folia medica

Folia Medica 65(3):355-361
DOI: 10.3897/folmed.65.e96440

Review

A Concise Review of Flow Cytometric Methods for Minimal Residual Disease Assessment in Childhood B-Cell Precursor Acute Lymphoblastic Leukemia

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Received: 17 Oct 2022 ♦ **Accepted:** 4 Jan 2023 ♦ **Published:** 30 June 2023

Citation: Baldzhieva A, Burnusuzov HA, Murdjeva MA, Dimcheva TD, Taskov HB. A concise review of flow cytometric methods for minimal residual disease assessment in childhood B-cell precursor acute lymphoblastic leukemia. Folia Med (Plovdiv) 2023;65(3):355-361. doi: 10.3897/folmed.65.e96440.

Abstract

Minimal residual disease refers to a leukemia cell population that is resistant to chemotherapy or radiotherapy and leads to disease relapse. The assessment of MRD is crucial for making an accurate prognosis of the disease and for the choice of optimal treatment strategy. Here, we review the advantages and disadvantages of the available genetic and phenotypic methods and focus on the multiparametric flow cytometry as a promising method with greater sensitivity, speed, and standardization options. In addition, we discuss how the application of automated data analysis outweighs the use of complex combinations of windows and gates in classical analysis, thus eliminating subjective evaluation.

Keywords

immunophenotyping, BCP-ALL, minimal residual disease, multiparameter flow cytometry

INTRODUCTION

Minimal residual disease (MRD) refers to a population of leukemia cells in the bone marrow and, less commonly, in the peripheral circulation after treatment. These cells may be primary residual blasts before therapy or transformed secondary blasts, which differ from the primary ones. The genesis of relapsed acute lymphoblastic leukemia (ALL) cells can be observed as early B- or T-cell transformations before they develop into overt leukemia. Drug agents can leave behind small populations of leukemic MRD cells. They may be clones of pre-existing leukemia cells or populations of mutated leukemia cells that either have altered cell markers compared to those of the original blast cells at diagnosis or have mutated genotypes.[1,2] Detection of MRD is crucial for selecting the best therapeutic approaches, predicting clinical outcomes, and striking the important balance between anti-leukemia efficacy and long-term toxicity. Measurement of MRD is performed at different time points during and after treatment with prognostic value at

the cutoff level of 0.01% or more MRD cells indicating a risk for leukemia relapse.[3,4] The study is an overview on the latest research methods and trends for minimal residual disease diagnostics in acute lymphoblastic leukemia by molecular approaches or flow cytometry with an emphasis on the latter.

Methods for evaluation of MRD

There are several laboratory methods for MRD assessment, grouped into two major categories: genetic and phenotypic (**Table 1**).

Genetic methods

They access the genetic elements from chromosomal DNA, allowing the identification of the mutations related to the lymphoproliferative disease as well as the aberrant expression patterns (such as fusion genes, overexpression, etc.) at the RNA level, with possibility for quantification of the latter.

Real-time quantitative polymerase chain reaction (RQ-PCR)

This method allows quantification of DNA amplification products – immunoglobulin and T cell receptor gene rearrangements. It is characterized by a high sensitivity (10^{-5}–10^{-6}), but has some disadvantages such as lack of standardization, complex methodology, high cost, and application in less than half of the cases.[4-6]

Reverse transcription polymerase chain reaction (RT-PCR)

Fusion transcripts that occur after a translocation or deletion are processed by reverse transcriptase to produce complementary DNA. It is characterized by speed and high sensitivity (10^{-5}–10^{-6}). Limitations: mRNA instability, quantitative errors, high cost, low specificity, cross-contamination of products, false-positive results in up to 20% of cases, and need for detection of chromosomal abnormalities at diagnosis for follow up.[4,5,7]

Table 1. Comparison between the methods for detection of MRD

	Sensitivity	Advantages	Disadvantages
FC	10^{-4}	1. Sensitive 2. Relatively economical 3. Rapid (turnaround time is 3-4 hours) 4. No need to use patient specific reagent 5. Quantification of targeted antigen expression 6. Distinct cell populations can be analyzed 7. Archival data can be easily stored 8. Applicable in >95% of cases	1. Standardized in different consortia 2. Continuous education of technicians 3. Difficulties distinguishing blasts from normal precursors 4. Possibility of immunophenotypic shifts 5. Needs fresh samples
RQ-PCR	10^{-5} to 10^{-6}	1. Sensitive quantifications 2. Accurate 3. Detection of MRD in all types of cases of B/T-ALL 4. Stable targets for detection	1. Complex methodology 2. Not applicable in every case (<50% of cases) 3. Need of significant expertise 4. Time-consuming 5. Relatively expensive 6. Limited standardization 7. Amplification of DNA from dead cells
RT-PCR	10^{-5} to 10^{-6}	1. Sensitive 2. Rapid 3. Good readout accuracy	1. Quantification errors 2. Instability of mRNA 3. Time-consuming 4. Complex methodology 5. Limited standardization 6. Amplification of DNA from dead cells
ddPCR	10^{-6}	1. Ultrasensitive 2. Relatively fast (turnaround time is 5-8 hours) 3. Absolute quantification of target DNA samples 4. Requires patient specific reagent 5. Applicable in >95% of cases	1. Limited standardization 2. Requires patient specific reagent 3. Time-consuming 4. Labor-intensive
NGS	10^{-6}	1. Ultrasensitive 2. Possibility for detection of unique genetic patterns, small clonal populations and clonal evolution 3. No need to use patient specific reagent 4. Only US FDA-approved assay	1. Limited standardization 2. Requires pretreatment sample 3. Minimal clinical validation 4. Expensive 5. Turnaround time is ~1 week

FC: flow cytometry; RQ-PCR: real-time quantitative polymerase chain reaction; RT-PCR: reverse transcription polymerase chain reaction; ddPCR: digital droplet polymerase chain reaction; NGS: next generation sequencing

Digital droplet polymerase chain reaction

A modern method that allows for absolute quantification of the target DNA without the need of calibration curves. It is applicable in 95% of cases, but there is no standardization.[5,8]

Next Generation Sequencing

Small DNA fragments are sequenced in parallel multiple times (immunoglobulin and T-cell receptor gene recombination). Advantages: high sensitivity (10^{-6}), speed, detection of different clones and clonal evolution, and in-depth analysis of variations that could lead to relapse. Disadvantages: lack of standardization, need of a sample before starting therapy, high cost, slow release of results (1 week), lack of validation and need for bioinformatic analysis.[5,8]

Immunophenotyping

It is a technique in which specific fluorescent-labeled antibodies identify the expression of surface or intracellular molecules. Currently, it is performed mainly by flow cytometry (FC).

Principles of the FC detection of MRD

MRD is sequentially monitored in bone marrow or peripheral blood samples at several time points during the treatment of children with BCP-ALL: at diagnosis, at 8, 15, and 33 post-therapeutic days (according to BFM-type protocols); before the beginning of consolidation; before the beginning of reinduction; in the end of intensive therapy; during maintenance therapy – on clinical indications.[9] The main challenge is to distinguish blasts from normal precursors during hematopoietic regeneration. There are three main stigmata distinguishing blasts from normal B-cell progenitors: 1) Insufficiency or overexpression of certain markers from the maturation palette of hematogonia. For example, overexpression of CD34 and/or CD10 and underexpression of CD45 and/or CD38 on blasts; 2) Aberrant expression of markers on B-cell precursors, characteristic of other hematopoietic lineages. For example, CD13, CD33, CD56, CD13 (myeloid lineage), CD11c, CD11b (monocytes); 3) "Asynchronous" blast maturation compared to the normal maturation process of B-cell precursors. For example, identification of markers whose expression is not expected for a certain stage of B-cell development such as CD21.[10-14]

An important diagnostic point is the immunophenotypic modulation. For example, Burnusuzov et al.[15] found statistically significant changes in the mean fluorescence intensity (MFI) levels in four of the CD markers expressed by leukemic blasts on days 15 and 33 compared to those at diagnosis: down-modulation of CD10, CD19 and CD34 and up-modulation of CD20.

One of the biggest challenges for reliable detection of MRD by multiparametric flow cytometry (MFC) is the requirement for a well-selected panel of leukocyte markers and well-trained experts in data interpretation. The modern trend, aiming to increase the sensitivity of FC is, on the one hand, to increase the parameters of clinical flow cytometers and, on the other hand, to identify new markers. Today, clinical flow cytometers have 10-12 fluorescence channels, which allow the use of panels with 10-12 and more antibodies. In 2019, Tembhare et al.[16] demonstrated the application of a highly sensitive FC-MRD testing in BCP-ALL. They established an easily reproducible 10-color panel allowing for a high sensitivity of two residual cells per 10^6 cells. It allows the detection of low MRD levels in samples that otherwise could be reported as negative. The main challenges in constructing multi-marker panels are: a) selection of the correct combination of immunophenotypic markers; b) selection of suitable fluorochromes so that they do not interfere with each other; c) finding the optimal concentration of antibodies so that they do not interfere sterically and that there is no excitation of several fluorochromes at the same time. Current recommendations, including those published by ELN[17] and utilized in ALL-REZ-BFM 2002[18] contain the following markers: a) backbone markers – CD10, CD19, CD20, CD34 and CD45; b) mandatory markers – CD38, CD58, and nuclear dye Syto41; c) additional markers – CD9, CD73, CD86, CD123, CD200, and CD304[18-21] Other potentially important markers have also been reported. EuroFlow consortium showed that MFC-MRD can be equally sensitive to RT-PCR with the use of a multicolor assay (8–9 colors or more), newer markers, and acquisition of large numbers of cells. The addition of new markers such as CD73, CD86, and CD123 to traditional backbone markers can improve the discriminability of leukemic blasts from normal B-cell progenitors.[9,16,19,20-25]

Flow cytometric approaches for MRD monitoring

MRD can be assessed by two approaches: a) determination of "leukemia-associated immunophenotypes" (LAIPs) at the time of diagnosis and then tracking the appearance of blasts with that specific phenotype in subsequent samples during follow-up; b) "Different from normal" approach relies on constructing a template of normal bone marrow and the detection of new immunophenotypes deviating from normal cells during follow-up.[26-28] The two approaches are not mutually exclusive, but complementary.

Quantification of MRD

Currently, levels of residual cells from 1×10^{-4} to 1×10^{-5} cells are accepted as having a prognostic significance.[3,4] Achieving a sensitivity of at least 10^{-4} requires acquisition of 1 *million* cells and the presence of at least 100 leukemia cells to define the sample as positive. To reach a sensitivity

of 10^{-5}, it is necessary to collect at least 4-5 million cells. Another issue is the debris events. They can make the MRD assessment difficult and can be caused by air bubbles, doublets, and dead cells. That could be overcome by delineating viable cells by an FSC/SSC gate, by selecting CD45+ cells, or by using viability dyes.[29]

Advantages and disadvantages of FC

Current FC methods for MRD have a lower sensitivity (up to 10^{-4}) than RT-PCR, but are applicable in more than 90% of cases. This is the reason why FC is the method of choice in the practice, along with the lower cost and faster speed. However, there are some disadvantages: sample processing must be done within 24 hours after collection; regenerating post-induction bone marrow may lead to false-positive results; interpretation in hypocellularity is difficult; continuous training is required. The currently emerging "Next Generation Flow Cytometry" is applicable in more than 95% of cases, it is fast, economical, and highly informative but it is a great challenge to analyze the results.[4,5,8,30]

MFC data analysis

There are two approaches for data analysis: classical manual analysis and automated analysis.

In the **classical bivariate analysis**, the operator visually determines the cell populations on two-dimensional plots of markers and selects them through gates. Different combinations of markers are then analyzed using the hierarchical analysis strategy. This approach works well for up to 6 parameters.[31] However, in the past decade, MFC with 10-12 parameters has been rapidly introduced into hematological laboratories. In the presence of multiple parameters, manual analysis takes significant time and is a source of considerable variation due to the subjectivity of gate placement.[32] This bottleneck leads to the need of switching to automated analysis of FC data.

The **automated multivariate data analysis** includes: 1) pre-processing, 2) automated analysis with visualization, and 3) interpretation. Computational tools have been developed for each of these stages. During the first step, the raw data is processed sequentially in several steps with appropriate software for each of them. These include elimination of debris and dead cells and compensation with FLOWJO; data transformation with FLOWCORE; data cleaning with FLOWCLEAN[33], FLOWAI[34] and data normalization with CYTONORM or FLOWSTATS[35].

In the last decade, the number of computational tools for automated analysis of FC data has rapidly increased (extensively reviewed elsewhere[36,37]) They can be divided into two groups: supervised learning methods and unsupervised methods. Supervised methods algorithms require training data with known data sets, and the strength of the algorithms depends on the quality of the source data. With unsupervised methods, no training data sets are needed. These can be divided into six groups: automated sequential gating, Boolean combination gates, multivariate analysis, clustering, dimensionality reduction, and trajectory inference.[36,37] Currently, clustering and dimensionality reduction are the two methods that are mainly used for diagnosis of leukemia.

In clustering, cells with similar profiles are grouped into clusters. They can be visualized by minimum spanning trees (MST), heatmaps, and dimensionality reduction plots.[37] Many computational tools with different algorithms have been developed such as: hierarchical clustering SPADE1, SPADE2, and SPADE3[38]; K-means clustering software FLOWMEANS[39,40] and FLOWPEAKS[41]; density-based clustering FLOWDENSITY[40]; self-organizing map (SOM) FLOWSOM, and dimensionality reduction techniques that aim to map high-dimensional data into a lower-dimensional space by losing as little information as possible – T-SNE, VISNE and UMAP. In the diagnosis of leukemia, FLOWSOM, T-SNE and PHENOGRAPH are the tools that are used the most often.

Approaches for the combined usage of two methods have recently been published. For example, a combination of clustering (FLOWSOM) and dimensional reduction (T-SNE)[36] was used to determine B-cell subpopulations in vaccine studies, and the combination of FLOWSOM and KALUZA was reported to determine MRD in acute myeloid leukemia[42]. An unsupervised method for MRD evaluation in pediatric BCP-ALL is being investigated.[43] These approaches are still under development but they allow establishing disappearance or persistence of diagnostic subclones; emergence of subclones and level of bone marrow regeneration.

CONCLUSIONS

Assessment of MRD in childhood BCP-ALL is extremely important not only in risk stratification but also in determining the subsequent treatment strategies. This requires the development of highly sensitive analytical methods that can be performed rapidly in most patients. MFC is one of the promising methods. With the use of 10-12 phenotypic markers, sensitivity comparable to that of the genetic methods can be achieved with significantly greater speed and standardization of methods. The application of automated analysis overcomes the use of complex combinations of windows and gates and eliminates the subjective evaluation of positive and negative populations. This opens a new era in MRD diagnosis in pediatric BCP-ALL by MFC.

Acknowledgements

This research was funded by the following 1. Intra-university project "DPDP-03/2020", Medical University of Plovdiv; 2. Project "National University Complex for Biomedical Applied Research linked to participation in BBMRI-ERIC" (NUKBPI-BBMRI.BG), Contracts D01-285/17.12.2019

and D01-395/18.12.2020, within the National Road Map of scientific infrastructure (2020-2027); 3. Project Contract No. BG05M2OP001-1.002-0005 – Personalized Innovative Medicine Competence Center (PERIMED); 4. EU, Operational Programme "Science and Education for Smart Growth" (OP SESG) 2014-2020.

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Краткий обзор методов проточной цитофлуорометрии для оценки минимальной резидуальной болезни при остром лимфобластном лейкозе с В-клеточным прекурсором в детском возрасте

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Дата получения: 17 октября 2022 ♦ **Дата приемки:** 4 января 2023 ♦ **Дата публикации:** 30 июня 2023

Образец цитирования: Baldzhieva A, Burnusuzov HA, Murdjeva MA, Dimcheva TD, Taskov HB. A concise review of flow cytometric methods for minimal residual disease assessment in childhood B-cell precursor acute lymphoblastic leukemia. Folia Med (Plovdiv) 2023;65(3):355-361. doi: 10.3897/folmed.65.e96440.

Резюме

Минимальная резидуальная болезнь (МРБ) относится к популяции лейкозных клеток, которая устойчива к химиотерапии или лучевой терапии и приводит к рецидиву заболевания. Оценка МРБ имеет решающее значение для точного прогноза заболевания и выбора оптимальной тактики лечения. Здесь мы рассматриваем преимущества и недостатки доступных генетических и фенотипических методов и сосредотачиваемся на многопараметрической проточной цитометрии как на многообещающем методе с большей чувствительностью, скоростью и вариантами стандартизации. Кроме того, мы обсуждаем, как применение автоматизированного анализа данных перевешивает использование сложных комбинаций окон и ворот (windows and gates) в классическом анализе, что устраняет субъективную оценку.

Ключевые слова

иммунофенотипирование, BCP-ALL, минимальная остаточная болезнь, мультипараметрическая проточная цитофлуорометрия