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Review Article

Minimal residual disease detection using flow cytometry: Applications in acute leukemia

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ABSTRACT

Minimal residual disease (MRD) describes disease that can be diagnosed by methodologies other than conventional morphology, and includes molecular methods (like polymerase chain reaction (PCR)) or flow cytometry (FCM). Detection and monitoring of MRD is becoming the standard of care, considering its importance in predicting the treatment outcome. MRD aids in identifying high-risk patients and hence therapy can be intensified in them while deintensification of therapy can prevent long-term sequelae of chemotherapy in low-risk category. FCM is considered as a less labor-intensive and faster MRD technique as compared to PCR although it has its own share of disadvantages. Current immune-based methodologies for detection of MRD depend on establishing leukemia-associated aberrant immunophenotype (LAIP), at diagnosis or relapse and use this information at specified time points for detection of MRD, or apply a standardized panel of antibody combinations for all MRD cases, in a different-from-normal approach. This review highlights MRD detection by FCM and its application in acute leukemia.

Introduction

Remission in acute leukemia is considered when neoplastic cells percentage falls below 5% of marrow nucleated cells. Cases of acute leukemia taken to be in remission by above-mentioned criteria may still harbor a large number (up to 10^{10}) of undetectable malignant cells.[1] This has often led to disease relapse in cases presumed to be in remission according to above-mentioned parameter. Minimal residual disease (MRD) describes disease that can be diagnosed by methodologies other than conventional morphology, and includes molecular methods (like polymerase chain reaction (PCR)) or flow cytometry (FCM). It can further be categorized as immunologic MRD (detected by FCM) or molecular MRD (detected by PCR).[2] Around 40% of patients with AML have no genetic markers suitable for PCR monitoring. For those, FCM remains the only option. For others, a combination of the two is ideal and gives maximum information.

Over a period of 25 years, several PCR-based and flow cytometric MRD technologies have stepwise developed into routinely applicable MRD tools, particularly because of

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long-term international collaboration with open exchange of knowledge and experience and collaborative experiments. However, each technique is associated with their inherent advantages and disadvantages.[3–10] Sensitive techniques to detect MRD are expected to give accurate estimation of burden of leukemia and formulation of better therapeutic protocols. MRD detection on FCM platform requires detection of immune phenotype markers with selective positive expression on leukemic cells vis-a-vis negative expression on normal cells of hematopoietic lineage. Such selectivity ensures a very high detection rate (1 leukemic/10,000 normal hematopoietic cells of bone marrow), and up to 2/3 of acute leukemia patients are expected to be benefitted out of it. Also, according to various studies, MRD levels are strongly associated with treatment outcome and clinical remission.[11,12]

Biology and treatment of acute leukemia

In present day scenario, better survival rates for acute leukemia patients treated with intensified regimes have emerged, partly due to much improved supportive care. Current multiagent regimens can offer cure in majority (up to 80%) of pediatric acute lymphoblastic leukemia (ALL) patients.[13,14] Pediatric acute myeloid leukemia (AML) and adult AML/ALL have also shown encouraging trends with multiagent regimens, albeit to a lesser degree. However, intensified chemotherapy is itself fraught with the risk of developing secondary malignancies, cardiomyopathy, and neuropsychiatric manifestations.[15,16] Hence, there was a requirement for identification of the subset of patients with minimal disease burden in them so that the late sequelae of high dose intensified chemotherapy can be avoided. Similarly, the patients with resistant disease below the resolution of morphology can be given intensified therapy or stem cell transplant at an early date to enhance the disease-free survival.

Response to various therapeutic regimes is also affected by the heterogeneous biologic features of each subgroup of leukemia. For example, most often than not, stem cell transplant is the only curative option for pediatric ALL with the *t*(9; 22) translocation or *MLL* gene rearrangements due to less than adequate response to chemotherapeutic agents. Similarly, response to chemotherapy has been quite well in pediatric patients with leukemic cells containing 51–65 chromosomes or rearrangements of *TEL*. In cases of AML, a similar better response to chemotherapy has been seen in cases with 16q22 translocations, *t*(15; 17)/*t*(8; 21). Hence, biologic features and certain clinical parameters (age, leukocyte count, etc.) are used for formulation of therapeutic protocols, in view of a significant correlation seen between them and the clinical outcome in acute leukemia patients.[17]

However, any of the current clinical or biologic features purported is far from being ideal. On one hand, we have seen relapses in patients with 'good risk' features, and on the other hand, unnecessary high intensity treatment has been provided in certain cases. Such inadvertent events may be avoided by MRD studies, as MRD studies during clinical remission are aimed at improving the total leukemic cells burden estimation. Appropriate treatment stratification can be done if one has this MRD information, as it can give an indication to the sensitivity to chemotherapeutic agents and disease aggressiveness.

MRD and its clinical applications

Classically, there are three different approaches used in monitoring MRD which include multiparameter flow cytometric immunophenotyping (FCM), real-time quantitative polymerase chain reaction (RQ-PCR)-based detection of clonal immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangements and RQ-PCR-based detection of fusion transcripts or breakpoints or aberrant/overexpressed genes. The principles and characteristics and the pros and cons of these MRD techniques are summarized below (Table 1).

Immunologic detection of MRD by FCM

Identification of leukemic cells

Flow cytometry was explored as a less labor-intensive and faster MRD technique, when 4- and 6-color cytometers became available in 1998–2002. These multicolor approaches followed classical concepts with emphasis on the detection of aberrant immunophenotypes in the ''empty spaces'' (not overlapping with normal leukocytes) in 2-dimensional dot plots.[3] However, relative nonavailability of monoclonal antibodies to markers on blasts, which were also shared by normal hematopoietic cells had hampered the earlier attempts to study MRD immunologically. A point in case is the concomitant expression of terminal deoxynucleotidyltransferase (TdT) and cluster of differentiation (CD)10 on most leukemic lymphoblast as well as normal B cell precursors (hematogones). Hematogones are seen in plenty in pediatric bone marrow and regenerating marrow following chemotherapy or hematopoietic stem cell grafting.[18] Leukemic cells scattered in bone marrow cannot be detected by using expression of TdT and CD10 in isolation, even though abnormal levels of these markers have been found in some leukemic cases. However, certain differences do exist regarding the expression of these immunophenotypic markers on leukemic cells vis-a-vis normal cells. The abnormal expression of immunophenotypic markers distinguishes the leukemic cells termed as leukemia-associated aberrant immunophenotype (LAIP), which could be cross-lineage antigen expression, maturational asynchrony, and under/overexpression or loss of particular antigen. There may be quantitative or qualitative or both types of the antigenic expression differences among normal progenitor and leukemic cells. Combination of immunophenotypes selectively expressed by blasts with very rare expression on normal marrow cells constitutes the qualitative differences. Notable examples are CD34/CD19/CD21 and CD34/CD56 expression in some cases of B-ALL and AML, respectively. These immunophenotypic combinations, even if they are expressed on normal cells (which is extremely rare), have a very weak expression in comparison to leukemic blasts cells expression levels. T-ALL cells also express a unique combination CD3/TdT, which apart from T cells developing in the thymus, is almost never found on normal hematopoietic cells.

Table 1 – Characteristics of the 3 classical MRD methods in ALL.

MRD technique	Conventional flow cytometry	RQ-PCR of IG/TCR genes	RQ-PCR of fusion transcripts and other aberrances
Estimated sensitivity	3–4 colors: 10^{-3}–10^{-4} 6–8 colors: 10^{-4}	10^{-4}–10^{-5}	10^{-4}–10^{-6}
Applicability	BCP-ALL: .90% T-ALL: .90%	BCP-ALL: 95% T-ALL: 90–95%	BCP-ALL: 25–40% (age dependent) T-ALL: 10–15%
Advantages	Fast analysis at cell population level or single cell level Easy storage of data Information about the whole sample cellularity	Applicable in virtually all BCP-ALL and T-ALL Sensitive Well standardized + regular international QA rounds	Relatively easy Sensitive Applicable for specific leukemia subgroups, such as BCR-ABL or MLL-AF4
Disadvantages	Variable sensitivity, because of similarities between normal (regenerating) cells and malignant cells Limited standardization, no QA results	Time-consuming Expensive Requires extensive experience and knowledge	Limited standardization (only harmonization) Limited QA rounds (with conversion factors) Limited applicability in ALL (absence of targets in 50% of cases) Risk of contamination

Adapted from van Dongen et al. *Blood*. 2015;125(26):3996–4009.
BCP-ALL, B cell phenotype acute lymphoblastic leukemia; T-ALL, T cell phenotype acute lymphoblastic leukemia; QA, quality assurance.

Currently, immune-based methods for diagnosis of MRD depend on establishing LAIP at diagnosis or relapse and use this information at specified time points for detection of MRD, or apply a standardized panel of antibody combinations for all MRD cases, in a different-from-normal approach (Tables 2 and 3).[19,20] In LAIP approach, the log ratio of LAIP-positive cells at follow-up compared to the initial proportion of cells carrying the respective LAIP is most informative with respect to clinical outcome. Hence, LAIP suitable for disease monitoring must be established at the time of diagnosis using comprehensive antibody panels for all cases and the same information be used at the time of MRD estimation. In different-from-normal approach, a standardized panel of antibody combinations and using healthy control bone marrow samples, the patterns of expression of antigens are delineated and templates are made. The biaxial dot plots of chosen combinations of markers will contain consistently blank areas when normal bone marrow cells are analyzed. MRD will be indicated by positive readings in these areas. Normal marrow cells and leukemic blast cells showing similar immunophenotypes can be distinguished using their quantitative differences. For example, the overexpression of CD10 and CD34, underexpression of CD45 and CD38 in CD19 positive blasts are abnormal features in some B-lineage ALL

Table 2 – Immunophenotypic markers used to study MRD in children with ALL.

ALL lineage	Type of phenotypic abnormality	Markers	Frequency (%)[a]
B	Overexpression or underexpression of markers also expressed in normal B-cell progenitors	CD19/CD34/CD10/TdT	30–50
		CD19/CD34/CD10/CD22	20–30
		CD19/CD34/CD10/CD38	30–50
		CD19/CD34/CD10/CD45	30–50
		CD19/CD34/CD10/CD58	40–60
	Expression of markers not expressed in normal B-cell progenitors (**aberrant marker**)	CD19/CD34/CD10/**CD13**	10–20
		CD19/CD34/CD10/**CD15**	5–10
		CD19/CD34/CD10/**CD33**	5–10
		CD19/CD34CD34/CD10/**CD65**	5–10
		CD19/CD34/CD10/**CD56**	5–10
		CD19/CD34/CD10/**CD66c**	10–20
		CD19/CD34/CD10/**7.1**	3–5
	Expression of markers expressed at different stages of normal B-cell maturation	CD19/CD34/CD10/CD21	5–10
		CD19/CD34/TdT/cytoplasmic heavy chain μ	10–20
T	Phenotypes normally confined to the thymus	TdT/CD3	90–95
		CD34/CD3	30–50

Adapted from Dario Campana. *Leukemia and Lymphoma: Detection of Minimal Residual Disease*. 2nd ed. 2003. p. 21–36.

[a] Proportion of childhood ALL cases in which 1 leukemic cell in 10^4 normal bone marrow cells can be detected with the listed immunophenotypic combination. Most cases express more than one combination useful for MRD studies.

Table 3 – Leukemia-associated Aberrant Immunophenotypes (LAIP) Classification in myeloblasts.[20]

LAIP class	Examples
Cross-lineage expression of lymphoid antigens	CD33+CD2+CD34+CD34+CD13 +CD19+
Overexpression	HLA−DR++CD33++CD34++CD64+ +CD4++CD45++
Lack of expression of antigen	HLA−DR−CD33+CD34+
Asynchronous expression of antigens	CD15+CD33+CD34+CD65+CD33 +CD34+

Adapted from Kern et al. *Cancer*. 2008;112(1):4–16.
+, expression; ++, overexpression; −, no expression.

cases.[21] There is an inter-laboratory variation when it comes to the proportion of cases where flow cytometric monitoring of MRD can be undertaken and is influenced by a number of factors. These factors could be variety of markers included for analysis, inclusion of chemotherapy induced regenerating marrow samples in order to specify normal ranges, and the degree of adherence to immunophenotypes associated with leukemia as defined by the laboratory.[22]

T-ALL MRD

As already stated, the combination of nuclear TdT and cytoplasmic or surface CD3 or CD5 is highly pathognomic for T-ALL. CD34 can also be used in cases with weak or negative expression of TdT expression. The detection of T-ALL blasts can be further augmented by use of CD19 and MHC-Class II markers that have strong positive expression on most normal bone marrow TdT+ cells but a negative expression on blasts of T-ALL.

B-ALL MRD

A relatively larger panel of antibodies is required when we are dealing with MRD in ALL of B lymphocytes lineage. Simultaneous expression of CD19, CD10, and CD34 or TdT is normally used to identify immature B cells. In almost 30–50% of cases, the quantitative differences in antigenic expression can also be used to differentiate between leukemic and normal cells. Also CD38, CD45, and CD22 are some other useful markers with different quantitative expression among normal immature B cells and leukemic cells. Qualitative differences between normal and leukemic cells can be detected by using antibodies to myeloid- and NK-associated molecules or to molecules expressed by mature but not immature normal B cells. Up to 85% of B-ALL can be studied at 10^{-4} level of sensitivity with combination of these markers.

AML MRD

Two-thirds or more of AML cases have reportedly found with abnormal expression of these cell markers.[1] The sensitivity of these abnormal immunophenotypes in detection of MRD is not clear, although the CD34/CD56 combination seems to be best suited for the detection of MRD. These 2 CD markers are expressed simultaneously in almost 20% of AML seen in children and they are also related with *t*(8; 21) (q22; q22). The combination of these 2 CD markers can monitor MRD at a sensitivity of 10^{-4}. CD34 and CD87 is the other combination that may be of some use as approximately 70–80% of AML patients are CD87+; however, it is expressed by only 0.2% CD34 + cells normally.[23] However, the immunophenotyping of AML as compared to that of ALL is more heterogenous.

At diagnosis, numerous immunophenotypic subsets may be observed, thereby complicating the selection of the most suitable markers and gates to use for MRD studies. Flow cytometric discrimination of leukemic and normal hematopoietic cells can be improved if multiple cells related parameters are examined. Within the last decade, most diagnostic laboratories shifted rapidly from 3- and 4-color flow cytometers to 8- and 10-color flow cytometers.[3] Moreover, this approach by reducing the usage of number of test tubes per sample allows efficient usage of reagents and cells.

Immunophenotypic analysis: sensitivity and reliability

Number of cells analyzed holds the key to the sensitivity of MRD detection methods. 5×10^7 or even less mononuclear cells may be available in bone marrow specimen from a case of acute leukemia. Technical limitations may decrease the cells for study to less than 1×10^6. Provided that at least 10^8 cells are studies and the fluidics system is comprehensively cleansed, FCM can perceive at least 1 target cell in 10^8 or more cells.[24,25] Practically however, the unavailability of such large samples in usual MRD studies in leukemic patients makes the sensitivity of approximately 1 target cell in 10^4 or 10^5 cells more pragmatic.[19] Therefore, such a high sensitivity can only be achieved by means of markers strongly related with leukemic cells.

Conclusion

Recent prospective studies have reported that in children with ALL, the estimation of MRD is an influential and independent prognostic factor of outcome of therapy. MRD analysis done in early therapeutic phases may also predict value of other prognostic indicators in the early response to therapy, such as the circulating blast cells at day 7 of therapy, response to prednisone, and the recognition of blast cells on days 15 and 21. The results of MRD studies in cases of AML are also strongly indicative of their diagnostic value. Thus, intensification of treatment in patients showing slow early response and having detectable MRD during clinical remission seems to be rational. On the other hand, the exceptional clinical outcome of MRD-negative cases increases the likelihood of using MRD assays to recognize candidates for experimenting reductions in treatment intensity. Currently, virtually all pediatric ALL patients and a large part of adult ALL cases in Western countries are being monitored with MRD techniques to assess treatment effectiveness and assign patients to MRD-based risk groups.

Based on multivariate analysis, the EuroFlow consortium has introduced new high-throughput concepts in flow MRD. The availability of new fluorochromes and 4- to 6-laser flow

cytometers will make 15 colors possible for routine settings in the forthcoming decade. This would contribute to improved applicability and improved specificity of flow MRD measurements. However, the major disadvantage of classical flow MRD is the lack of uniformity in the applied immunostaining protocols, antibody panels, and gating strategies, which differ significantly between centers and between treatment protocols. Hence, the results of flow-based MRD methods between different laboratories are less comparable than PCR-based methods. A concentrated effort needs to be made by all research groups to follow unified standardized protocol, so that the results are comparable and reduce the inter-laboratory variability. Multiple interactive workshops and deliberations for exchange of protocols, as well as consensus on the definition of MRD cutoff levels for risk group assignment would achieve this goal.

Conflicts of interest

The authors have none to declare.

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