosis in 13q deletions is more due to its association with other high-risk markers such as t(4;14) and deletion 17p/*TP53*. [6]

Role of *MYC*

MYC translocations are seen in 13 to 15% of newly diagnosed or relapsed cases of MM. These are commonly associated with older age group, plasmablastic morphology, hypercalcemia, 1q amplifications, and a poor outcome.[61–63] PARP1 inhibitors have been suggested as a therapeutic option in patients with *MYC* rearrangements based on the evidence that *MYC* acts as a promoter of PARP1 mediated repair in MM.[64] *MYC* gene rearrangements involving the enhancer elements of the kappa and lambda light chain genes, i.e., t(8;22)(q24;q11) and t(2;8)(p12;q24) are rare and are associated with light chain type of monoclonal gammopathy[65] (►**Fig. 2A**).

Data from India on Frequencies of Cytogenetic Abnormalities

There are a few studies on cytogenetic characterization of myeloma patients from India, one of the largest one describes the frequency of cytogenetic abnormalities in 475 patients identified on FISH analysis on enriched plasma cells. The study reported abnormalities in 66% of patients with high-risk abnormalities in 52% of patients. The study also reported smaller clonal size of secondary abnormalities compared to primary abnormalities. The frequency of t(4;14) was reported to be higher than t(11;14).[37]

Role of FISH in Other Plasma Cell Dyscrasias

Monoclonal Gammopathy of Undetermined Significance (MGUS)

MGUS is characterized by the presence of serum monoclonal protein < 3 g/dL and the bone marrow shows < 10% PCs and have a 1% per year risk of progression to MM.[19,66]

Deletion 17p/*TP53*, t(4;14), and trisomies are associated with a shorter time to progression (TTP) as compared to the other cytogenetic markers in MGUS. In these patients, it was suggested that trisomies may be an indicator of genomic instability, hence resulting in shorter TTP.[67]

Smoldering Multiple Myeloma (SMM)

SMM represents a stage between MGUS and MM and is characterized by a serum monoclonal protein ≥ 3 g/dL, bone marrow PCs ≥ 10% without CRAB features (hypercalcemia, renal impairment, anemia, or lytic bone lesions). The risk of progression to MM is 10%, 3%, and 1% per year during the first 5 years, next 5 years, and subsequent 10 years after diagnosis respectively.[68] In a large study with cytogenetic

data available in 689 SMM patients t(4;14), t(14;16), +1q, 13q deletion/monosomy 13q and deletion 17p/*TP53* were associated with a shorter probability of progression at 2 years.[69] In other studies, the presence of t(4;14), deletion 17p/*TP53*, and 1q gain/amp were found to be associated with shorter TTP.[70,71]

Plasma Cell Leukemia

Plasma cell leukemia (PCL) is an aggressive form of plasma cell dyscrasia and can present as de novo primary PCL (pPCL) or can be derived from a pre-existing plasma cell dyscrasia, as a secondary PCL(sPCL). In PCL *IgH* translocations, deletion 17p/*TP53*, 1q gain/amp, and *MYC* gene rearrangements are associated with a high risk. pPCL is frequently associated with t(11;14) and sPCL with t(4;14).[72] Translocation t(11;14) a standard risk marker in MM is associated with an aggressive course in patients of pPCL.[72–74] The two genes reported to be involved in t(11;14) are *CCND1* and *MYEOV*. *MYEOV* is not commonly seen involved in MM patients.[75]

Summary

Plasma cell neoplasms are characterized by primary founding chromosomal abnormalities that includes *IgH translocations* and aneuploidies. Secondary abnormalities acquired at a later stage of the disease evolution includes 17p/13q deletions, 1q gain/amp,1p deletion and *MYC* rearrangements.

FISH is the most effective efficient cost-sensitive genomic technique to identify the cytogenetic abnormalities that are essential for risk-stratified therapy in modern treatment protocols. It is essential to enrich the plasma cells before FISH analysis to ensure accurate identification of the abnormality.

Authors' Contributions

A.D. and M.P. wrote the original draft, S.D. prepared the figures; M.P. and A.D. have full access to all data and the final responsibility for publication. All authors reviewed the manuscript draft submitted for publication.

Conflict of Interest

None declared.

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Voyage of Measurable Residual Disease Assessment in Multiple Myeloma Using Multiparametric Flow Cytometry

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Abstract

Measurable residual disease (MRD) in multiple myeloma (MM) has emerged as one of the strongest and independent biomarkers to evaluate therapeutic response for the prediction of long-term treatment outcome. With the incorporation of MRD in response assessment criterion by International Myeloma Working Group, it has become the routine parameter to be assessed at various time points after therapy. Among various techniques to assess MRD, multiparametric flow cytometry (MFC)-based MRD estimation has evolved dramatically over the last two decades achieving sensitivity comparable to molecular methods. Next-generation flow cytometry with the incorporation of innovative tools in MRD detection including consortium-based guidelines for preanalytical and analytical factors led to the overall improvement in MFC-based MRD detection. However, flow cytometry assays suffer from inherent challenges ranging from procedural hemodilution to lack of harmonization and standardization across the centers.

This review article outlines and summarizes the essential laboratory prerequisites for reproducible MRD analysis by flow cytometry. Furthermore, a brief account of the utility of MRD evaluation in clinical practice as predictor of response and long-term treatment outcome has also been discussed. Considering the evolution of MFC-based MRD over two decades from a scientific research tool to a routine clinical diagnostic assay, it needs to be explored further in studying complex phenomenon like clonal evolution, clonal switches, and identification of treatment refractory clones for guiding more effective therapies improving overall survival.

Keywords

- multiple myeloma
- flow cytometry
- next-generation flow
- measurable residual disease

Introduction

Multiple myeloma (MM) is a hematological malignancy characterized by the proliferation of clonal plasma cells (PC) in the bone marrow (BM) and presence of monoclonal protein (M protein) in serum and/or urine and lytic bone lesions. Though the development of novel therapeutic strategies has improved the overall prognosis, a substantial number of patients relapse despite achieving good clinical response. Following the current guidelines of response assessment in MM, over two-thirds of the patients achieving complete remission (CR) relapse within 2 years.[1,2] Thus, in order to refine the response assessment in MM, International Myeloma Working Group (IMWG) included the measurable residual disease (MRD) assessment as additional response criteria in 2016.[3] MRD in MM is defined as the residual small

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number of cancer cells surviving after treatment that is not identifiable by routine clinical and laboratory parameters.

IMWG recommends either intramedullary or extramedullary MRD detection based on BM aspirate (BMA) samples and imaging techniques respectively.[3] Extramedullary MRD detection includes imaging techniques, such as positron emission tomography with computed tomography using ^{18}F-deoxyglucose or magnetic resonance imaging.[4]

Earliest evidence of MRD assessment in MM came in late 1980s when an attempt was made to detect residual PC in patients' BM biopsies using immunohistochemistry, though the technique was too crude to estimate MRD.[5] Following this, researchers in the initial years estimated MRD in MM by qualitative polymerase chain reaction (PCR) followed by quantitative allele-specific oligonucleotide PCR (ASO qPCR).[6,7] Use of multiparametric flow cytometry (MFC) for MRD detection in MM was attempted in 1999, when Almeida et al explored the role of flow cytometric immunophenotyping (FCMI) and DNA ploidy status for the investigation of residual neoplastic PC in MM patients.[8] Since then, MFC has contributed significantly to the understanding of the aberrant/clonal PC (APC) compartment and has now found a place in the diagnosis, prognosis, and in treatment monitoring of MM. In this review, we will critically appraise the role of MFC for MRD detection in MM and make an attempt to identify the caveats present in MM MRD estimation by FCMI.

Flow Cytometry in Myeloma MRD—Transitioning from Conventional Flow Cytometry to Next-Generation Flow cytometry

MFC has become the most valuable tool to monitor MRD and evaluate the depth of response in MM patients. MFC offers an edge over other techniques of MRD detection, that is, ASO qPCR and next-generation sequencing (NGS) due to its rapid turn-around time, wider applicability, and cost-effectiveness. Furthermore, it plays a crucial role in treatment monitoring and response assessment in oligo-secretory and nonsecretory myeloma where monoclonal protein estimation has no role. FCMI has also shown a promising role in demonstrating rare phenomenon like clonal switch and light chain escape. The very first step in the FCMI for MRD evaluation is optimal panel design and validation before using it routinely in clinical diagnostics.[9] Four set of markers are required for a panel to precisely determine MRD:

A. Gating marker—CD38, CD138, and CD45
B. Markers of aberrancies—CD19, CD56, CD27, CD81, CD117, CD20, CD28, etc.
C. Markers to identify clonality—cyt kappa and cyt lambda
D. Markers for adequacy—CD117. The major limitation of four to six color panels is lack of incorporation of all sets of markers in a single tube that led to the transition to higher versions with added benefits of increased sensitivity.

Conventional 4–6 Color Panel

Though the conventional four to six-color flow-MRD is applicable in majority of patients (≦95%), the sensitivity of conventional flow cytometry remains lower ($<10^{-4}$) than that of molecular techniques, namely ASOqPCR ($<10^{-5}$) and NGS ($<10^{-6}$).[10–12] Due to the limited sensitivity of four to six color assays, there was high likelihood of missing the low APC burden. Limitations of such limited panel assay are high likelihood of missing the low APC burden and failure to combine immunophenotypic aberrancies with light chain restriction leading to difficulty in the characterization and differentiation of APC from non-neoplastic normal PC (NPC). Thus, overall, the four to six-color panel was not suitable for MRD assessment in MM and the transition to higher version was inevitable.

Conventional 8-Color Panel

A step forward in disease monitoring was conventional eight-color flow-MRD assays with an increased sensitivity of less than 10^{-4} to less than 10^{-5}, leading to a significantly improved prediction of outcome across different studies.[9,13,14]However, the two major limitations of the conventional eight-color antibody panel were limited sensitivity and lack of standardization in terms of variation in antibodies, number of cells evaluated, and lack of unanimous cutoff for MRD levels. To offset this challenge, standardization efforts were made by the Euroflow consortium for highly sensitive and standardized detection of MRD in MM using the NGF approach.[15]

Euroflow 2-Tube-8-Color Panel

Euroflow standardization study for NGF included an optimized 2-tube eight-color antibody panel, acquisition of more than or equal to10^7 cells/sample using bulk lysis procedure and construction of novel software tools for combined analysis of both the tubes for automated PC gating. In this multicenter evaluation of NGF for myeloma MRD, one-fourth (∼25%) patients classified as MRD-negative by conventional eight-color flow cytometry were found to be MRD-positive by NGF. This resulted in identification of a flow MRD-negative subset of patients within the CR group who had significantly superior progression-free survival (PFS). However, in the real-life clinical settings especially in the resource constraint countries, the inherent challenges associated with Euroflow two tube panel are as follows:

A. High sample demand with wastage: Two eight-color tubes with the acquisition of more than 1 million cells per tube require a higher volume of BMA that may not be always available due to the primary fibrotic nature of MM. Furthermore, it also leads to wastage of precious samples which can be utilized for cytogenetic and molecular studies.
B. Turnaround time: Comparatively longer due to increased sample processing and data acquisition time
C. Wastage of antibodies: Common gating markers in both the tubes
D. Increased technical demand: Heavy demand on sample quality control, and data storage
E. Analysis: Time-consuming with inferential reasoning between the tubes. As a result, investigators started looking for a better, cost-effective, and efficient panel for MRD evaluation in MM.

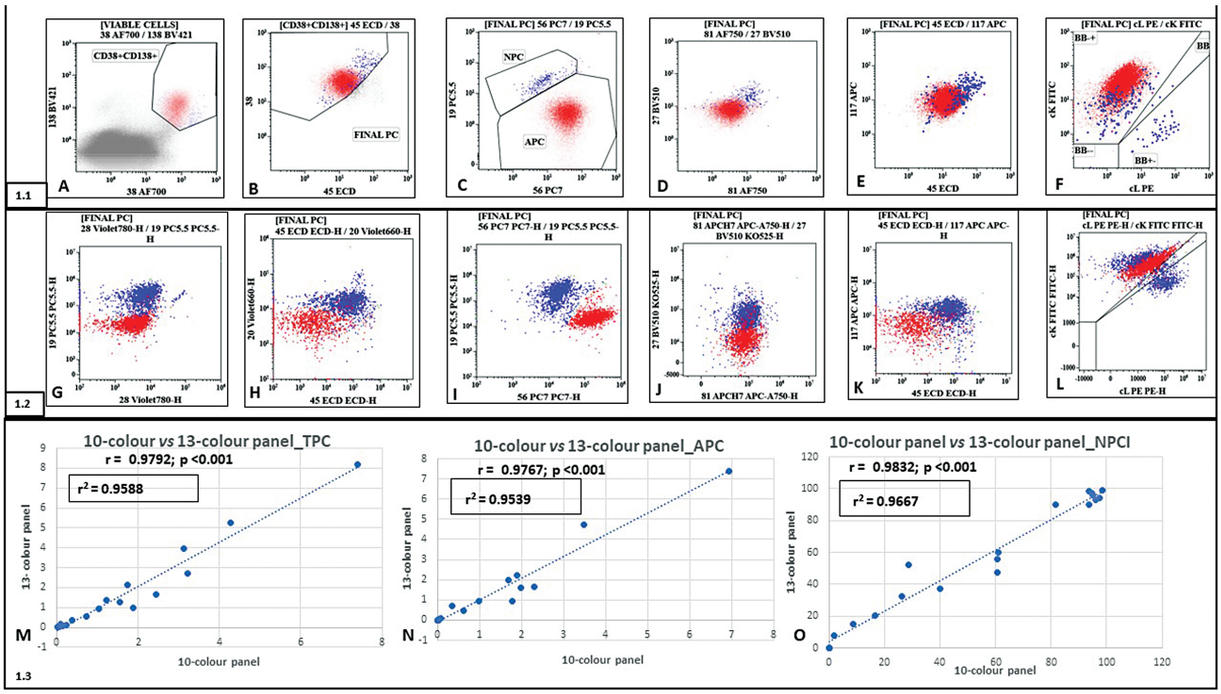


Fig. 1 Flow cytometric dot plots showing PC quantification using 10- and 13-color panel, respectively (1.1 and 1.2); Bland-Altman correlation analysis showing linear positive correlation between 10-color versus 13-color panel (1.3). APC, aberrant PC; NPC, normal PC; PC, plasma cell.

Single-Tube-10-Color Panel

In an attempt to develop less expensive, low sample requisite method to detect MRD with equivalent sensitivity to that of Euroflow-NGF, single-tube-10-color panel was designed across few centers.[16,17] In a study by Sato et al,[17] the analytical ability of the10-color panel was compared to that of Euroflow-NGF that demonstrated a good correlation between the two methods. This study also demonstrated a comparable performance of 10-color-MFC and Euroflow-NGF in terms of median percentages of total PC (0.2148 vs. 0.2200%, $r = 0.950$) as well neoplastic PC (0.0012 vs. 0.0007%, $r = 0.954$). Thus, as per the published literature, the single tube10-color-panel is a promising alternative to Euroflow two tube eight-color panels for MRD detection.

Beyond 10-Color Panel in MM

In constructing the panel for MRD detection, the next relevant question is the need of additional immunophenotypic markers for MRD evaluation. In a recent study, fair discrimination between normal and clonal PC was demonstrated in most of the plasma cell proliferative disorders (PCPD) samples (~99.5% of samples) using eight markers (Gating:CD38, CD138, CD45; aberrancy:CD19, CD56, CD27; clonality: Cy-kappa & Cy-lambda).[18] This confirmed the high efficacy of single tube-10-color-panel in APC identification and thus, MRD detection. Our in-house data for 13-color panel with addition of CD20, CD28 and CD3 (unpublished) showed a good correlation between single-tube-10-color panel (median TPC: 0.98%, range: 0.02–5.24; median APC: 0.59%, range: 0.009–4.72; median neoplastic plasma cell index [NPCI]: 54.25%, range: 8.16–99.11) and single-tube-13-color panel (median TPC: 1.12%, range: 0.03–4.28; median APC: 0.48%, range: 0.01–4.39; median NPCI: 60.55%, range: 1.65–98.46; ►**Fig. 1**) with no added benefits of more than 10 color panel in MRD analysis. Nonetheless, a higher version of panel can be explored for ancillary immunophenotypic studies, namely B-cell subset analysis; immune cell profiling in MM patients owing to the benefits of additional markers. ►**Supplementary Table 1** (available in the online version) shows various antibody panels used for flow cytometry based MRD assay in MM.

Preanalytical Considerations for MFC-Based MRD Detection

The basic step in the MRD monitoring of MM is to ensure optimal sample quality till the processing of sample (►**Supplementary Fig. 1**, available in the online version).

Sample Type and Collection

BMA sample is the prerequisite for MRD estimation; however, peripheral blood sample has been explored as an alternative for circulating PC identification and quantification using sequencing studies across the literature.[19–21] The major drawbacks associated with the use of bone marrow sample for MRD detection are use of quasi-invasive BM aspiration procedure, inadequate PC representation due to the inherent marrow fibrosis associated with MM, and procedural hemodilution. The first two issues can be addressed with the use of non-BMA-based alternate strategy to detect MRD, that is, estimation

of M-protein and imaging.[22,23] Hemodilution is one of the major challenges in MRD estimation and can be reduced using first pull BMA sample for MFC assay as shown across various studies.[24–26] For sample collection, ethylenediaminetetraacetic acid (EDTA) is the anticoagulant of choice. Although heparin can be used as an alternative to EDTA in MRD estimations in other hematolymphoid tumors, its use in MM is best avoided as it interferes with assessments of CD138 that is a key gating marker in myeloma MRD. The use of sodium citrate is also avoided as it reduces the cell viability.[27]

Sample Storage and Transportation

Apart from the type of anticoagulant used, the handling and transportation of the specimen are critical. The peripheral blood and BM specimen must be insulated from the external temperature. While transportation, the age of the specimen also plays an important role, therefore, the specimens must be labeled with the date and time of collection. A 24-hour cutoff is considered appropriate for sample processing to ensure PC viability for accurate MRD determination.[9] However, for all practical purposes a 4 to 6-hour cutoff from sample collection to sample processing is preferred. The viability of the specimen should be assessed with the flow cytometric evaluation and the samples with less than 85% viability should be reported stating that the viability of the sample was suboptimal for testing.[28] Factors indicating a compromised specimen are hemodilution, clots, small BMA volume, and cold or excessively warm samples.

Sample Processing

BMA sample for MRD detection should be preferably processed within 4 to 6 hours of collection to get maximum PC yield; however, it may be stored for 2 days at 4 to 8 degrees. Lysing the sample before staining is preferred especially in samples for MRD analysis as this allows the delivery of maximum number of cells per tube in the panel. However, this method also has a drawback that certain antibody-fluorochrome **c**onjugates become unstable. Cell fixation post-staining is recommended for resolving such issues.[29] The samples can be gently prelysed by incubating with ammonium chloride-based lysis solution. Another method is used is lysis of the sample post-staining, but it is associated with lower stability issues and decreased sensitivity due to lower number of cells. Euroflow consortium also supports the prelysis method for MRD detection with high cellular yield in MM and the same method can be used for PC enumeration in other PCPDs.[30]

Analytical Considerations for MFC-Based MRD Detection

Determinants of Reproducible MRD Enumeration

Multicenter data has demonstrated that the level of 0.01% is of clear prognostic value for MRD monitoring in MM.[31] With the advancement in the technology, the assay sensitivity has increased to 10^{-5} to 10^{-6} and with the improvements in therapy, the current minimum acceptable sensitivity is 0.001% and the recommended threshold for abnormal PC in the determination of MRD ranges from 20 to 100 cells.[32,33] A number of studies have demonstrated that a cluster of 20 cells is a conservative value for the smallest number of a homogenous and clustered population of cells that can be reliably detected by an experienced Hematopathologist.[9,15,17] The limit of blank (LOB), the limit of detection (LOD) and the lower limit of quantitation (LLOQ) are further determined to establish the sensitivity and reproducibility of the flow cytometric assay for MRD assessment.[9] LOB is calculated as LOB = mean of blank values plus 1.645 x (Standard deviation [SD] of blank values).[34] The LOD is calculated as LOB plus three times SD of blank values.[34] LOD determined using the values from the LOB experiment may further be validated with a dilution and spiking experiment. In the experiment for LLOQ determination, spiked and diluted cells with the same concentration (number) of neoplastic PC are processed and acquired multiple times separately to determine the minimum number of clonal PC present in the sample that can provide assay results with CV less than 20%.[16] Next critical determinant in MRD calculation is assigning appropriate numerator and denominator for MRD estimation. For all practical purposes, numerator is clonal PC showing light chain restriction with/without aberrant immunophenotype designated as APC in the subsequent sections. The denominator used for MRD studies is total viable cells that are total events after removal of doublets and debris and thus, MRD = clonal PC/ total viable cells. Another parameter that has been shown to be relevant in MRD estimation in MM by our group is total PC. The use of total PC as denominator is based on the premise that in a hemodiluted BM, both the normal and neoplastic PC are equally diluted and thus this statistic offsets the effect of hemodilution to some extent. Thus, using clonal PC as numerator and total PC as denominator, we defined NPCI, as NPCI = clonal PC/total PC.[35]

Data Acquisition and Analysis Strategy

A number of clinical studies have supported the fact that the total number of events acquired is a key step in the interpretation of the specimen quality for MRD negative cases with the acquisition of at least two million cellular events as the acceptable minimum number in the absence of MRD.[9] If MRD is not detectable and the total cellular events acquired are fewer than two million, both LOD and a qualifying statement as to the decreased level of sensitivity should be mentioned in the final MRD report.[28] Data acquisition is considered to be accomplished when the acceptable total cell collection is achieved, and the specimen meets the criteria of quality. For samples in which MRD is not detected, the quality of the specimen must be checked by an assessment of normal cell population such as mast cells, B cell progenitors, NPC, nucleated red blood cells, and/or myeloblasts (►**Fig. 2**).[26,36,37] After ensuring BMA adequacy and acquiring enough events, the next critical step is the gating strategy for identification of the PC compartment. The backbone gating markers for the PC analysis strategy are CD38, CD138, and CD45.[38,39] However, in the era of targeted therapy especially in the setting of use of anti-CD38 monoclonal antibody in the

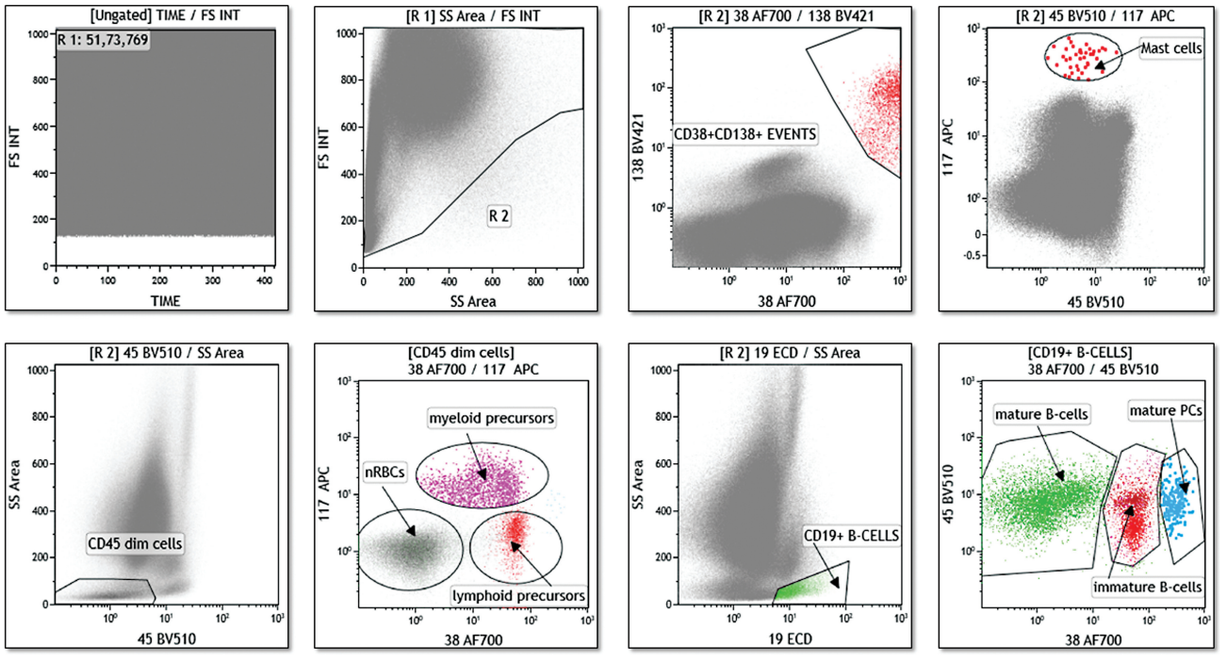


Fig. 2 Flow cytometric dot plots showing various components used to measure adequacy of BM in MRD assessment. BM, bone marrow; MRD, measurable residual disease.

treatment protocols, a search for alternate gating markers for PC continues. In quest of newer markers for PC identification, Pojero et al carried out a detailed analysis of several PC-associated markers and demonstrated that CD54 and CD319 had limited utility for PC identification because of significant overlap of the staining on PCs and other myeloid cells in the sample.[40] Furthermore, CD229 was found to be reliable marker for the identification of PCs in patients undergoing anti-CD38 or anti-CD138 therapy. Recently, Broijl et al explored the role of CD38-multiepitope antibody and the VS38c antibody for MRD assessment in MM patients and concluded that both antibodies allow reliable MRD detection with 100% concordance with added benefits of VS38c in daratumumab-treated patients due to its intracellular expression.[41] ►**Fig. 3** demonstrates the gating strategy for PC identification using four gating markers and shows that best discrimination between PC and non-PC compartment is achieved with the combination of CD38 and CD138. The utility of CD229 as a gating marker for PC identification reaffirms its role in MRD detection in patients receiving anti-CD38 therapy.[42] Overall, the combination of CD38, CD138, and CD45 remains the mainstay for PC gating and additional markers may help in specific circumstances.[39]

Characterization and Discrimination of Normal from Aberrant PC

After ensuring the optimal gating strategy, the next critical step in MRD evaluation by MFC is the discrimination of nonmalignant polyclonal NPC from the malignant APC. There is no single surface marker that would enable 100% discrimination of benign PCs from myeloma PCs, highlighting the need of multiple markers to differentiate NPC from APC. A major challenge in the immunophenotypic characterization of APC is the presence of a polyclonal reactive PC that closely mimics the immunophenotype of neoplastic PC. With the progressive increase in the number of markers and events analyzed, we are increasingly becoming aware of the existence of polyclonal PCs with immunophenotypic aberrancies and therefore, an in-depth understanding of the immunophenotypic heterogeneity of the background NPC is essential. Recent studies have shown evidence of wider immunophenotypic variation in NPC than earlier studied, thus, revealing a partial overlap between the NPC and APC.[43,44] Immunophenotypic aberrancies in polyclonal PCs have demonstrated for all the markers using 10-color panel, signifying that an aberrancy of immunophenotypic marker alone does not define malignancy in otherwise polyclonal population of PCs.[16] Thus, in view of the high frequency of immunophenotypic aberrations observed in polyclonal PCs, presence of aberrant immunophenotype alone can no longer be used to define clonal APC and, thus antigen aberration must be supported by light chain restriction for correct assessment of MRD.

Impact of Therapy on MRD Assessment in MM

Data on immunomodulation of the PCs subsequent to antimyeloma therapy is sparse.[45,46] However, it may be noted that the discriminative power of an individual antigen for delineating neoplastic from non-neoplastic PC varies from case to case and is not stable during the disease course of treatment. Almost a decade ago, we reported immunophenotypic changes in clonal APC following antimyeloma therapy in as many as78% cases.[47] In this context, it is important to note that the high degree of immunomodulation renders the baseline diagnostic immunophenotype redundant for the

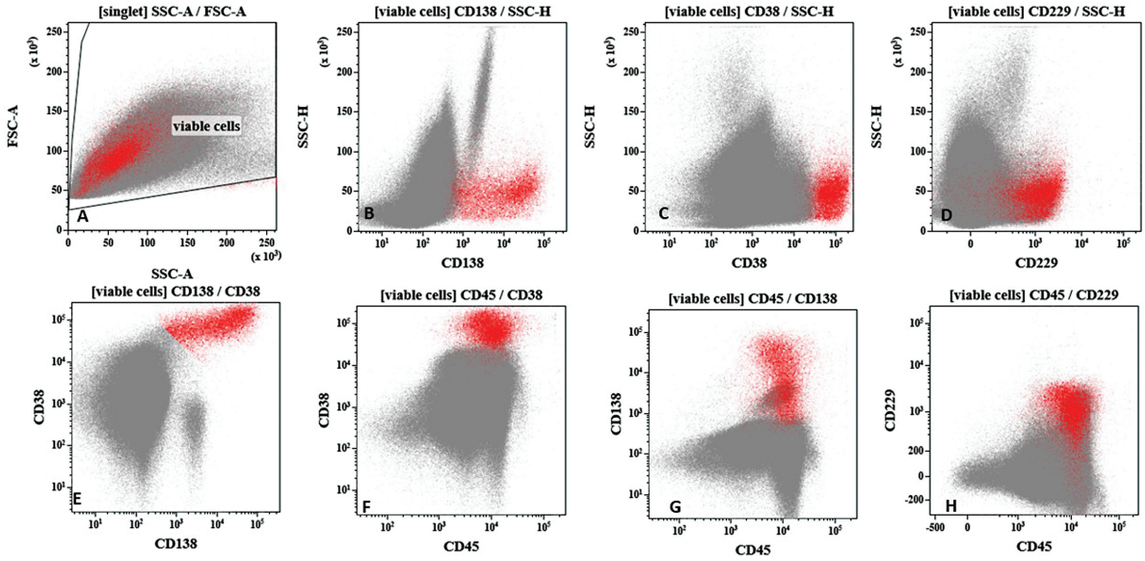


Fig. 3 Flow cytometric dot plots showing gating strategy; maximum PC yield with CD38 versus CD138 (E) followed by CD38 versus CD45 (F); CD229 show overlap with non-PC compartment (D) and (H) reconfirming CD38 and CD138 as PC specific marker. PC, plasma cell.

purpose of MRD assessments in MM. CD45, CD38, and CD138 are gating markers and thus change in their expression is likely to affect the MRD as recently described.[16] Thus, more prospective studies in a larger cohort of patients may unravel the mechanism of post-therapy immunomodulation under the aegis of various treatment modalities and lines of therapy administered.

Caveats and Pitfalls of Flow-Based MRD Assessment in MM

Since MRD emerged as one of the strongest and independent prognostic indicators relating to long-term treatment outcomes across various studies, accurate and precise determination of MRD is crucial for disease monitoring.[48] There are multiple factors influencing the MRD status in MM patients on therapy.

Hemodiluted Bone Marrow Aspirate

This is the major pitfall in assessing MRD across laboratories as it limits the minimum number of neoplastic cells required to detect MRD. To increase the challenge, high throughput techniques like NGF require high amounts of starting material. Several measures have been taken in the past to overcome this challenge including use of first-pass sample for MRD assessment, bulk lysis as the optimal preanalytical procedure, and evaluating sample cellularity (e.g., by quantification of erythroblasts more than 5% and mast cells by flow cytometry or smear) before proceeding with MRD evaluation. However, none of these methods found to be beneficial in all cases. Thus, recently we demonstrated the utility of NPCI, that is, the percentage of clonal APC of the total PC in for MRD evaluation as a parameter to document MRD.[35] Since both neoplastic and NPC are proportionately reduced in a hemodiluted BMA, NPCI may prove to be a better parameter to assess MRD and may be explored further in larger cohorts.

Multiple Institutional and Nonstandardized Protocols

This is one of the major pitfalls in the MRD detection in MM. A survey of multi-institutional practices for MFC based MRD detection in MM revealed that MRD estimation suffers from major heterogeneity with considerable variation in the number of BM cells analyzed (events) and the number of APC needed to identify the MRD.[49] Nonetheless, the variation also existed in the definition of APC by MFC affecting ability to differentiate normal from neoplastic PC. To overcome this challenge, the College of American Pathologists in 2014 recommended to include the LOD and the LLOQ for flow-based MRD assays in the final diagnostic report along with the documentation of methods for MRD assay's LOD measurement.[50] Furthermore, the International Clinical Cytometry Society, European Society for Clinical Cell Analysis, and the Euroflow Consortium recommend the harmonized use of different reagents, antibody panels, sample processing protocols, instrument standardization and LOD to improve the sensitivity and reproducibility of MFC-MRD detection in MM.[28,30] Recently, an international harmonized approach for data analysis has been evaluated across seventeen participants from thirteen countries using a consensus gating protocol to reduce inter-laboratory variation in MRD reporting.[51] Taken together the precise definition of LOD and LOQ is mandatory for reliable and reproducible MRD reporting.

Optimal Timing to Assess MRD

There is no data to depict exact frequency of MRD monitoring. In this context, it is important to mention that sustained MRD negativity has survival benefits over MRD negative status documented at single time point. In a recent study, Sharma et al demonstrated that MM patients with MRD negativity had survival benefit at the end of induction chemotherapy or at day 100 post-transplant in terms of PFS ($p = 0.0002$) and overall survival (OS; $p = 0.009$).[52] San-Miguel et al also showed that patients with newly diagnosed

multiple myeloma who achieved sustained MRD negativity lasting more than or equal to 6 and more than or equal to 12 months had deep remission and improved clinical outcomes.[53] Suggested time points for MRD assessment in MM based on literature review are as follows:

A. At first CR—to evaluate the depth of response.
B. Post-therapy—after a predefined number of chemo-cycle
C. Prior to autologous stem cell transplant (ASCT)—to assess transplant eligibility.
D. Post-ASCT—at day 100 (most of the literature on MRD is based on this time point)
E. During maintenance therapy—at periodic intervals to assess duration of response.

Of all these time-points day 100 post-ASCT is the most studied in clinical trials and demonstrated superior survival of MRD-negative patients on day 100 post-ASCT as compared to MRD positive patients. Recently, we too have demonstrated that day 100 post-transplant MRD positive status is associated with poor survival outcome compared to MRD negative status with progressive worsening of both PFS and OS with increase in MRD levels.[35]

Depth of MRD Monitoring

Latest IMWG guidelines define the MFC-based MRD negative status as the absence of phenotypically aberrant clonal PC by NGF on BMA using the Euroflow standard operative procedure (or validated equivalent method) with a minimum sensitivity of 1 in 10^{-5}.[53] Thus, IMWG recommends minimal sensitivity of 10^{-5} or higher for the BMA-based MRD testing. A few publications in the literature correlated the log reduction in MRD assessment with survival where researchers studied MRD level as a continuous variable determined by flow cytometry and demonstrated survival benefits in MM patients per log depletion in MRD.[54,55]Likewise, our group also demonstrated that gradual fall in MRD levels from 0.1 to 0.001% is predictive of superior survival outcomes[35] (►**Fig. 4**). In order to get an optimal sensitivity for clinical relevance more prospective studies needed to define the depths precisely since disease progression and relapse evident in MRD negative subsets as well.

MRD to Make Therapy Decisions

Prognostic value of MRD evaluation in predicting long-term survival has been demonstrated in major clinical trials. A meta-analysis by Munshi et al demonstrated the strong prognostic impact of MRD negativity in MM patients and confirmed the utility of MRD as a relevant surrogate for PFS and OS in MM.[56] However, acceptance of MRD as a surrogate endpoint for treatment requires correlation with PFS and OS benefit in larger cohort of patients in multiple trials.

Future Perspectives

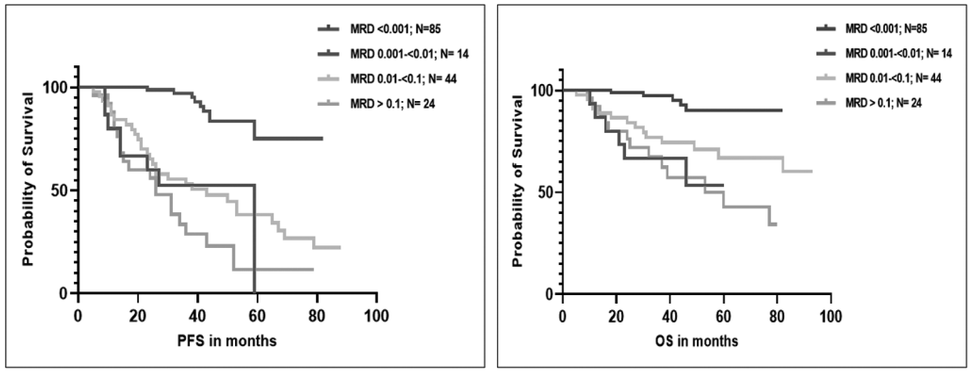
With the growing evidence of MRD as the strong and independent predictor of survival in MM, it is recommended to evaluate MRD more precisely and accurately to benefit the patients in the long run.

Role of Flow Cytometry in Clonal Evolution

Though the phenotype and antigenic variability of malignant PC can be evaluated by flow cytometry, there is limited evidence of the identification of intratumoral heterogeneity by FCMI. Intratumoral heterogeneity can be described as the heterogeneous distribution of genetically distinct MM clones and subclones within a single patient and may complicate diagnostic and prognostic groups posing a significant challenge for disease monitoring. However, FCMI can identify multiple subclones of malignant PC within the same patient based on differential expression of various surface markers on different subpopulations of PC (►**Fig. 5**). This highlights the potential role of FCMI in identifying clonal evolution in MM cases when studied at multiple time points and needs to be explored further in clinical trials to see the impact of these clones on treatment response to explore if a particular subclone is treatment refractory or is responsible for possible relapse.

Emerging Techniques for MRD Evaluation

NGS-based approach for MRD investigation has drawn the attention of researchers in the recent past, however, limited by the costing, labor-intensive technique with analytic expertise. A few studies have also investigated the role of gene expression



MRD

Fig. 4 Kaplan–Meier survival curves displaying sequential improvements in PFS and OS for each log depletion in MRD level. OS, overall survival; MRD, measurable residual disease; PFS, progression free survival.

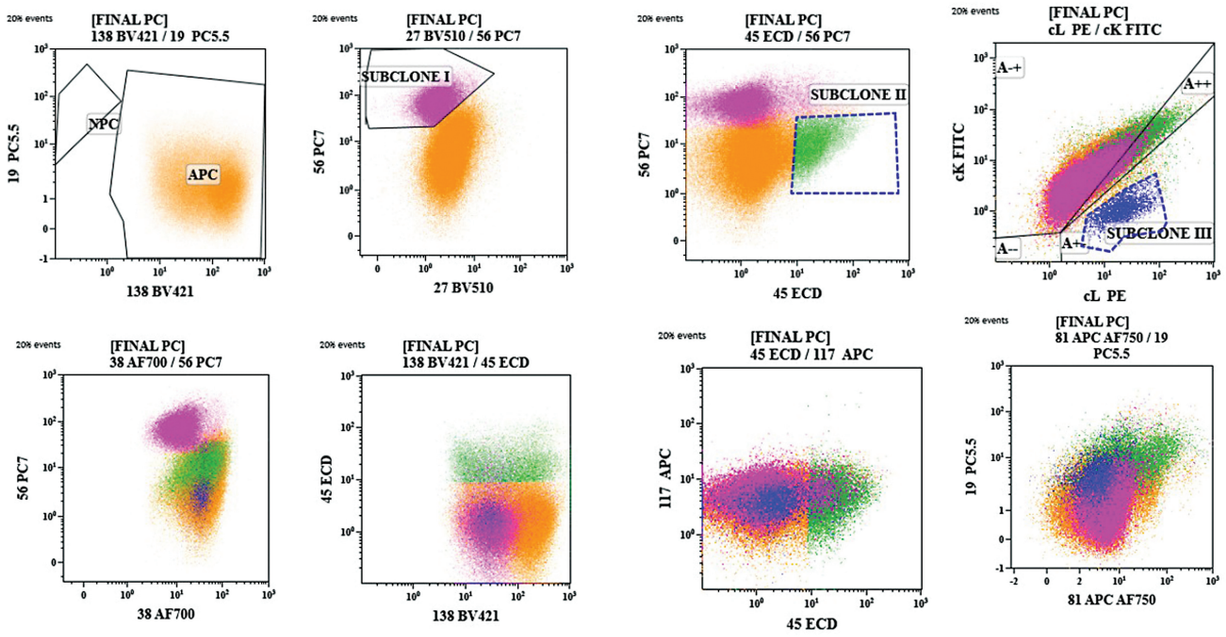


Fig. 5 Flow cytometric dot plots showing multiple subclones of APC (major myeloma clone: orange events; subclone I: magenta events; subclone II: green events; subclone III: blue events) in a case of NDDM. APC, aberrant plasma cell; NDMM, newly diagnosed multiple myeloma.

profiling in myeloma MRD estimation and revealed the role of differentially regulated genetic pathways in MRD landscape of MM.[57,58] Furthermore, considering the patchy nature of disease and spatial heterogeneity in MM, BMA sample may not always be the representative for MRD assessment by MFC or molecular techniques. Nonetheless, in a substantial proportion of extramedullary disease/relapse, BMA is not the ideal sample and may lead to false-negative MRD status. Imaging studies for residual disease estimation may be useful in such scenarios, however, limited by failure to identify the disease present at microscopic/submicroscopic levels. In this regard, utilizing peripheral blood for MRD evaluation has been investigated recently using cell free DNA (cfDNA).[23] In a long-term study on blood based MRD monitoring in MM, cfDNA detection of VDJ rearrangement by ASO-qPCR was used to demonstrate its utility as a prognostic marker.[59]

Conclusion

In conclusion, flow cytometry offers a promising role in MRD estimation in MM. However, it suffers from the inherent challenge of standardization and expertise in analysis. Thus, adoption of a unanimous harmonized approach for MRD analysis by flow cytometry as well as integration of MRD reporting in clinical trials is the need of the hour to utilize MM MRD as a surrogate clinical endpoint.

Authors' Contributions

R.G. and N.D. conceived the study, wrote the manuscript, and approved the final manuscript.

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Conflict of Interest

None declared.

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Flow Cytometric Ploidy Analysis in Acute Lymphoblastic Leukemia and Plasma Cell Myeloma

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Abstract

Identification of underlying cytogenetic (CG) aberrancies plays a significant role in risk stratification of hematological malignancies. These abnormalities can be due to aberrancies that affect the number or structure of chromosomes. Numerical chromosomal abnormalities are called aneuploidies, which result from either gain or loss of whole chromosomes. Ploidy assessment by CG is a laborious and less sensitive technique. With the aid of fluorescent nucleic acid binding dyes, the total DNA content and different phases of the cell cycle specific to any population of interest can be deciphered and analyzed by flow cytometry (FCM). DNA index (DI), a parameter derived by FCM DNA analysis, is equivalent to conventional CG-based ploidy assessment. In this study, the technical aspects and implications of FCM DNA assessment among patients diagnosed with acute lymphoblastic leukemia and plasma cell myeloma are discussed.

Keywords

- ploidy
- flow cytometry
- DNA Index
- S-phase fraction

Introduction to Cell Cycle

Cell cycle is a strictly regulated sequential series of events that result in two offspring cells from a single parent cell. Cells enter into a cell cycle in response to either biological or pathological stimuli.[1] Until such stimuli occur, the cells remain in a dormant phase called the G0 phase. The sequential series of events in a cell cycle is described in ►**Table 1** and the techniques available to assess the cell cycle are described in ►**Table 2**.

Concept of Flow Cytometric Cell Cycle Analysis

The cells in G0/G1 (pre-replicative), S (replicative), and G2/M (post-replicative) phases differ by their intracellular DNA content. Flow cytometry (FCM) can segregate these cells based on the amount of fluorescent DNA binding dye present in each cell.[2–5]

Fluorescent Nucleic Acid Binding Dyes

These dyes emit fluorescence upon binding to their nucleic acid targets.[6] To be eligible for FCM DNA analysis, a fluorescent dye must fulfill the following prerequisites[6]:

- It must have specific binding to nucleic acids.
- It must acquire strong fluorescence after binding to its nucleic acid target.
- The dye's fluorescence emission must be stoichiometric to the target cell's nucleic acid content.
- The dye must have preferential binding to either DNA or RNA. Dyes that have an equal affinity to both DNA and RNA are less preferred. However, undesired but minimal RNA binding in DNA preferential dyes can be overcome by RNAse treatment.

Commonly used dyes for FCM-based nucleic acid analysis and their preferential DNA and RNA binding properties are

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Table 1 Sequential stages of cell cycle and intracellular events at each stage[53]

	Phase	Intracellular events
Interphase	G0	• Dormant stage between two cell cycles • Cells perform their physiological function, but there is no synthesis of RNA and ribosomal protein machinery needed for DNA synthesis
	G1	• Time gap between a recently concluded mitosis and the onset of the next DNA synthesis process • Cells undergo synthesis of RNA and ribosomal protein machinery needed for DNA synthesis
	S	• Synthesis and replication of nuclear DNA content before division • Indicates proliferative intent of a cell
	G2	• The interval between completion of DNA synthesis and onset of mitosis • Opportunity to repair any DNA damage that might have occurred in preceding cell cycles • Reorganization of DNA structure, enabling equal division of DNA between ensuing daughter cells
Mitosis	M	• The actual process of cell division • Equal distribution of DNA, RNA, protein, and cellular organelles between the daughter cells

Table 2 Laboratory techniques to assess cell cycle

Technique		Brief Description
Immunohistochemistry-based cell cycle detection (iCCD)[53,54]		• Geminin: Highest expression in S/G2/M phase and degrades after M phase • Chromatin licensing and DNA replication factor 1 (Cdt1): Highest expression in G1 phase and degrades before S phase • Gamma H2A.X: Marker for DNA double-strand breaks (DSBs)[55]
Fluorescence in situ hybridization (FISH)[56]		• Ploidy assessment by centromere and α-satellite DNA assessment probes
Fluorescent ubiquitination-based cell cycle indicator (FUCCI)[57]		• Fluorescence-labelled proteins tagged with Cdt 1 and Geminin • Time-lapse photographs are used to identify both temporality and spatial organization of cells in culture according to their stage in cell cycle
Radioisotope-labeled DNA[58]		• Incorporating radioisotope (now replaced by nucleoside analogues) into the DNA to calculate the time spent by a cell across the cell cycle stages
Flow cytometry[2,3,8,9,52]	Univariate	• Using DNA binding dyes that enable only cellular DNA content assessment • Applicable in samples that have only/predominantly tumor cells
	Bivariate	• Combined use of a DNA binding dye along with another dye/antibody specific to RNA, cyclins, or cell proliferation-related antigens (e.g., Ki67) • Enable better understanding of cell cycle kinetics
	Multivariate	• Combination of cell surface antigen and intracellular DNA staining • Cell surface antigens aid in gating cells of interest, enabling cell population-specific DNA analysis
Mass flowcytometry (FCM)[59]		• Combination of conventional FCM and mass spectrometry • Metal-labeled antibodies specifically bind to the proteins to be detected • Enables concurrent detection of multiple metal-labeled antibody–antigen complexes by mass spectrometry, facilitating simultaneous analysis of more than 40 antigens per cell
Microfluidics-based cell cycle analysis[60]		• Detecting intracellular DNA content and cell cycle–specific proteins • Can be performed on low-volume samples
Raman spectroscopy[61]		• Label-free technique for cell cycle analysis • Functional capability of cells is retained
Cell cycle chromobody (CCC)[62]		• Uses fluorescent protein-linked intracellular functional single-domain antibody • Demonstrates cell cycle–dependent distribution of proliferating cell nuclear antigen (PCNA) in living cells

depicted in ►**Supplementary Table S1** (available in the online version).

Components of DNA Histogram

In FCM, the cell cycle is analyzed as a frequency histogram where the fluorescence intensity of DNA binding dye and the number of cells is plotted across the X-axis and Y-axis, respectively.[4] In a cell cycle histogram, the fluorescence intensity of DNA binding dyes is expressed in a linear scale. The linear scale simplifies interpretation of DNA histogram as each unit in this scale represents an equal interval in increments of fluorescence (i.e., DNA content per cell). As the data is well spread in a linear scale, individual phases of cell cycle can be grossly identified by visual inspection itself. Also, the linear scale facilitates identification of artefacts and sub-G0/G1 peaks, which might be readily missed in a log-scale.

Due to the stoichiometric binding of DNA-specific dyes, all cells in any given phase of the cell cycle are expected to have the same amount of DNA and the "expected" cell cycle must be as the green, blue, and red colored bars depicted in ►**Fig. 1**. However, due to cell-to-cell variability in dye binding, a gaussian curve with normal distribution is generated for each phase of the cell cycle, and a multimodal histogram is generated as depicted in ►**Fig. 1**.

Following are the components of a DNA histogram. The *G1/G0 curve* represents cells in G0/G1 phases of the cell cycle. Using DNA-specific dyes, a distinction between G0- and G1-phase cells is not feasible as both G0- and G1-phase cells have the same DNA content. In contrast to G0 cells, which have only DNA, cells in the G1 phase also have RNA (refer ►**Table 1**). Combined use of RNA binding dyes like pyronin Y along with a DNA-specific dye (having a different emission spectrum) can aid distinction between G0- and G1-phase cells. The *G2/M curve* represents cells in the G2/M phase of cell cycle. In theory, the G2/M-phase cells must have exactly double the DNA content of cells in the G0/G1 phase. However, the extensive DNA–protein interactions in the G2/M-phase cells result in extensive chromatin compaction, which hinders stoichiometric nucleic acid binding by the dyes. Due to this, the dye fluorescence intensity of cells in the G2/M phase is 1.97 times that of the cells in the G0/G1 phase.[7] The *S curve* is a broad-based histogram between G0/G1 and G2/M curves. This curve reflects the gradual increase in the DNA content of a cell until it reaches a content equivalent to a cell in the G2/M phase.

In a DNA histogram, the "origin point" indicates the point on x-axis where the fluoresce of the DNA binding dye starts. In a typical DNA histogram, the peak staring from this origin point has the least DNA content, that is, the G0/G1 peak. This origin point on the x-axis must be clearly defined in the DNA histogram as it facilitates data normalization for comparing DNA histograms from different experiments or samples or different cell populations within the same sample. The origin point is also the end result of variations in sample age, sample processing, and measurement techniques. The exact start and end points of G1/G0, S, and G2/M histograms are always arbitrary by manual gating. To identify the correct proportion of cells representing each phase of a cell cycle, mathematical modeling-based curve fitting algorithms are available (Dean–Jett model of 1974, Fox-modified Dean–Jett model of 1980, Watson's pragmatic model of 1987, etc.).

Clinically Relevant Parameters Derived from FCM DNA analysis

In patients diagnosed with B-lineage acute lymphoblastic leukemia (B-ALL) and plasma cell myeloma (PCM), the DNA index (DI) and S-phase fraction (SPF) are the two parameters of clinical relevance obtained by FCM DNA analysis.

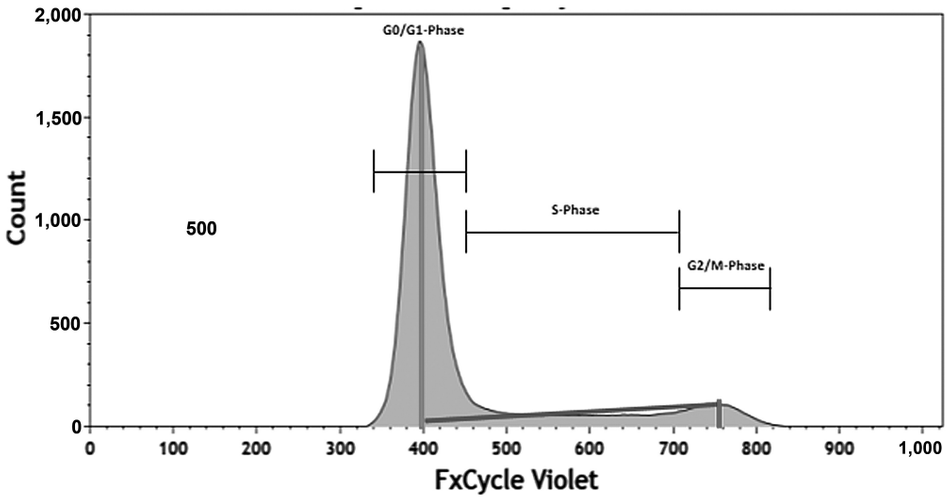

Fig. 1 Flowcytometric DNA assay depicted as a multimodal histogram (*shaded curves*). The *green*, *blue*, and *red colored lines* represent the theoretical G0/G1, S, and G2/M phases, respectively. Y-axis: number of cells. X-axis: intensity of staining with FxCycle violet, a DNA-specific fluorescent dye.

DNA Index

The DI is calculated as the ratio of the G0/G1 phase of malignant cells to that of the G0/G1 phase of normal diploid control cells.[4] There is near 90% correlation between the ploidy status assessed by DI and cytogenetics (CG).[8–10] There are published cutoffs (refer to ►**Supplementary Table S2**, available in the online version) that have correlated the range of DI with the corresponding number of chromosomes in a population of cells.[8,11–13] The advantages of ploidy assessment by DI are that the technique is more rapid and sensitive than CG, and can aid in ploidy identification even in samples with less than 1% cells of interest.[8] This technique can identify cell population–specific ploidy even in samples with markedly heterogeneous cellular composition (e.g., bone marrow). However, ploidy evaluation by FCM is not sensitive in assessing aneuploidies involving smaller chromosomes.[9]

Role of DNA Index in B-Lineage Acute Lymphoblastic Leukemias

Hyperdiploidy (51–65 chromosomes) is observed in nearly 30% of pediatric patients diagnosed with B-ALL and is associated with a favorable prognosis.[14–16] These hyperdiploid blasts are highly susceptible to apoptosis and hence require stringent conditions for their ex vivo survival. This is reflected in frequent metaphase failures and/or poor morphology of chromosomes during conventional CG evaluation of these patients. Under ambient culture conditions used for conventional karyotyping, the normal diploid cells in the sample can overwhelm the hyperdiploid blasts and result in pseudodiploidy. Ploidy assessment by DI is effective in overcoming these limitations of conventional CG-based ploidy assessment.

The relevance of DI in pediatric patients with B-ALL was observed as early as 1985.[17] A DI of ≥1.16 in these patients is consistently associated with hyperdiploidy and hence better outcomes.[11,13,17–21] Although there is more than 95% concordance in hyperdiploidy identified by DI and karyotyping, not all B lymphoblasts with 51 to 65 chromosomes have DI ≥1.16.[8,9,22] In this context, it has been proposed to consider B lymphoblasts having 51 to 55 chromosomes and DI of 1.10 to 1.15 as "low DI-high hyperdiploid" (LDI-HHD). These patients with LDI-HHD have a poor prognosis as compared to patients with DI ≥1.16 and 51 to 65 chromosomes, that is, the "high DI-high hyperdiploid" category.[23] In fact, patients with DI ≥1.24 (corresponding ≥58 modal chromosomes) have the best prognosis.[23] ►**Fig. 2** demonstrates the FCM DNA analysis histograms of individual patients with diploid (►**Fig. 2a–c**) and hyperdiploid (►**Fig. 2d–f**) B lymphoblasts.

Hypodiploidy (<46 chromosomes) is observed in less than 5% of pediatric B-ALL patients.[15] In this category, patients with 31 to 39 chromosomes (low hypodiploid) and 24 to 29 chromosomes (near haploid) have dismal prognosis.[15,24,25]

Endoreduplication is a phenomenon where a cell undergoes DNA replication without undergoing cell division.[24] This results in the cell having multiple copies of its chromosomes within a single nucleus. Although endoreduplication is a physiological process in certain human cells (e.g., megakaryocytes), its occurrence in blasts is pathological and complicates ploidy assessment.[24] Nearly 65% of patients with near haploidy and 45% of patients with low hypodiploidy undergo endoreduplication.[24] Due to this phenomenon, CG can misinterpret endoreduplicated near-haploid and

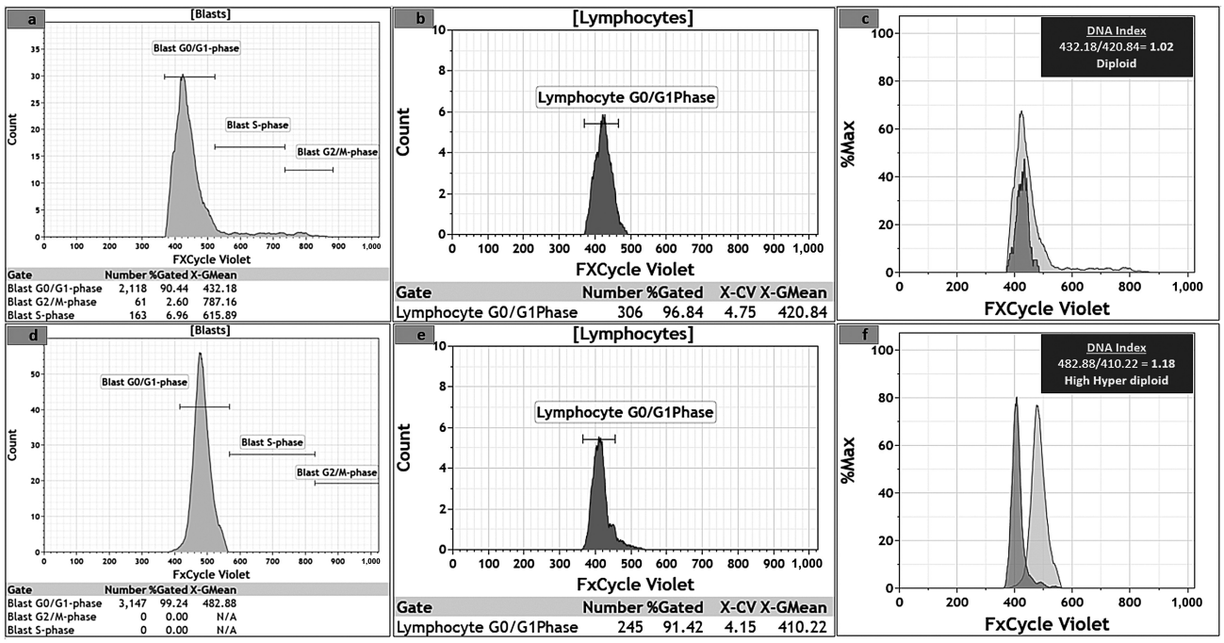

Fig. 2 DNA index assessment in B-lymphoblastic acute lymphoblastic leukemia using FxCycle violet, a DNA-specific dye. (**a–c**) From a sample with diploid blasts. (**d–f**) From a sample with high hyperdiploid blasts. Overlay histograms (**c**,**f**) show the G0-/G1-phase cells of both blasts and lymphocytes. The fluorescence intensity of FxCycle violet is depicted as Geometric mean (GMean) of expression.

low-hypodiploid lymphoblasts as hyperdiploid (50–60 chromosomes) and near-triploid (up to 78 chromosomes) lymphoblasts, respectively.[24] Recently, both low-hypodiploid and near-triploid blasts have been recognized as a unified genetic entity involving *TP53* mutations.[24–26] Hence, misinterpretation of low hypodiploidy as near triploidy might not affect the patient's prognosis. However, misclassification of endoreduplicated near haploidy as high hyperdiploidy is detrimental.[24] In a sample where the near-haploid blasts have endoreduplicated, the ensuing hyperdiploid blasts are larger in size than their founder cell.[24–27] Using blast size–specific DI assessment, both hypodiploid and endoreduplicated hyperdiploid blasts in a sample can be identified in isolation.[9]

Role of DNA Index in Plasma Cell Myeloma

Malignant plasma cells in nearly 50% of patients with PCM are hyperdiploid due to trisomies involving odd-numbered chromosomes.[28–32] These hyperdiploid PCM patients have a better prognosis as compared to hypodiploid and *IgH* translocated PCM patients.[33,34] As plasma cells are terminally differentiated, conventional CG results in normal diploid metaphases (possibly of myeloid origin) in nearly 70% of PCM samples.[34] In this context, a multiparametric FCM DI analysis can identify ploidy in both normal and malignant plasma cells.[10,35] Using normal plasma cells as the reference population, malignant plasma cells with DI of 0.95 to 1.05, 1.06 to 1.50, 1.51 to 1.70, and greater than 1.71 are considered diploid, hyperdiploid, near tetraploid, and tetraploid, respectively.[35] Using lymphocytes as diploid control, an alternative cutoff of $\leq$0.95 (hypodiploid), 0.95 to $\leq$ 1.05 (diploid), 1.06 to 1.15 (near hyperdiploid), and $\geq$1.16 (hyperdiploid) to define DI-based ploidy has also been described.[10]

S-Phase Fraction of Malignant Cells

In a cell cycle, cells in the S phase are those that undergo active DNA replication (refer to ►**Table 1**). As cells in the S phase eventually result in mitosis (i.e., physical doubling of the cells), the SPF reflects the proliferative capability of the tumor.

Role of S-Phase Fraction Assessment in Acute Lymphoblastic Leukemias

The relevance of SPF assessment in acute leukemias dates back to 1980 when the presence of greater than 6% lymphoblasts in the S phase was associated with poor prognosis among pediatric patients diagnosed with ALL.[36] This 6% cutoff was established based on the median SPF (range of 1–60% and mean of 9%) among 65 ALL samples with greater than 80% lymphoblasts, irrespective of their B- or T-lineage origin.[36] The prognostic relevance of this 6% SPF was further confirmed by contemporary literature from both the Dutch and British groups.[11,36] However, further literature regarding the relevance of SPF in pediatric patients with ALL is sparse and has not claimed any prognostic relevance.[18,37,38] Due to the emergence of more robust risk assessment modalities, SPF is no longer used as a prognostic tool in any present-day pediatric ALL treatment protocols.

Role of S-Phase Fraction Assessment in Plasma Cell Myeloma

The proliferative capability of plasma cells, as indicated by the plasma cell labeling index (PCLI), is a time-tested prognostic factor in patients diagnosed with plasma cell dyscrasias.[39] Initial PCLI identification assay using radiolabeled thymidine incorporation followed by autoradiography was both technically challenging and hazardous. In 1985, PCLI by a smear-based immunofluorescence assay utilizing an anti-5-Bromo-2'-deoxyuridine (BrdU) antibody was introduced. A PCLI of less than 1, 1 to 3, and $\geq$3% identified by this technique was associated with low, intermediate, and high-risk diseases, respectively.[40–44] Although this BrdU-based PCLI technique is safer and relatively easier than the radiolabeled thymidine technique, it is not widely utilized in routine practice.[39]

In 1994, Orfäo et al described an FCM-based CD38/propidium iodide dual staining technique to analyze the DNA content of plasma cells in the bone marrow of patients diagnosed with PCM.[45] According to that study conducted among 120 treatment naïve PCM patients, those patients with $\geq$3% malignant plasma cells in the S phase had inferior relapse-free and overall survival.[30] To date, higher SPF of malignant plasma cells identified by FCM is considered one of the parameters associated with high-risk disease across the spectrum of plasma cell dyscrasias and at various phases of treatment.[39,46–48]

Laboratory Aspects of Flow Cytometric Ploidy Assessment

A successful FCM DNA analysis warrants fulfilling of the prerequisites discussed in the following sections.

Sample-Related Prerequisites

FCM DNA analysis pertinent to hematological malignancies is commonly performed on fresh, unfixed, fluid-state samples like peripheral blood, bone marrow, body fluids, and fine needle aspirates.[18] In indications requiring sample fixation (like transported samples), precipitating fixatives (acetone and alcohol) are preferred over cross-linking fixatives (formaldehyde and glutaraldehyde).[4] This is because the nucleic acid cross-linking resulting from these fixatives will interfere with the desired stoichiometric biding of the dyes.[4] In the era of univariate and bivariate FCM DNA analysis, it was recommended to perform the assay in samples with a minimum of 15 to 20% malignant cells of interest.[49] With the advent of multiparametric FCM DNA analysis, the assay could be successfully performed even in samples with less than 1% malignant cells.[8]

If the assay has to be performed on archived paraffin blocks, the following factors have to be taken care of. A cell suspension must be prepared from a section of the paraffin block that contains both the tumor cells and normal resident cells (selected by light microscopic evaluation). The thickness of the section is determined by the size of the tumor cell's nucleus (a 50-nm-thick section would be optimal).[5] The quality of DNA binding dye staining is relatively inferior as the tissue is already exposed to formaldehyde during

Table 3 Common issues encountered during flow cytometric DNA analysis and the possible reasoning and solutions to consider

Issue	Reasons	Solution
Doublets and cell aggregates	Higher relative centrifugal force (RCF)	The ideal RCF has to be calculated to achieve an optimal centrifugation force of 500–540 *g*[52]
	Aged sample. Autolysis of cells cause release of sticky nuclear content, causing cell-to-cell adhesions	Sample should be processed as fresh as possible, ideally within 24–48 h from collection[52]
	Sample acquired at high acquisition rate	The sample must be acquired at a low event rate, ideally 100–200 events/s[52]
	Harsh handling of samples during processing	Mixing steps involved during processing must be gentle, preferably using a Pasteur pipette[52]
Nonstoichiometric staining (ideally >90% cells in a sample must be stained with the dye)[52]	Low concentration of dye used for staining	Re-standardize the assay with gradients of dye concentration to identify optimal dye concentration to be used. Note: the cell concentration and incubation time have to be kept constant
	Excess of cells in the cell suspension being stained	Re-standardize the assay with gradients of cell concentrations to identify optimal cell concentration to be used. Note: the dye concentration and incubation time have to be kept constant
	Insufficient incubation time	Re-standardize the assay with gradients of incubation time to identify optimal incubation time. Note: the dye and cell concentration have to be kept constant
	Samples from patients on DNA binding anticancer drugs	Repeat the assay in a treatment naïve sample (if available)
Undesired RNA binding[4]	The degree of undesired RNA binding depends on the dye's RNA binding propensity	• While using dyes with higher RNA binding propensity (e.g., propidium iodide), the sample has to be treated with RNAse before incubation with the dye[4] • A dye without RNA binding capability (e.g., FxCycle violet) can be used[9,52]
Sub-G0/G1 population[4] (the cells that have DNA content less than that of a normal diploid control)	Apoptosis: apoptosis results in DNA fragmentation of specific lengths. During sample processing, apoptotic cells leak out their intracellular contents (including smaller DNA fragments). Hence, apoptotic cells have a lower total DNA content than normal diploid control and manifest as a distinct sub-G0/G1 peak	• If a sub-G0/G1 peak is also present in diploid controls, then the sub-G0/G1 population present in the malignant cells can be considered apoptotic and ignored; however, this approach is subjective and risks exclusion of true hypodiploid malignant cells[9] • Concurrent staining with annexin V will aid objective identification of apoptotic cells[9]
	Necrosis: necrosis involves random-sized DNA fragmentation, resulting in a sub-G0/G1 "smearing pattern" against a perfect peak generated by apoptosis/hypodiploid cells	Samples should be fresh and processed within 24–48 h of collection[52]

fixation; hence, stoichiometric staining might not be optimal.[3,4] In this scenario, 4',6-diamidino-2-phenylindole (DAPI) is the preferred nucleic acid binding dye as it is least affected by formaldehyde-induced cross-linking.[4] Since the nucleus of cells is cut through during sectioning, it is common to get a sub-G0/G1 population during analysis. This population must not be interpreted as a hypodiploid clone of cells. In such situations, curve-fitting algorithms/software (Dean–Jett model of 1974, Fox-modified Dean–Jett model of 1980, Watson's pragmatic model of 1987, MultiCycle of 1988, etc.) can be used to analyze the cell cycle.

Assay-Related Prerequisites

For any FCM DNA assay, it is mandatory to validate the linearity, degree of resolution, and robustness of sample processing in a machine and panel-specific setting. Commercially

available chicken or trout (they inherently have a mixture of single, double, triple, and quadruple nucleated red blood cells [RBCs]) erythrocytes are used for this purpose.[4,10]

According to the 1993 DNA cytometry consensus conference's recommendations, indigenous normal cells (stromal cells in the case of paraffin-embedded tissues) present in a tumor-rich sample should be used as internal diploid controls. In scenarios where such internal controls are not available/sparsely present within the sample, normal diploid cells sourced from healthy controls can be spiked to the tumor sample before processing.

In the context of FCM DNA analysis pertinent to hematological malignancies, lymphocytes are used as diploid controls. Neutrophils and monocytes are not ideal controls as the former are highly fragile and the latter have increased stainability (especially for propidium iodide) due to their unique chromatin conformation.[4]

Sample Processing–Related Prerequisites

The protocols pertinent to sample processing toward FCM DNA analysis depend on the nature of the sample, the cell-permeant ability of the dye, and its extent of RNA binding capability (refer to ►**Supplementary Table S1**, available in the online version). Detailed discussion regarding the specific modifications required for each dye is beyond the scope of this review but is readily available in the literature.[2,3,50,51] The vital features are that the samples must be processed within 24 to 48 hours of collection. A stable dye binding equilibrium is achieved only if the concentration of dye used for staining is nearly 100 times above the concentration of the cells to be stained. Hence, the optimal concentrations of cells and the dye used must be standardized to achieve stoichiometric staining.[52] The processed samples must be acquired immediately and the acquisition must be at a low event rate, around 100 to 200 events per second.[52] A minimum of 200 diploid control cells must be acquired.[52]

Analysis-Related Prerequisites

For optimal FCM DNA evaluation, a minimum of 90% of cells must be stained with the DNA binding dye.[52] The quality of an FCM DNA assay is evaluated based on the coefficient of variation (CV) of the G0/G1 phase of diploid control cells present in the processed sample. This CV is the result of sample quality, sample processing errors, heterogeneity in individual cells' DNA content, and the degree of chromatin compaction. For optimal FCM DNA analysis, the CV of the G0/G1 phase of diploid control must be less than 6%, with CV less than 3% yielding the best results.[4,18,52]

The common pitfalls encountered during FCM DNA analysis are depicted in ►**Table 3**.[3,4,52]

Conclusion

FCM DNA assessment is an objective, reproducible, and sensitive assay to evaluate ploidy and SPF of any cells of interest. FCM-determined ploidy correlates with CG-determined ploidy and patient survival. The FCM DNA assay can be used even in scenarios where the load of tumor cells is low or conventional CG assessment is not feasible or fails.

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Conflict of Interest
None declared.

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An Approach to Nodal T- and NK-Cell Lymphomas—A Systemic Review

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Abstract

T-cell lymphomas are rare neoplasms that have complex pathology. The multiparameter approach has been recommended by World Health Organization (WHO) for the classification of T-cell lymphomas taking into account morphology, immunophenotype, genetics, and clinical features. This also includes division established on the possible cell-of-origin (COO) from T regulatory or T-follicular helper (TFH) cells. The recent WHO-HAEM5 has classified entities as precursor T-lymphoblastic neoplasms, mature T-cell neoplasms, Epstein-Barr virus (EBV)-related T- and NK/T-cell lymphomas, and tumor-like lesions with T-cells predominance. Distinct entities have been recognized within the anaplastic large cell lymphoma (ALCL) family founded on the status of anaplastic lymphoma kinase (ALK) gene rearrangement: ALK-positive and molecularly heterogeneous ALK-negative. The family of lymphomas arising from TFH cells consists of three distinct nodal TFH cell lymphoma entities: angioimmunoblastic-type, follicular-type, and not otherwise specified. These three entities show significant clinical and immunophenotypic overlap. The cases that do not qualify for ALCL or nodal TFH cell lymphomas are labelled as peripheral T-cell lymphomas-not otherwise specified after ruling out nodal EBV-positive T- and NK- cell lymphoma. The new category termed tumor-like lesions with T cell predominance has a high chance to be misdiagnosed as lymphoma. This category includes entities such as Kikuchi-Fujimoto disease, indolent T-lymphoblastic proliferation, and autoimmune lymphoproliferative syndrome. For pathologists, diagnosing nodal T-cell lymphomas may be thought-provoking due to their broad histopathologic spectrum that mimics reactive as well as other neoplastic processes. This review provides a comprehensive diagnostic criterion of the most commonly encountered nodal T-cell and NK cell lymphomas in day-to-day training and an algorithmic approach.

Keywords
- pathology
- T-cell lymphoma
- NK cell lymphomas
- T-follicular helper cells
- WHO-HAEM5

Introduction

The nodal T-cell lymphoma classification is difficult because of its rarity and broad spectrum of pathological aspects. T-cells are derived from their precursors in the bone marrow and attain maturation in the thymus.[1] The thymocytes mature in the medulla and acquire either surface CD4 or CD8. These mature thymocytes migrate to peripheral lymph nodes and extranodal regions (spleen, gastrointestinal tract, skin, etc.) and participate in adaptive and innate immune responses. Most nodal and extranodal T-cell lymphomas are derived from T cells of the adaptive and innate immune systems, respectively.[2] Mature T-cell lymphomas, both nodal

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Table 1 The classification of commonly encountered nodal T- and NK-cell lymphomas based on WHO-HAEM5[3]

A) Mature T-cell lymphomas
a) Anaplastic large cell lymphoma (ALCL):
• Anaplastic lymphoma kinase (ALK)-positive ALCL
• ALK-negative ALCL
• Breast implant-associated ALCL
b) Nodal T-follicular helper (TFH) cell lymphoma:
• Nodal TFH cell lymphoma, angioimmunoblastic type
• Nodal TFH cell lymphoma, follicular type
• Nodal TFH cell lymphoma, not otherwise specified (NOS)
c) Peripheral T-cell lymphoma, not otherwise specified
d) Epstein-Barr virus (EBV)-positive nodal T- and natural killer (NK)-cell lymphoma
B) Precursor T-cell neoplasms
• T-cell lymphoblastic leukemia/lymphoma, NOS
C) Tumor-like lesions with T-cell predominance
• Kikuchi-Fujimoto disease
• Indolent T-cell lymphoblastic proliferation
• Autoimmune lymphoproliferative syndrome

and extranodal, comprise only 15% of all non-Hodgkin lymphomas (NHLs).[3] Recently, a new World Health Organization (WHO) classification of lymphomas has listed many distinct entities derived from T-cells.[4] The focus of this review is to provide an inclusive approach to the diagnostic morphological features and clinicopathological features of the most commonly encountered nodal T-cell lymphomas (►**Table 1**).

A) Mature T-cell lymphomas

a) Anaplastic large cell lymphoma

Anaplastic large cell lymphomas (ALCL) are characterized by large pleomorphic cells having a significant amount of cytoplasm, horseshoe to reniform nuclei, and CD30 showing strong uniform expression.[5] Lymphoma cells tend to grow in cohesive sheets and have a predilection for the lymph node sinuses. Anaplastic lymphoma kinase (ALK) expression and their location help in classifying the ALCLs as[6]:

- Nodal ALCL:
  - ALCL, ALK-positive: chromosomal abnormalities involving chromosome 2p23 (*ALK* locus)
  - ALCL, ALK-negative: negative for *ALK* rearrangements, and are heterogeneous at a molecular level
- Extranodal ALCL:
  - Breast implant-associated ALCL
  - Primary cutaneous ALCL (cALCL)

ALK-positive ALCL: It accounts for 10–15% of pediatric and adolescent NHL and approximately 3% of adult NHL.[7] Preferentially affects children and young adults (< 40 years) with a predilection for males and frequent extranodal involvement.[8]

Morphology: In lymph nodes, the lymphoma cells grow in cohesive sheets and disseminate within lymph node sinuses. The prominent involvement of the sinuses mimics metastatic solid tumors. The common variant contained characteristic atypical large hallmark cells that have eccentrically placed horseshoe or reniform or wreath-shaped nuclei, finely clumped chromatin, many distinct basophilic nucleoli, and copious eosinophilic cytoplasm (►**Fig. 1A**). Additionally, multinucleated cells similar to Hodgkin/Reed-Sternberg (HRS) cells of classic Hodgkin lymphoma may be seen. The lymphohistiocytic variant, as the name suggests, is characterized by numerous reactive lymphocytes and histiocytes with admixed few hallmark cells clustered around vessels. The small cell variant lacks hallmark cells and consists of mainly small-sized cells with perivascular aggregation and eosinophilic cytoplasm. ALCL with a Hodgkin's pattern resembles nodular sclerosis classic Hodgkin lymphoma and they are distinguished using an appropriate immunohistochemistry panel.

Immunophenotype: All ALCLs show expression for CD45 (variable) and CD30 (diffuse, strong, membranous, and perinuclear Golgi) (►**Fig. 1B**). Sometimes, it is termed a "null" immunophenotype as the neoplastic cells show a defect in the TCR/ CD3 complex expression and lack expression for T cell antigens. Among pan T cell immunomarkers, more than 75% of cases are negative for CD3. CD5 and CD7 immunostains are not expressed often, whereas CD2 and CD4 expression is noted in some cases. *ALK* rearrangement is pathognomonic for ALK-positive ALCL; 84% cases involve a fusion t(2;5)(p23;q35) *NPM*/*ALK* gene, followed by t(1;2)(q25;p23) with fusion of *TPM3*/*ALK* (13% cases).[9,10] The rare fusion partners are *PurH* gene (*ATIC*), clathrin heavy chain (*CLTC*), *TRK* fused gene (*TFG*), myosin heavy chain 9 (*MYH9*), Moesin (*MSN*), Ring finger protein 213 (*RNF213*/*ALO17*), and tropomyosin 4 (*TPM4*).[6] The immunostaining pattern of ALK protein helps in determining its fusion partner such as nuclear and cytoplasmic ALK expression is seen in cases involving *NPM*/*ALK* fusion (►**Fig. 1C**), strong cytoplasmic and membranous ALK expression corresponds to *TPM3*/*ALK* fusion (►**Fig. 1D**), and the "membranous pattern" uses moesin as a partner. However, the variant translocations do not have prognostic implications.

ALK-negative ALCL: This entity represents 5.5 to 15% of mature T cell neoplasms.[11,12] It is similar to ALK-positive ALCL in terms of the hallmark cells, histological patterns, and uniform diffuse CD30 expression but lacks ALK protein expression or *ALK* rearrangement (►**Fig. 1E** and **F**). In comparison to ALK-positive ALCL, it occurs more commonly in the elderly, has a more aggressive clinical behavior, and carries a dismal survival outcome.[11] This group is more heterogeneous at a molecular level and the next-generation sequencing analysis has revealed two subsets of ALK-negative ALCLs.[13,14]

- DUSP22 rearrangements at chromosome 6p25.3 (~30% of cases): most cases had a better prognosis, lack STAT3 activation, and LEF-1 expression, and showed characteristics of "doughnut cells" on morphology (►**Fig. 1G**)
- TP63 rearrangement (8% of cases): associated with aggressive behavior (►**Fig. 1H**)
- Activating mutations of JAK and/or STAT3 pathway in a subset of cases.[15,16]

Differential diagnosis: Breast implant-associated anaplastic large cell lymphoma is present in association with breast implants and is a noninvasive neoplasm characterized by the presence of malignant cells infiltrating the periprosthetic

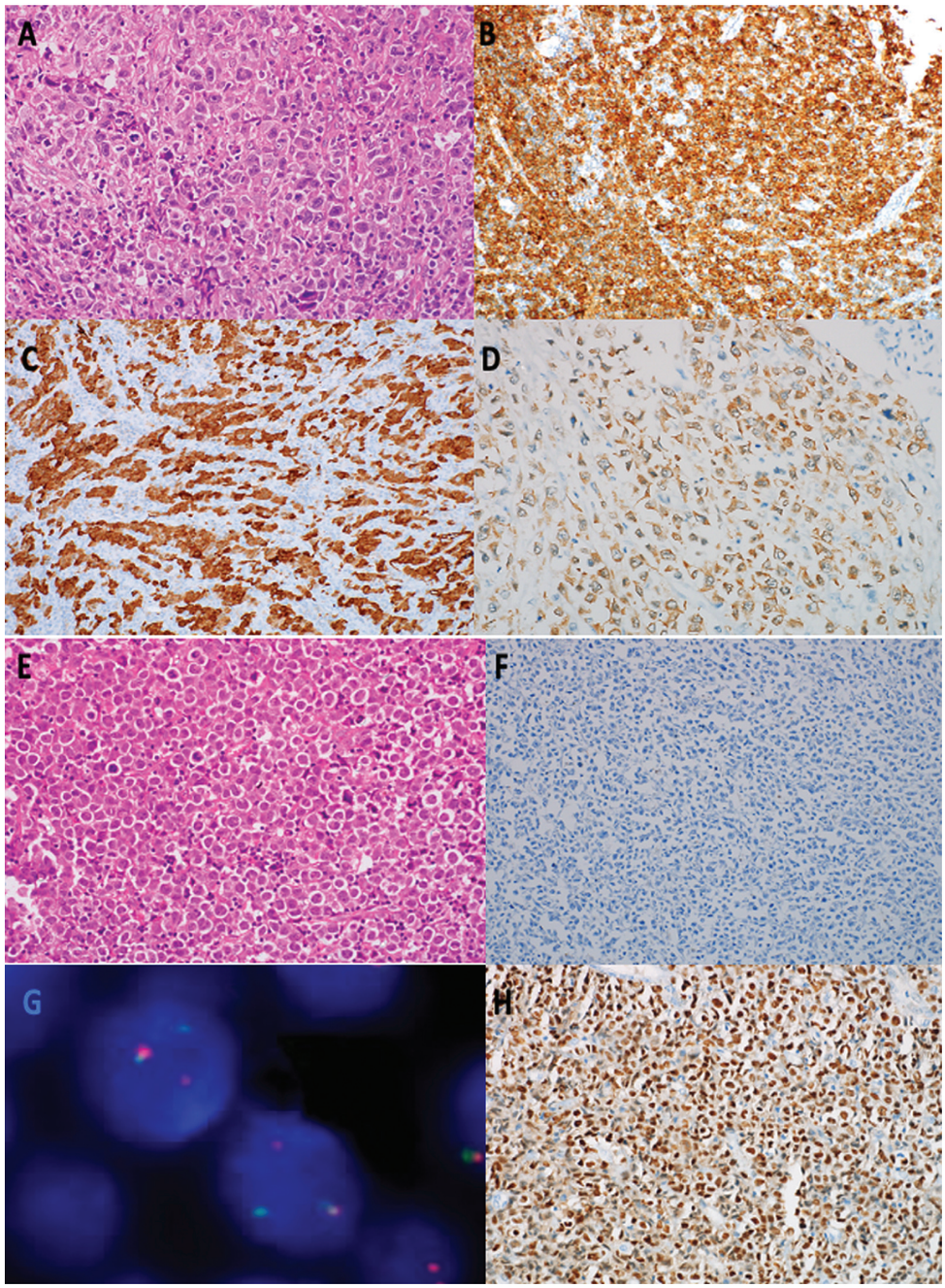

Fig. 1 Anaplastic lymphoma kinase (ALK) positive, anaplastic large cell lymphoma—(**A**) Large hallmark cells with a pleomorphic, horseshoe, or wreath-shaped nucleus and abundant cytoplasm, (**B**) CD30 expression with a membranous and perinuclear (Golgi) pattern, (**C**) Nuclear and cytoplasmic ALK expression, (**D**) cytoplasmic ALK expression. ALK negative, anaplastic large cell lymphoma—(**E**) Hallmark cells along with doughnut cells, (**F**) negative for ALK expression. (**G**) On fluorescence in situ hybridization, the IRF4-DUSP22 dual color break apart probe shows one normal fused signal and one split green-orange signal indicating translocation affecting the DUSP22 locus, (**H**) ALK-ve ALCL showing nuclear expression for P63.

capsule or as a periprosthetic seroma. The clinical behavior is indolent and has excellent outcomes; however, extension to the neighboring structures and contiguous lymph nodes deteriorates the prognosis and also needs to be differentiated from systemic ALCL.[17] Similarly, primary c-ALCL that shares the morphological and immunophenotypic features with the nodal ALCL but exhibits a superior prognosis (5-year overall survival of 90%) needs to be differentiated from cutaneous involvement of systemic ALCL by thorough clinical and radiological examination.[18]

b) Nodal T-follicular helper cell lymphoma

Nodal T-follicular helper cell lymphoma (nTFH) cell family includes three distinct entities that show significant clinical,

immunophenotypic, and TFH gene expression signatures.[19,20] It consists of nTFH cell lymphoma, angioimmunoblastic type (nTFHL-AI), nTFH cell lymphoma, follicular type (nTFHL-F), and nTFH cell lymphoma, not otherwise specified (nTFHL-NOS).[4]

nTFH cell lymphoma, angioimmunoblastic type: It constitutes 15-20% of peripheral T-cell lymphomas (PTCLs) and presents with a median age of 60 years.[21–25] This lymphoma has unique clinical characteristics of generalized lymphadenopathy, hepatosplenomegaly, polyclonal hypergammaglobulinemia, pruritic skin rash, and hematologic abnormalities like Coombs-positive hemolytic anemia.

Morphology: Lymph node in nTFHL-AI usually shows pattern 3 where there is a complete loss of normal architecture with depleted follicles. There is an extensive prominence of arborizing high endothelial venules (HEV) and follicular dendritic cells (FDCs) with a diffuse collection of latter cells surrounding the small vessels (►**Fig. 2A**).[26] The cellularity is variable and composed of small to intermediate neoplastic lymphocytes, clear cytoplasm, and minimal cytologic atypia (►**Fig. 2B**). The background is polymorphous with the admixture of variable numbers of reactive lymphocytes, eosinophils, plasma cells, and histiocytes. The lymphoma cells cluster around HEV and as perifollicular collections. Scattered large B-cell immunoblasts mimicking HRS cells are commonly seen, but sometimes they are plentiful (B-cell-rich nTFHL-AI).[27] In addition, architectural patterns of hyperplastic follicles and depleted /regressive follicles can also be noted.[26,28]

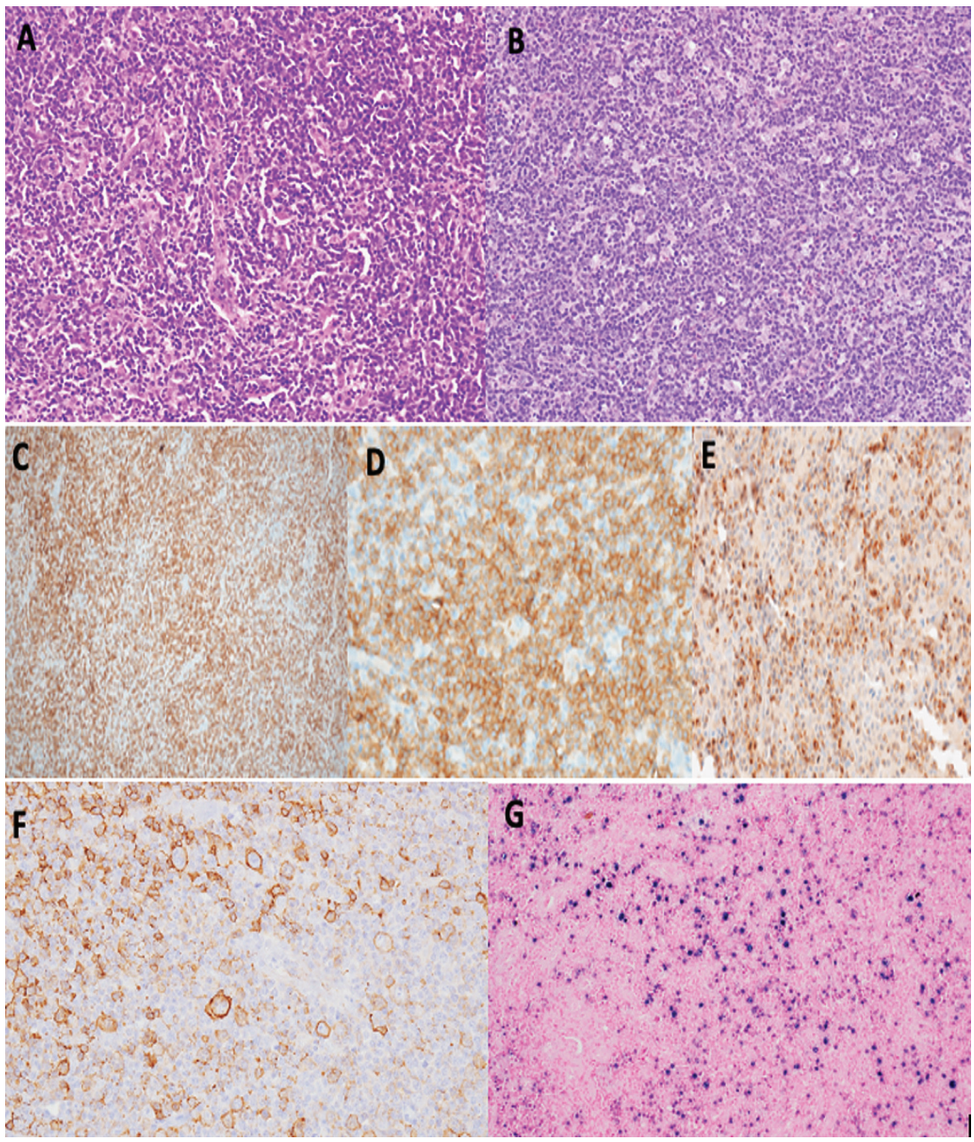

Fig. 2 Angioimmunoblastic T-cell lymphoma—(**A**) normal lymph node architecture is completely effaced with marked proliferation of arborizing high endothelial venules. (**B**) Neoplastic cells are composed of small to medium-sized lymphocytes, with clear to pale cytoplasm, and minimal cytologic atypia. (**C–E**) Neoplastic cells are positive for CD3, programmed cell death protein 1, and CXCL13. (**F**) CD20 highlights the residual follicle center cells as well as many of the large transformed immunoblasts. (**G**) Epstein-Barr encoding region in situ hybridization positivity in CD20-positive immunoblasts.

Immunophenotype: The neoplastic cells are CD3+ T cells, which show downregulation of CD7 and coexpress CD4 (►**Fig. 2C**).[29] Neoplastic cells express characteristic TFH markers, such as CXCL13, programmed cell death protein 1 (PD1) (CD279), and CD10 (►**Fig. 2D** and **E**). The expression of CD10, as compared to other PTCLs, is highly specific. CXCL13 expression is evident in both TFH cells and FDCs. Inducible T-cell costimulator (ICOS) is another sensitive TFH marker. The use of a minimum of two TFH markers is prudent for diagnosis.[30] To summarize, CXCL13 and CD10 are more specific and PD1 and ICOS are more sensitive markers in identifying the neoplastic TFH cells.[31,32] The B-cell markers, such as CD20 and CD79a, highlight the residual follicles and largely transformed immunoblasts in the interfollicular areas (►**Fig. 2F**). Epstein-Barr virus (EBV)-positive B-cells are noted due to reactivation of EBV in the background of a compromised immune system (►**Fig. 2G**).[33] Expansion of the FDC meshworks is highlighted by CD23, especially around the paracortical small vessels.

Genetics: The mutational landscape includes hotspot mutations in *RHOA*, *TET2*, *IDH1*, *IDH2 R172,* and *DNMT3A*.[34] Of these IDH2R172 is specific for the nTFH-AI type, whereas others are also reported in other PTCLs.[35] Cases carrying *RHOAG17V* mutation have classic clinicopathological features, more FDC proliferation, high microvessel density, and pronounced TFH immunophenotype.[36,37] The nTFH-AI type harboring *IDH2* mutations have a distinct morphology in the form of intermediate-to-large sized cells, clear cytoplasm, enhanced TFH phenotype, and strong expression of CD10 and CXCL13.[38]

nTFH cell lymphoma, follicular type: Morphologically, it is typified by follicular pattern and lack of features of nTFHL-AI type, that is, extrafollicular proliferating FDC and HEV.[4,12,39,] CD3 and CD4 are expressed in the neoplastic cells, and also enhanced immunoexpression of TFH markers (PD1 +, CXCL13 +, ICOS +, BCL6 +, and CD10 +/−) is also noted.

nTFHL-NOS: This terminology is used for lymphomas with TFH phenotype and is CD4+ but devoid of features that are characteristics of nTFHL-AI or nTFHL-F. In addition to CD4 positivity, the expression of at least two TFH markers in the neoplastic cells is mandatory for diagnosing nTFHL-NOS. The mere presence of TFH immunophenotype along with some AITL-like features warrants a diagnosis of nTFHL-NOS but not nTFHL-AI. Instead of nTFHL-NOS, the term nTFHL is recommended for small core biopsies to avoid miscategorization as a result of insufficient sampling.[4,39]

Differential diagnosis: Due to diverse histopathologic features of nTFH cell lymphomas, they mimic both benign and malignant lymphoid proliferations.[26] For separating reactive paracortical hyperplasia from early AITL, immunohistochemical staining for PD1 is of utmost importance. In reactive lymph nodes, PD1-expressing cells are limited to the germinal centers, whereas a perifollicular staining pattern is noted in AITL. Prominent EBV-positive B cells, forming confluent foci of large transformed B cells, also pose diagnostic challenges and can mimic large B-cell lymphoma. However, the background of neoplastic T cells with TFH phenotype, FDC meshwork expansion, prominence of HEVs, and monoclonal TCR rearrangement favors the diagnosis of AITL.

c) Peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS)

Heterogeneous entity comprising nodal and extranodal PTCLs that do not qualify for any specific PTCL subtypes and account for 30% of all PTCLs.[40] The latest WHO edition recommends the omission of nodal PTCLs with a TFH phenotype, before labelling it PTCL-NOS.[4] The COO is activated T-cell, mainly CD4+ of the adaptive immune system.

Morphology: Diffuse nodal architecture effacement or rare cases showing an interfollicular or para-cortical infiltrate can be noted. The cellular infiltrate is polymorphous, with varied cytological features. Most cases have medium-to-large-sized cells with irregular nuclei, prominent nucleoli, and many mitotic figures (►**Fig. 3A**). The milieu shows non-neoplastic lymphocytes, plasma cells, eosinophils, and histiocytes. In the lymphoepithelial variant (Lennert's lymphoma), the atypical lymphoid cells are mainly buried under the confluent sheet of numerous epithelioid histiocytes, and this variant has a superior prognosis compared to other forms of PTCL.[41]

Immunophenotype and genetics: PTCL-NOS generally shows the expression of pan-T-cell antigens (CD3, CD2, CD5, CD7); however, decreased or absent expression of one or more T-cell markers might be noted (commonly CD5 or CD7) (►**Fig. 3B**). Most of them are CD4 +/CD8-, although certain cases particularly the lymphoepithelial variant are CD4-/CD8 +. Less often, double positive (CD4 +/CD8 +) or double negative (CD4-/CD8-) can be seen. The immunoreactivity of TFH markers (CD10, PD1, CXCL13, or BCL6) is not noted. One or more cytotoxic markers (TIA-1, granzyme) can be expressed in 20 to 35% of cases. Most nodal cases have αβ TCRs, whereas few are either gamma/delta positive or both negative (TCR-silent). Gene expression profiling divides PTCL-NOS into subgroups based on the enhanced expression of GATA3 or TBX21 transcription factors that are chief regulators of T helper 1 (Th1) and 2 (Th2) cells, respectively.[42] In view of their clinical relevance, these subgroups are now considered under the WHO classification. In contrast to TBX21-positive PTCL-NOS with *CXCR3*, *IL2RB*, *CCL3*, and *IFN*-gamma as target genes, GATA-3-positive PTCL-NOS cases with target genes *CXCR7*, *IL18RA*, *CCR4*, *IK* carries a dismal outcome.[43,44]

d) Nodal EBV-positive T- and NK-cell lymphoma

Nodal EBV-positive T- and natural killer (NK)-cell lymphoma, formerly clubbed with PTCL-NOS, is now a definite neoplasm in recent WHO-2022.[4] Patients exhibit advanced stage presentation with or without extranodal involvement. Morphologically, it appears high-grade and lacks classic histopathological findings of extranodal positive T- and NK-cell lymphomas, that is, angioinvasion and necrosis.[45] The cytotoxic immunophenotype is more of a T-cell lineage rather than NK-cell, and in the majority, EBV-encoded small RNA is seen by in situ hybridization.

►**Fig. 4** illustrates the algorithm for diagnosing peripheral T-cell lymphomas.

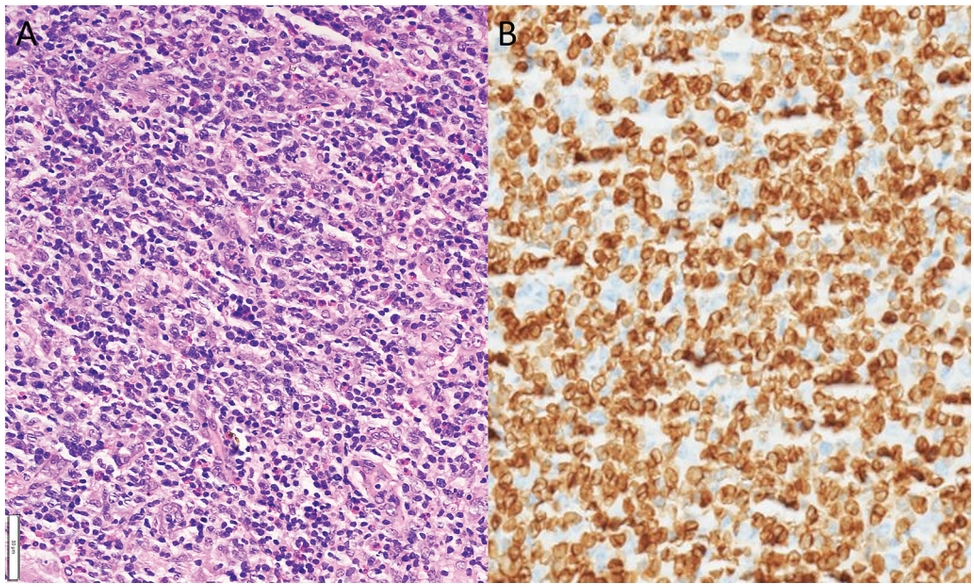

Fig. 3 Peripheral T-cell lymphoma, not otherwise specified—(**A**) shows diffuse effacement of the architecture by polymorphous lymphoid infiltrate with medium to large cells with irregular and hyperchromatic nuclei. (**B**) Lymphoma cells are positive for CD3.

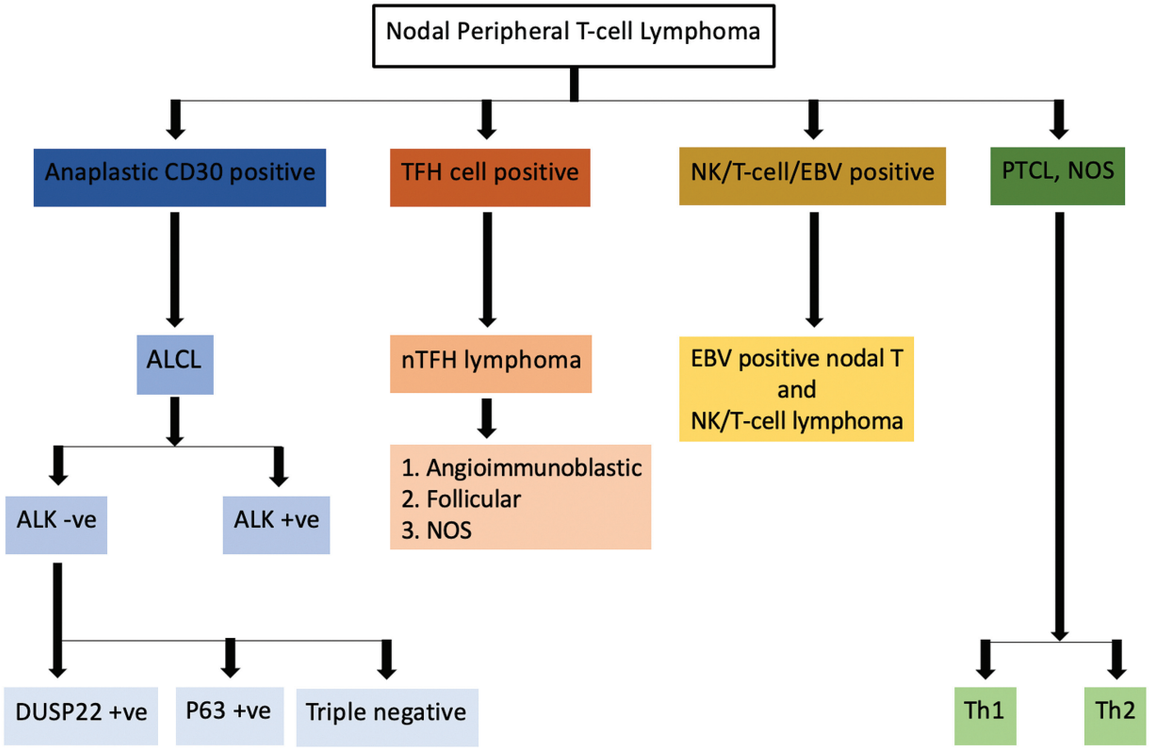

Fig. 4 The algorithm for diagnosing nodal peripheral T-cell lymphomas. ALCL, Anaplastic large cell lymphoma; ALK, anaplastic lymphoma kinase; EBV, Epstein-Barr virus; NK, natural killer; NOS, not otherwise specified; PTCL, peripheral T-cell lymphomas; TFH, T-follicular helper.

B) Precursor T-cell Neoplasms

T-cell lymphoblastic leukemia/lymphoma, NOS: T-lymphoblastic leukemia/lymphoma, NOS, is a neoplasm arising from immature T-cells. It mainly develops in children and adolescents as an aggressive disease with extensive peripheral blood and bone marrow involvement (acute T- lymphoblastic leukemia T-ALL).[46] It is termed as T- lymphoblastic lymphoma (T-LBL) when primary involvement is the thymus, lymph node, or extranodal sites with blasts count in the marrow $\leq$25%.

Morphology: Characterized by small-sized lymphoid cells, finely granular chromatin, and small pinpoint nucleoli in a diffuse growth pattern. Cells may sometimes expand only

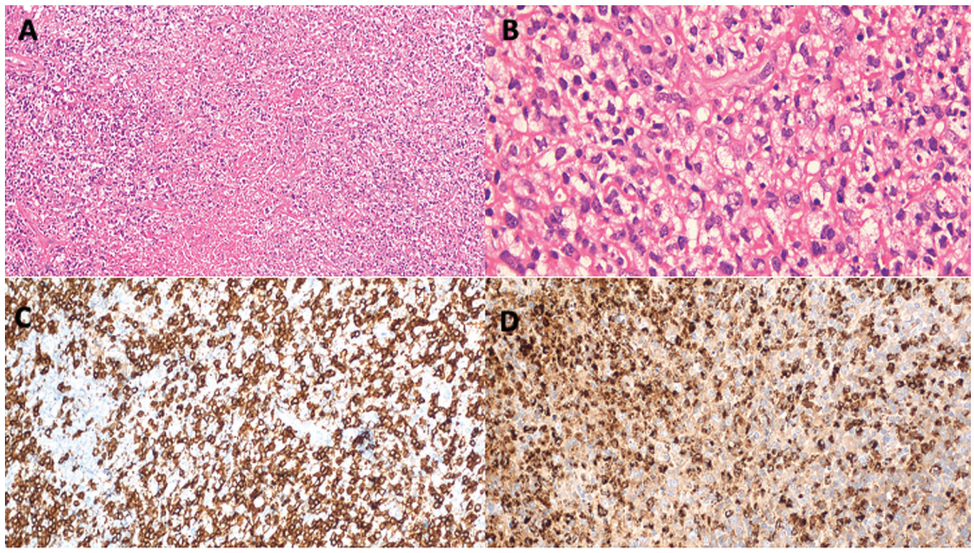

Fig. 5 Kikuchi lymphadenitis—(**A**) showing partial preservation of the lymph node architecture with necrosis, apoptotic cells and nuclear debris; (**B**) admixed histiocytes and large transformed lymphocytes; (**C**) CD3 expression in large, transformed cells; (**D**) Myeloperoxidase expression in crescentic histiocytes.

the interfollicular area in subtotally replaced lymphoid organs and occasionally may have prominent nucleoli (such as in the L2 subtype).

Immunophenotype: Lymphoma cells express markers of T-lineage (CD2, CD3, CD4, CD8, CD5, CD7) and markers of immaturity (CD34, CD99, HLA-DR, and TdT). Like normal cortical thymocytes, they often have a double-positive (CD4+, CD8+) immunophenotype along with CD1a positivity or can express either CD4 or CD8 resembling medullary thymocytes.

C) Tumor-Like Lesions with T-cell Predominance

The fifth edition of WHO (WHO-HAEM-5) incorporated the new family of tumor-like lesions with T-cell predominance.[4] This includes Kikuchi-Fujimoto disease (KFD), autoimmune lymphoproliferative syndrome (ALPS) and indolent T-lymphoblastic proliferation (IT-LBP).

a) Kikuchi-Fujimoto disease

KFD comprises three phases: the proliferative, necrotizing, and xanthomatous phases. It is characterized by paracortical expansion of activated cytotoxic T cells that appear morphologically atypical along with histiocytes and apoptotic debris that may be confused with PTCL (►**Fig. 5A-B**).[47] However, KFD in comparison to lymphoma shows preserved overall lymph node architecture and lacks the diffuse monotonous population of neoplastic cells. The myeloperoxidase-positive histiocytes with crescent-shaped nuclei are typical of KFD. KFD contains essentially CD8+ proliferating T-cells and may have numerous plasmacytoid dendritic cells, whereas PTCLs are CD3+/CD4+ in most cases (►**Fig. 5C–D**).

b) Indolent T-lymphoblastic Proliferation

Indolent T-lymphoblastic proliferation occurring as tiny clusters or confluent sheets of lymphoid cells morphologically consistent with thymocytes has been noted by itself or in association with other benign and malignant lesions.[4,48] These thymocyte-like cells express TdT and may be misdiagnosed as T-lymphoblastic leukemia/lymphoma. However, IT-LBP does not obscure the architecture of nodes and is devoid of monoclonal TCR gene rearrangement.

c) Autoimmune Lymphoproliferative Syndrome

Autoimmune lymphoproliferative syndrome (ALPS) is a lymphoproliferative disorder with heterozygous mutations noted within the first apoptosis signal (FAS) receptor signaling pathway.[49] Clinically, it is characterized by cytopenia, splenomegaly, lymphadenopathy, and autoimmune disorders. Morphologically, it shows florid paracortical expansion by small proliferative lymphocytes that are double negative (CD4-/CD8-) T-cells, immunoblasts, and plasma cells. Overall, the preserved nodal architecture and expression of the pan–T-cell antigens (CD2, CD5, CD7) help to differentiate it from T-cell lymphoma.

Conflict of Interest
None declared.

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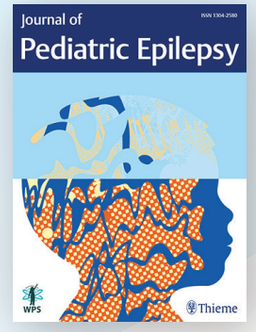

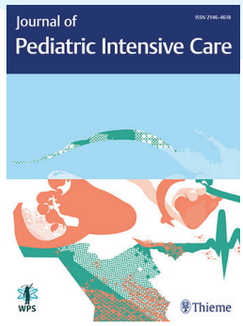

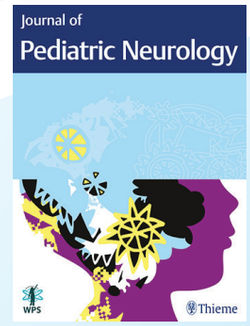

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