

Minimal residual disease in acute lymphoblastic leukemia: optimal methods and clinical relevance, pitfalls and recent approaches

Fatemeh Salari · Mohammad Shahjahani ·
Saeid Shahrabi · Najmaldin Saki

Received: 5 September 2014 / Accepted: 20 September 2014 / Published online: 7 October 2014
© Springer Science+Business Media New York 2014

Abstract After advances in experimental and clinical testing, minimal residual disease (MRD) assay results are considered a determining factor in treatment of acute lymphoblastic leukemia patients. According to MRD assay results, bone marrow (BM) leukemic burden and the rate of its decline after treatment can be directly evaluated. Detailed knowledge of the leukemic burden in BM can minimize toxicity and treatment complications in patients by tailoring the therapeutic dose based on patients' conditions. In addition, reduction of MRD before allo-HSCT is an important prerequisite for reception of transplant by the patient. In direct examination of MRD by morphological methods (even by a professional hematologist), leukemic cells can be under- or over-estimated due to similarity with hematopoietic precursor cells. As a result, considering the importance of MRD, it is necessary to use other methods including flow cytometry, polymerase chain reaction (PCR) amplification and RQ-PCR to detect MRD. Each of these methods has its own advantages and disadvantages in terms of accuracy and sensitivity. In this review article, different MRD assay methods and their sensitivity, correlation of MRD assay results with clinical symptoms of the patient as well as pitfalls in results of these methods are

evaluated. In the final section, recent advances in MRD have been addressed.

Keywords Minimal residual disease · Acute lymphoblastic leukemia · Methods

Introduction

Today, an important feature of treatment management especially in acute lymphoblastic leukemia (ALL) is proportionality of treatment rate to the risk of disease relapse, in order to adjust the intensity of treatment and avoid the risk of relapse and treatment-associated cytotoxicity in such patients. To this end, we need to pursue the methods and approaches to improve efficacy of treatment and avoid the risk of relapse [1]. To achieve this goal, the topic of minimal residual disease (MRD assay and measurement of residual cancer cells not detectable by morphological methods) has been addressed, which is independent of clinical predictors.

In view of recent progress in prediction of treatment and its outcome based on MRD assay results, the need for studies and accurate integrated presentation of MRD data has been raised to improve the treatment rate and quality. Multiparameter flow cytometric immunophenotyping (FCM) and quantitative polymerase chain reaction (PCR) are the most common MRD assay methods in ALL with their own advantages and disadvantages [2–4]. In this review article, we first explain the importance of MRD evaluation in ALL. Then, after a brief review of each method, the advantages and disadvantages, gold markers, standardization method and optimum time for MRD assay, the pitfalls of this method will be reviewed, and the best method will be discussed. Eventually, we will take a look at recent developments in the field of MRD assay methods.

F. Salari · M. Shahjahani · N. Saki (✉)
Health Research Institute, Research Center of Thalassemia and Hemoglobinopathy, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
e-mail: najmaldinsaki@gmail.com

S. Shahrabi
Department of Biochemistry and Hematology, Faculty of Medicine, Semnan University of Medical Sciences, Semnan, Iran

Why should we detect MRD in ALL?

Nowadays, with progress in treatment of childhood ALL, over 80 % of children with ALL can be cured with a combination of modern chemotherapy and bone marrow (BM) transplantation (in case of high-risk patients) [5]. However, 20 % of ALL children and 30 % of patients undergoing allo-HSCT experience disease relapse after treatment. The relapse rate can reach up to 60 % in high-risk patients [6]. Furthermore, despite increased overall survival, ALL is the second cause of cancer-related mortality in US children [7]. Moreover, prediction of relapse by current indicators such as the number of blasts at diagnosis and the presence of specific chromosomal abnormalities [e.g., t(4,11) and t(9,22)] is not favorably adequate [8].

In recent years, MRD assay in the early stages of treatment provides more constructive prognostic information than classical prognostic factors. Moreover, maximum decline in MRD before transplantation is currently considered a prerequisite for achievement of allo-HSCT, and patients with a high pre-transplant level of MRD are at a high-risk of relapse [9, 10]. Therefore, MRD assay before allo-HSCT is an important parameter determining the appropriate time and outcome of transplant, which can contribute to post-transplant clinical management [10, 11].

Consequently, MRD is a valuable tool for faster detection of patients who are more likely to relapse. Moreover, the clinical impact of MRD before HSCT and during treatment has made it a valuable parameter for association with clinical markers [12]. However, a number of fundamental problems should be solved before routine use of MRD in clinical decisions, which will be explained below.

Methodological approaches

Multiparametric flow cytometry (FCM) immunophenotyping

FCM is able to detect leukemia-associated immunophenotypic patterns (LAIPs). LAIPs can be detected in over 95 % of ALL patients by FCM [13, 14]. In this method, a combination of fluorochrome-labeled monoclonal antibodies against specific cell surface markers is used to identify the immunophenotypic pattern of the cell surface. Application of this method in MRD assay is increasingly developing due to introduction of new specific markers.

Polymerase chain reaction

Since each lymphocyte has a specific V-D-J set encoding various domains of TCR and Ig, the junctional region of clonal Ig and TCR gene rearrangements provide

fingerprint-like sequences for each lymphocyte. As ALL is usually derived from a lymphoid progenitor, all the leukemic cells derived from a lymphoid precursor are detected by the same rearrangement in Ig and TCR genes [15, 16], which can be identified using the primers standardized in collaboration with BIOMED-1 and -2 framework in over 95 % of patients. This method can detect most common rearrangements of Ig/TCR including IGH, IGK, TCRG, TCRD and SIL-TAL as well as light chain IGK (VK-JK) and IG lambda (IGL, VL-JL) rearrangements [17].

MRD can be assessed in lymphoblastic leukemia through the expression of chromosomal abnormalities and transcripts such as MLL-MFFI, TCF3-PBX1, ETV6-RUNX1, E2A-PBX and BCR-ABL as well as other fusion genes [18, 19]. Each of these methods has its own limitations and capabilities for MRD monitoring, which will be discussed below. Further, optimization of the markers used in each method can result in increased application of the method.

Why and how do we standardize each method for MRD detection?

Given the importance of MRD, the entire process of PCR and FCM requires standardization. In fact, all the stages of PCR screening (including sequencing, primer design and results analysis) and flow cytometry (sample preparation and conditions as well as the choice of appropriate antibodies) require standardization in interlaboratory and international levels. ESG-MRD-ALL organization and the European Commission have presented methods and information for standardization between laboratories at the international level so as to obtain MRD assay with the same quality in different laboratories [20].

The following principles should be observed in PCR-based MRD:

1. The quantity of sample DNA should be sufficient.
2. At least two targets for Ig/TCR-PCR should be available.
3. Sensitivity of the selected targets should be at least 10^{-4} .
4. An authenticated method should be used to assess MRD results [4].
5. Specific amplification should be effectively separated from any unspecific amplification (which is possible by using DNA of healthy subjects as control) [20].
6. Positive control should be used to confirm or rule out false positive results [4].

The following points should be considered in standardization of FCM-based MRD:

1. Standardization of sample preparation and staining.

2. Monoclonal antibodies should be coordinately used as a combination of 4 or 6 colors between the laboratories.
3. The use of different flow cytometry brands and statistical analysis software does not seem to have a negative impact on interpretation of results.
4. A few immunophenotyping compounds should be used to distinguish leukemic cells from healthy immature cells [21].

What are the golden markers for MRD detection by PCR and FCM?

Considering the fact that sensitivity and specificity of a method can significantly affect the interpretation of its results, different methods are used to determine MRD during patient monitoring. The main variable in determining sensitivity and specificity of a test is the marker used in the experiment, which should have high sensitivity, specificity and stability during the course of disease to avoid false negative results. The results of Ig/TCR-PCR rearrangements in patients with B-lineage ALL have indicated that almost all these patients have a rearranged immunoglobulin heavy chain (IGH) [22]. In most cases, rearrangements of IGH are in the form of complete recombination, with incomplete rearrangement of DH-JH detectable in 20 % of patients [23]. It is noteworthy that incomplete rearrangement of IGH is most common in infants with ALL [24]. In fact, IGH gene loci is the most sensitive marker of B-lineage ALL. However, due to ongoing and secondary rearrangement processes, it is prone to oligoclonality (presence of more than one clone of leukemic cells) in 30–40 % of cases [25]. However, if IGH is used in combination with the highly stable “endpoint rearrangements” (that is IGK-Kde rearrangements), it will be a good marker for monitoring MRD in these patients [26]. There is 60 % prevalence of Igk rearrangements in ALL patients [27]. Incomplete TCRD rearrangements (especially V δ 2-D δ 3 and D δ 2-D δ 3) are also good markers for MRD monitoring due to good stability and high sensitivity [28], but their prevalence is low [29]. Rearrangements of TCRG are not good candidates in these patients due to low sensitivity [28]. As a result, IGH rearrangements are the most appropriate candidates for MRD monitoring in B-ALL. In patients with T-ALL, rearrangements of TCRG (especially VH3) [29] and IGH, with a respective prevalence of 84 [30] and 94 % [31], are the most common targets in these patients. However, TCRB rearrangements are less common (54 %) and are often used as a supplement for MRD assay in these patients, being added to screening protocols through BIOMED-2 multiplex TCRB

PCR assay guidelines [32]. In general, the use of TCRA and TCRB as an independent marker has been limited due to the large number of gene segments [29].

In FCM, combined and optimized panels have been introduced in various studies. In more than 90 % of T-ALL patients, the blasts normally express nucleotide TdT/cytoplasmic CD3 (a combined marker limited to the thymus), but B-ALL blasts normally express the phenotypes of B cell precursors in BM [33]. In an extensive study by Borowitz et al. [34] on 2,143 children with B-ALL, the two panels of CD20/CD10/CD45/CD19 and CD19/CD34/CD45/CD9 were introduced as optimal for MRD assay in these patients. Other studies have also suggested the following common panels for MRD assay in B-ALL patients: CD20/CD10/CD19/CD34, CD58/CD10/CD19/CD34, CD10/CD34/CD19/CD45, CD10/CD11a/CD19/CD25 and CD10 \pm CD20/CD38/CD19/CD34 [35–42].

Three combinations were introduced for T-ALL: CD99/CD7/CD5/surface(s), CD3, CD99/CD71/sCD3/c CD3 and TdT/CD7/s CD3/c CD3. In general, TdT/c CD3 (with a prevalence of 90–95 %) is the most common marker of T-ALL [43].

When should we monitor MRD to achieve a better diagnosis and treatment?

Children with undetectable MRD always have excellent prognosis at the end of induction, are good candidates for treatment attenuation and should not be subject to severe treatments (especially BM transplantation) [36]. Overall, clinical impact of MRD is highly dependent upon the therapeutic time point in which it is studied. This means that the initial time point is very important in terms of prognosis and increases the application of MRD in clinical stages. The children oncology group suggested MRD assay 8 days after induction in peripheral blood by FCM as an early time point method for identifying low-risk patients by studying 2,143 children with B-ALL. This period has been suggested to be 28–35 days for BM samples [44]. MRD assay at day 29 in BM by this method was suggested as the most important prognostic time point in multivariate analyses [34]. MRD assay by FCM in BM is recommended on day 15 for estimation of relapse [35]. Recently, MRD assay in BM has been enabled on day 15 in 94 % of ALL patients using 6-color flow cytometry. Moreover, MRD assay is recommended on day 78 to correct the risk estimate [36, 45]. In a study by Basso et al., day 33 was found to be the optimal time to perform PCR on BM sample [46]. Some studies have specified the patients with rapid decrease in the content of leukemic cells on day 19 (by PCR on BM sample) as candidates to reduce the toxicity of

Table 1 Golden time and sample based on clinical value in B- and T-ALL with PCR and FCM protocol

| ALL | Patient number | Time point | Sample | markers | MRD negative (%) | Relapse (%) | References |
|-------|----------------|---|----------------|---|--|----------------------|------------|
| B-ALL | 3184 | Days 33 and 78 of treatment | BM | <i>IGK-Kde</i> (Vk-Kde, intron-Kde), <i>TCRD</i> ; (Vd-(Dd)Jd1, Dd2-Jd1, Vd2-Dd3, Dd2-Dd3), <i>TCRG</i> ; (Vg-Jg1.3/2.3, Vg-Jg1.1/2.1), Complete and incomplete <i>IGH</i> rearrangements (using the BIOMED-1 primer sets), <i>TCRB</i> ; (Dβ-Jβ and Vβ-Dβ-Jβ), <i>IGK</i> (Vκ-Jκ) rearrangements (using BIOMED-2 multiplex PCR primer sets) | 44 % have negative MRD in day 33 77 % have negative MRD in day 78 | 6.3 % 10.6 % | [48] |
| T-ALL | 464 | Days 33 and 78 of treatment | BM | Complete and incomplete TCR-δ, <i>TCRD</i> (Vd-(Dd)Jd1, Dd2-Jd1, Vd2-Dd3, Dd2-Dd3), TCR-γ, <i>TCRG</i> (Vg-Jg1.3/2.3, Vg-Jg1.1/2.1) rearrangements, and SIL-TAL deletion, Complete and incomplete <i>IGH</i> rearrangements (VH-(DH)-JH, DH-JH), (using the BIOMED-1 primer sets), Complete and incomplete TCRB (Vb-(Db)-Jb, Db-Jb) rearrangements (using BIOMED-2 multiplex PCR primer sets) | 16 % in day 33 48 % in day 33 | 7.5 % 8.5 % | [49] |
| B-ALL | 284 | Day 33 | BM | IgH rearrangements and TCR rearrangements | 62 % | 6.2 % | [50] |
| B-ALL | 2143 | Day eight of induction Day 29 (end of induction) Week 22–30 (consolidation) | PB BM BM | The two antibody combinations CD20-FITC/CD10-PE/CD45-PerCP/CD19-APC and CD9-FITC/CD34-PE/CD45PerCP/CD19-PE | 54 % in day 8 74 % in day 29 54 % in week 22–30 | 10 ± 2 % 12 ± 1 % | [34] |

treatment [47]. On the other hand, the importance of MRD timing also depends on the type of leukemia. For example, MRD assay at end induction is valuable for B-ALL, but has a greater value for T-ALL in end consolidation, detecting extramedullary relapse in addition to general relapse [29] (Table 1).

What is the optimal sample for each method?

The choice of optimal sample for MRD assay depends on type of leukemia, patients' conditions, the method used and the time of the survey. In patients with T-ALL, MRD assay is easier to perform on peripheral blood than on BM, enabling more accurate monitoring. On the contrary, MRD assay in patients with B-lineage ALL indicates that despite the presence of leukemic cells in BM, there may be no trace of leukemia in PB [51]. As a result, the sample preferred in patients with B-ALL and T-ALL is BM and PB, respectively. Furthermore, another feature in FCM is freshness of the samples, and delayed samples can adversely affect the results and cause false negative results, while this is not the case for PCR. In addition, MRD assay can be done by FCM on PB samples containing a few blasts for patients whose BM sample is not available [52, 53].

Can MRD be used as a surrogate marker for risk classification in clinical study?

In the past, risk assessment in ALL patients was based on clinical characteristics including age and WBC count as well as biological characteristics of leukemic cells such as immunophenotyping, karyotype and molecular genetic methods [54]. Over the past 15 years, MRD assay in BM of children with ALL has enabled identification of patients with different prognoses [55] and has become one of the most potent predictors of outcome in childhood ALL. The 4-year study by MRD-I-BFM 91 ALL showed that MRD-based classification of ALL patients is the most important criterion to classify approximately 80 % of patients in the multicenter international level [56]. The study conducted by this center as well that of Conter et al. on, respectively, 129 and 3,148 BM samples from patients with ALL in which PCR was done at two time points (day 33 and 78), and MRD completely replaced the common risk factors. In these studies, patients with negative MRD on day 33 were placed in standard risk group, and MRD-negative patients on day 78 were placed in high-risk group [48, 57]. Another study has shown that using MRD results (end induction, i.e., 5 weeks after TP1 diagnosis, and before consolidation treatment, i.e., 3 months after diagnosis of TP2), the

patients can be divided into groups with different relapse risks. Accordingly, MRD-negative patients in both time points (with a sensitivity of at least 10^{-4}) were placed in low-risk group, and MRD-positive patients at both time points ($\geq 10^{-3}$) were placed in the high-risk group [57]. Godoy et al. [44] also studied Ig/TCR on 59 BM samples from ALL patients and found that a positive MRD result (with a sensitivity of 10^{-4}) on day 28–35 in high-risk patients had a prevalence of 78 %. However, in another study, a positive MRD result in BM sample (with a sensitivity of 10^{-2}) on day 15 of induction was classified in the high-risk group [58]. Flow cytometry-based classification is such that each patient having more than 10 % leukemic blasts on day 15 after induction is placed in high-risk group, and any B-ALL patient with MRD $>10^{-3}$, which is still positive on day 78, is classified in the high-risk group [59]. In addition, the presence of MRD by FCM in peripheral blood on day eight and in BM on day 29 is associated with an adverse outcome [34].

What are the important pitfalls in MRD detection?

The change in applied targets due to instability of some rearrangements (clonal evolution) [60] and selection and expansion of subclones not identified upon diagnosis (oligoclonality) are important pitfalls of PCR. This problem may occur between diagnosis and relapse and may cause false negative results [61]. To overcome these pitfalls, detailed analysis of all molecular markers upon diagnosis and selecting two specific and sensitive targets of Ig/TCR rearrangement among them is recommended for each patient [62]. Thus, the origin of the dominant clone can be easily identified, and false negative results are avoided. After determining PCR targets upon diagnosis, clonality should be confirmed by homo-/heteroduplex analyses or by gene scanning to confirm their malignant origin and rule out contamination of normal cells with Ig/TCR gene rearrangements [63].

Changing expression of antigen during treatment, which is often the result of BM regeneration during this stage, is an important pitfall of FCM, which can lead to false positive results in 50–60 % of B-ALL cases [53, 64], and often occurs 78 days after BFM-type induction [36, 65]. Although checking the actual date of sampling is helpful in this regard, delayed treatment may cause untimely the expression of markers. In these cases, the use of two specific immunophenotypic compounds is helpful to reliably distinguish normal immature cells from malignant cells [2, 66]. In addition, 11b has been recently proposed as a good candidate marker to solve this problem, so that its expression is increased in the early stages of childhood ALL and remains high during treatment induction and

consolidation (day 78). In more than 30 % of MRD-positive cases, expression of 11b is higher in blast cells than in memory cells, improving separation of remaining leukemic cells from rearranging BM cells [67].

CD49f is another good candidate to overcome this pitfall in B-ALL, such that detection of MRD using this marker is consistent with results of a standard panel of antibodies (with a sensitivity of 0.02 %), and its expression is indistinguishable in normal BM samples [68]. In addition, decreased expression of CD81 has been considered a determining factor for MRD, and fourfold reduction of it has been reported as an indicator of relapse after treatment induction with 87 and 99 % sensitivity and specificity to detect MRD in B-ALL, respectively [69]. To avoid false negative results in FCM-based studies, assessment of two specific immunophenotypes is recommended. Recently, the sensitivity of this method has been improved to some extent ($\leq 10^{-6}$) with the advent of 6-color flow cytometry.

What is the optimal method for MRD detection?

Since MRD assay is very important especially for predicting disease prognosis, we should seek the most appropriate method to monitor it. This method should be specific for patient characteristics or leukemia type and should have such parameters as satisfactory sensitivity (at least 10^{-4} , i.e., one malignant cell per 10,000 normal cells), extensive use, easy implementation and standardization as well as internal and between-laboratory reproducibility. In the largest study conducted to date, Neil et al. suggested 97 % consistency between the results of both methods by examining 1,300 PB and BM samples by PCR and FCM by regarding 0.001 % sensitivity [70]. However, each method has its own advantages and disadvantages (Table 2).

Although at least one cluster containing at least ten leukemic cells is required for a positive MRD result, positive result of PCR needs a single copy of target sequence in a background of normal cells [56]. As a result, both methods may be similar in detection of MRD in 10^{-4} level. In fact, the difference between PCR and FCM is observed more frequently in samples with low levels of MRD (lower than 10^{-4}), but high-risk patients are not affected, and both methods give the same results [48]. However, MRD monitoring by PCR sometimes indicates a positive result in PCR and a negative result with FCM. This inconsistency can be due to the fact that during PCR, dead cells are evaluated, but FCM cannot detect these cells. In general, we face less false negative results in PCR, and it is a sensitive method after MRD treatment to classify childhood ALL especially in the first 3 months after treatment [77]. However, among MRD assay methods, FCM is the one most affected with BM remission conditions [78].

Table 2 Advantages and disadvantages of PCR versus FCM

| | PCR | FCM | References |
|---------------|---|---|--------------|
| Sensitivity | 10^{-4} – 10^{-6} | 4-color: 10^{-4} 6-color: 10^{-6} | [33, 44] |
| Frequency | B-ALL: 93–98 % | ≤ 90 % | [71] |
| Advantages | High sensitivity High stability of DNA High prevalence | Time- and cost-effective Rapid Detectable in earlier time after induction Applicable for all patients Reproducibility | [47, 72, 73] |
| Disadvantages | Time-consuming Expensive Clonal evolution and false negative B cell precursor regeneration Oligoclonality | Fresh sample Drug-induced immunophenotyping modulation Only detect alive cells Low cellularity during treatment and after it | [44, 74–76] |

The choice of method is also dependent upon patients' conditions. For example, MRD assay by PCR is not suitable for CD10-negative B-ALL and ALL with t(4,11) (both of which show pro-B-ALL phenotype), as a PCR target with high prevalence is not found in these patients. However, FCM interestingly enables easy detection of MRD in all these patients. Therefore, FCM is a proper substitute for Ig/TCR-PCR in this group of ALL patients [79]. Moreover, despite high consistency between FCM and PCR results, FCM is used more frequently in some subsets of ALL, including childhood ALL and immature B- and T-ALL phenotypes [79]. In recurrent BFM-based protocols, FCM and PCR cannot be easily substituted in single time point, and percent consistency between them is mainly dependent on the time of their use [80] (Table 1). On the other hand, eliminating the false negative results using Ig/TCR rearrangement methods due to clonal expansion is inevitable [81], and only 40 % of ALL cases have specific chromosomal abnormalities detectable by PCR [82]. As a result, FCM can be used as a complementary method for PCR. Most studies emphasize the role of these two methods to optimize risk stratification in clinical procedures for reduction of treatment failure risk in children with ALL.

Future perspectives

MRD is one of the most interesting research topics in which sophisticated basic research is transmitted into advanced methods of laboratory diagnosis. Today, given the crucial role of Ig/TCR rearrangements in MRD assay, advanced methods including allele-specific oligonucleotides (ASOs), ASF-sequencing PCR-gene scan sequencing and high-throughput sequencing are used to identify these rearrangements. In ASO, the primers are designed in such a

way that they complement the unique short binding regions of VJ (IGK & TCRG) or VDJ (IGH) rearrangements (as most common targets in ALL) and are used in combination with common primers and complementary probes to join J segments to quantitatively determine MRD by RQ-PCR. Sensitivity of this method is reproducibly $\leq 10^{-4}$ using a single primer and is increased five times by using the second primer [56, 83].

Generating sequencing technology can now give valuable information about clonality of ALL, changing our viewpoint to MRD. Recently, the use of PCR-gene scan sequencing analysis in Ig/TCR gene rearrangement has improved relapse detection on BM samples from B-ALL patients [84]. Next generation sequencing based on high-throughput sequencing globally amplifies and detects antigen receptors, and considers all clonal gene rearrangements in diagnosis [85, 86].

ASF sequencing is a highly sensitive tumor-specific sequencing-based method, with a sensitivity level enough to identify patients with at least one detectable genomic amplification. In PCR-based methods, it is not known whether the signal arises from specific amplification of DNA from leukemic cells or non-specific reproduction of DNA from normal cells, but ASF sequencing is a highly tumor-specific method and can provide useful information for MRD assay.

The sensitivity and reliability of flow cytometry is increasing by application of FCM 6-color. Due to the fact that this method is cheaper than other methods, with improvements that have occurred in this method [87], it can become a rapid and efficient method, which can be a good candidate to evaluate MRD according to patients' conditions [75].

By far, MRD has been highly reflective of treatment response in vivo, providing more information to doctors to

control clinical symptoms in the patients. As a result, it is fair to assume that such individualized medicine approaches will ultimately lead to improved outcome of patients with ALL.

Acknowledgments We wish to thank all our colleagues in Shafa Hospital and Allied Health Sciences School, Ahvaz Jundishapur University of Medical Sciences.

Conflict of interest The authors declare no conflict of interest.

References

- Nyvold C, Madsen HO, Ryder LP, Seyfarth J, Svegaard A, Clausen N, et al. Precise quantification of minimal residual disease at day 29 allows identification of children with acute lymphoblastic leukemia and an excellent outcome. *Blood*. 2002;99(4):1253–8.
- Campana D. Determination of minimal residual disease in leukaemia patients. *Br J Haematol*. 2003;121(6):823–38.
- Szczepański T, van der Velden VH, van Dongen JJ. Flow-cytometric immunophenotyping of normal and malignant lymphocytes. *Clin Chem Lab Med*. 2006;44(7):775–96.
- Cazzaniga G, Biondi A. Molecular monitoring of childhood acute lymphoblastic leukemia using antigen receptor gene rearrangements and quantitative polymerase chain reaction technology. *Haematologica*. 2005;90(3):382–90.
- Hoelzer D, Gokbuget N, Ottmann O, Pui C-H, Relling MV, Appelbaum FR, et al. Acute lymphoblastic leukemia. *Hematology*. 2002;2002(1):162–92.
- Bunin N, Johnston DA, Roberts WM, Ouspenskaia MV, Papusha VZ, Brandt MA, et al. Residual leukaemia after bone marrow transplant in children with acute lymphoblastic leukaemia after first haematological relapse or with poor initial presenting features. *Br J Haematol*. 2003;120(4):711–5.
- Cazzaniga G, Gaipa G, Rossi V, Biondi A. Minimal residual disease as a surrogate marker for risk assignment to ALL patients. *Rev Clin Exp Hematol*. 2003;7(3):292–323.
- Hoelzer D. Monitoring and managing minimal residual disease in acute lymphoblastic leukemia. *Am Soc Clin Oncol Educ Book*. 2013;33:290–3.
- Van der Velden V, Joosten S, Willemse M, Van Wering E, Lankester A, Van Dongen J, et al. Real-time quantitative PCR for detection of minimal residual disease before allogeneic stem cell transplantation predicts outcome in children with acute lymphoblastic leukemia. *Leukemia*. 2001;15(9):1485.
- Bader P, Hancock J, Kreyenberg H, Goulde N, Niethammer D, Oakhill A, et al. Minimal residual disease (MRD) status prior to allogeneic stem cell transplantation is a powerful predictor for post-transplant outcome in children with ALL. *Leukemia* (08876924). 2002;16(9):1668–72.
- Lankester AC, Bierings M, Van Wering E, Wijkhuijs A, de Weger R, Wijnen J, et al. Preemptive alloimmune intervention in high-risk pediatric acute lymphoblastic leukemia patients guided by minimal residual disease level before stem cell transplantation. *Leukemia*. 2010;24(8):1462–9.
- Yeoh AEJ, Ariffin H, Chai ELL, Kwok CSN, Chan YH, Ponudurai K, et al. Minimal residual disease-guided treatment deintensification for children with acute lymphoblastic leukemia: results from the Malaysia–Singapore acute lymphoblastic leukemia 2003 study. *J Clin Oncol*. 2012;30(19):2384–92.
- Van der Velden V, Hochhaus A, Cazzaniga G, Szczepanski T, Gabert J, Van Dongen J. Detection of minimal residual disease in hematologic malignancies by real-time quantitative PCR: principles, approaches, and laboratory aspects. *Leukemia*. 2003;17(6):1013–34.
- van Dongen J. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia*. 2003;17:2257–317.
- Lankester A, Bierings M, van Wering E, Wijkhuijs A, de Weger R, Wijnen J, et al. Preemptive alloimmune intervention in high-risk pediatric acute lymphoblastic leukemia patients guided by minimal residual disease level before stem cell transplantation. *Leukemia* (08876924). 2010;24(8):1462–9.
- Flohr T, Schrauder A, Cazzaniga G, Panzer-Grümayer R, van der Velden V, Fischer S, et al. Minimal residual disease-directed risk stratification using real-time quantitative PCR analysis of immunoglobulin and T-cell receptor gene rearrangements in the international multicenter trial AIEOP-BFM ALL 2000 for childhood acute lymphoblastic leukemia. *Leukemia*. 2008;22(4):771–82.
- Bassan R, Spinelli O, Oldani E, Intermeoli T, Tosi M, Peruta B, et al. Improved risk classification for risk-specific therapy based on the molecular study of minimal residual disease (MRD) in adult acute lymphoblastic leukemia (ALL). *Blood*. 2009;113(18):4153–62.
- Brüggemann M, Schrauder A, Raff T, Pfeifer H, Dworzak M, Ottmann O, et al. Standardized MRD quantification in European ALL trials: proceedings of the second international symposium on MRD assessment in Kiel, Germany, 18–20 September 2008. *Leukemia*. 2010;24(3):521–35.
- Caye A, Beldjord K, Mass-Malo K, Drunat S, Soulier J, Gandemer V, et al. Breakpoint-specific multiplex polymerase chain reaction allows the detection of IKZF1 intragenic deletions and minimal residual disease monitoring in B-cell precursor acute lymphoblastic leukemia. *Haematologica*. 2013;98(4):597–601.
- Van der Velden V, Cazzaniga G, Schrauder A, Hancock J, Bader P, Panzer-Grumayer E, et al. Analysis of minimal residual disease by Ig/TCR gene rearrangements: guidelines for interpretation of real-time quantitative PCR data. *Leukemia*. 2007;21(4):604–11.
- Dworzak MN, Gaipa G, Ratei R, Veltroni M, Schumich A, Maglia O, et al. Standardization of flow cytometric minimal residual disease evaluation in acute lymphoblastic leukemia: multicentric assessment is feasible. *Cytom Part B Clin Cytom*. 2008;74(6):331–40.
- Brüggemann M, Droese J, Bolz I, Lüth P, Pott C, Von Neuhoff N, et al. Improved assessment of minimal residual disease in B cell malignancies using fluorogenic consensus probes for real-time quantitative PCR. *Leukemia* (08876924). 2000;14(8):1419–25.
- Szczepański T, Willemse M, Van Wering E, Van Weerden J, Kamps W, Van Dongen J. Precursor-B-ALL with D H-J H gene rearrangements have an immature immunogenotype with a high frequency of oligoclonality and hyperdiploidy of chromosome 14. *Leukemia* (08876924). 2001;15(9):1415–23.
- Peham M, Panzer S, Fasching K, Haas OA, Fischer S, Marschalek R, et al. Low frequency of clonotypic Ig and T-cell receptor gene rearrangements in t (4; 11) infant acute lymphoblastic leukaemia and its implication for the detection of minimal residual disease. *Br J Haematol*. 2002;117(2):315–21.
- Szczepański T, Flohr T, van der Velden VH, Bartram CR, van Dongen JJ. Molecular monitoring of residual disease using antigen receptor genes in childhood acute lymphoblastic leukaemia. *Best Pract Res Clin Haematol*. 2002;15(1):37–57.
- Van der Velden V, Willemse M, Van der Schoot C, Hähnen K, Van Wering E, Van Dongen J. Immunoglobulin kappa deleting element rearrangements in precursor-B acute lymphoblastic leukemia are stable targets for detection of minimal residual disease by real-time quantitative PCR. *Leukemia* (08876924). 2002;16(5):928–36.

27. Beishuizen A, Verhoeven MA, Mol EJ, van Dongen JJ. Detection of immunoglobulin kappa light-chain gene rearrangement patterns by Southern blot analysis. *Leukemia*. 1994; 8(12):2228–36; discussion 37–9.
28. Van der Velden V, Wijkhuijs J, Jacobs D, Van Wering E, Van Dongen J. T cell receptor gamma gene rearrangements as targets for detection of minimal residual disease in acute lymphoblastic leukemia by real-time quantitative PCR analysis. *Leukemia* (08876924). 2002;16(7):1372–80.
29. Meleshko A, Lipay N, Stasevich I, Potapnev M. Rearrangements of IgH, TCRD and TCRG genes as clonality marker of childhood acute lymphoblastic leukemia. *Exp Oncol*. 2005;27(4):319–24.
30. Szczepański T, Langerak A, Willemsse M, Wolvers-Tettero I, Van Wering E, Van Dongen J. T cell receptor gamma (TCRG) gene rearrangements in T cell acute lymphoblastic leukemia reflect 'end-stage' recombinations: implications for minimal residual disease monitoring. *Leukemia* (08876924). 2000;14(7):1208–14.
31. Moreau E, Langerak A, van Gastel-Mol E, Wolvers-Tettero I, Zhan M, Zhou Q, et al. Easy detection of all T cell receptor gamma (TCRG) gene rearrangements by Southern blot analysis: recommendations for optimal results. *Leukemia* (08876924). 1999;13(10):1620–6.
32. Van Dongen J, Langerak A, Brüggemann M, Evans P, Hummel M, Lavender F, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia*. 2003;17(12):2257–317.
33. Krampera M, Perbellini O, Vincenzi C, Zampieri F, Pasini A, Scupoli MT, et al. Methodological approach to minimal residual disease detection by flow cytometry in adult B-lineage acute lymphoblastic leukemia. *Haematologica*. 2006;91(8):1109–12.
34. Borowitz MJ, Devidas M, Hunger SP, Bowman WP, Carroll AJ, Carroll WL, et al. Clinical significance of minimal residual disease in childhood acute lymphoblastic leukemia and its relationship to other prognostic factors: a Children's Oncology Group study. *Blood*. 2008;111(12):5477–85.
35. Basso G, Veltroni M, Valsecchi MG, Dworzak MN, Ratei R, Silvestri D, et al. Risk of relapse of childhood acute lymphoblastic leukemia is predicted by flow cytometric measurement of residual disease on day 15 bone marrow. *J Clin Oncol*. 2009;27(31):5168–74.
36. Dworzak M. Prognostic significance and modalities of flow cytometric minimal residual disease detection in childhood acute lymphoblastic leukemia. *Blood*. 2002;99:1952–8.
37. Lucio P, Gaipa G, Van Lochem E, Van Wering E, Porwit-MacDonald A, Faria T, et al. BIOMED-1 concerted action report: flow cytometric immunophenotyping of precursor B-ALL with standardized triple-stainings. *Leukemia* (08876924). 2001;15(8):1185–92.
38. Dworzak M, Fröschl G, Printz D, De Zen L, Gaipa G, Ratei R, et al. CD99 expression in T-lineage ALL: implications for flow cytometric detection of minimal residual disease. *Leukemia*. 2004;18(4):703–8.
39. Dworzak M, Fritsch G, Fleischer C, Printz D, Fröschl G, Buchinger P, et al. Comparative phenotype mapping of normal vs. malignant pediatric B-lymphopoiesis unveils leukemia-associated aberrations. *Exp Hematol*. 1998;26(4):305–13.
40. Campana D, Coustan-Smith E. Detection of minimal residual disease in acute leukemia by flow cytometry. *Cytometry*. 1999;38(4):139–52.
41. Weir E, Cowan K, LeBeau P, Borowitz M. A limited antibody panel can distinguish B-precursor acute lymphoblastic leukemia from normal B precursors with four color flow cytometry: implications for residual disease detection. *Leukemia* (08876924). 1999;13(4):558–67.
42. Veltroni M, De Zen L, Sanzari MC, Maglia O, Dworzak MN, Ratei R, et al. Expression of CD58 in normal, regenerating and leukemic bone marrow B cells: implications for the detection of minimal residual disease in acute lymphocytic leukemia. *Haematologica*. 2003;88(11):1245–52.
43. Coustan-Smith E, Sancho J, Hancock ML, Boyett JM, Behm FG, Raimondi SC, et al. Clinical importance of minimal residual disease in childhood acute lymphoblastic leukemia. *Blood*. 2000;96(8):2691–6.
44. Assumpcao JG, Paula FDF, Xavier SG, Murao M, Aguirre Neto JCd, Dutra AP, et al. Gene rearrangement study for minimal residual disease monitoring in children with acute lymphocytic leukemia. *Revista Brasileira De Hematol E Hemoter*. 2013;35(5):337–42.
45. Dworzak MN, Panzer-Grümayer ER. Flow cytometric detection of minimal residual disease in acute lymphoblastic leukemia. *Leuk Lymphoma*. 2003;44(9):1445–55.
46. De Haas V, Verhagen O, von dem Borne A, Kroes W, Van Den Berg H, Van Der Schoot C. Quantification of minimal residual disease in children with oligoclonal B-precursor acute lymphoblastic leukemia indicates that the clones that grow out during relapse already have the slowest rate of reduction during induction therapy. *Leukemia*. 2001;15(1):134–40.
47. Coustan-Smith E, Sancho J, Behm FG, Hancock ML, Razzouk BI, Ribeiro RC, et al. Prognostic importance of measuring early clearance of leukemic cells by flow cytometry in childhood acute lymphoblastic leukemia. *Blood*. 2002;100(1):52–8.
48. Conter V, Bartram CR, Valsecchi MG, Schrauder A, Panzer-Grümayer R, Möricke A, et al. Molecular response to treatment redefines all prognostic factors in children and adolescents with B-cell precursor acute lymphoblastic leukemia: results in 3184 patients of the AIEOP-BFM ALL 2000 study. *Blood*. 2010;115(16):3206–14.
49. Schrappe M, Valsecchi MG, Bartram CR, Schrauder A, Panzer-Grümayer R, Möricke A, et al. Late MRD response determines relapse risk overall and in subsets of childhood T-cell ALL: results of the AIEOP-BFM-ALL 2000 study. *Blood*. 2011;118(8):2077–84.
50. Zhou J, Goldwasser MA, Li A, Dahlberg SE, Neuberg D, Wang H, et al. Quantitative analysis of minimal residual disease predicts relapse in children with B-lineage acute lymphoblastic leukemia in DFCI ALL Consortium Protocol 95-01. *Blood*. 2007;110(5):1607–11.
51. Coustan-Smith E, Sancho J, Hancock ML, Razzouk BI, Ribeiro RC, Rivera GK, et al. Use of peripheral blood instead of bone marrow to monitor residual disease in children with acute lymphoblastic leukemia. *Blood*. 2002;100(7):2399–402.
52. Borowitz MJ, Pullen DJ, Winick N, Martin PL, Bowman WP, Camitta B. Comparison of diagnostic and relapse flow cytometry phenotypes in childhood acute lymphoblastic leukemia: implications for residual disease detection: a report from the children's oncology group. *Cytom Part B Clin Cytom*. 2005;68(1):18–24.
53. Gaipa G, Basso G, Maglia O, Leoni V, Faini A, Cazzaniga G, et al. Drug-induced immunophenotypic modulation in childhood ALL: implications for minimal residual disease detection. *Leukemia*. 2005;19(1):49–56.
54. Pui C-H, Evans WE. Treatment of acute lymphoblastic leukemia. *N Engl J Med*. 2006;354(2):166–78.
55. Borowitz MJ, Devidas M, Hunger SP, Bowman WP, Carroll AJ, Carroll WL, et al. Clinical significance of minimal residual disease in childhood acute lymphoblastic leukemia and its relationship to other prognostic factors: a Children's Oncology Group study. *Blood*. 2008 Jun 15;111(12):5477–85.
56. Flohr T, Schrauder A, Cazzaniga G, Panzer-Grümayer R, Van Der Velden V, Fischer S, et al. Minimal residual disease-directed risk stratification using real-time quantitative PCR analysis of immunoglobulin and T-cell receptor gene rearrangements in the international multicenter trial AIEOP-BFM ALL 2000 for childhood acute lymphoblastic leukemia. *Leukemia*. 2008;22(4):771–82.
57. van Dongen JJ, Seriu T, Panzer-Grümayer ER, Biondi A, Pongers-Willemsse MJ, Corral L, et al. Prognostic value of minimal

- residual disease in acute lymphoblastic leukaemia in childhood. *The Lancet*. 1998;352(9142):1731–8.
58. Ryan J, Quinn F, Meunier A, Boublíkova L, Crampe M, Tewari P, et al. Minimal residual disease detection in childhood acute lymphoblastic leukaemia patients at multiple time-points reveals high levels of concordance between molecular and immunophenotypic approaches. *Br J Haematol*. 2009;144(1):107–15.
 59. Basso G, Veltroni M, Grazia Valsecchi M, Dworzak MN, Ratei R, D Silvestri, et al. Risk of relapse of childhood acute lymphoblastic leukemia is predicted by flow cytometric measurement of residual disease on day 15 bone marrow. *J Clin Oncol*. 2009;27(31):5168–74.
 60. Guggemos A, Eckert C, Szczepanski T, Hanel C, Taube T, van der Velden V, et al. Assessment of clonal stability of minimal residual disease targets between 1st and 2nd relapse of childhood precursor B-cell acute lymphoblastic leukemia. *Haematologica*. 2003;88(7):737–46.
 61. Gawad C, Pepin F, Carlton VE, Klinger M, Logan AC, Miklos DB, et al. Massive evolution of the immunoglobulin heavy chain locus in children with B precursor acute lymphoblastic leukemia. *Blood*. 2012;120(22):4407–17.
 62. Nyvold C. Precise quantification of minimal residual disease at day 29 allows identification of children with acute lymphoblastic leukemia and an excellent outcome. *Blood*. 2002;99:1253–8.
 63. Cazzaniga G, Biondi A. Molecular monitoring of minimal residual disease. *Treatment of acute leukemias*. Berlin: Springer; 2003. p. 537–47.
 64. Borowitz M, Pullen D, Winick N, Martin P, Bowman W, Camitta B. Comparison of diagnostic and relapse flow cytometry phenotypes in childhood acute lymphoblastic leukemia: implications for residual disease detection: a report from the children's oncology group. *Cytom Part B Clin Cytom*. 2005;68(1):18–24.
 65. Dworzak M, Fritsch G, Fleischer C, Printz D, Fröschl G, Buchinger P, et al. Multiparameter phenotype mapping of normal and post-chemotherapy B lymphopoiesis in pediatric bone marrow. *Leukemia* (08876924). 1997;11(8):1266–73.
 66. McKenna RW, Washington LT, Aquino DB, Picker LJ, Kroft SH. Immunophenotypic analysis of hematogones (B-lymphocyte precursors) in 662 consecutive bone marrow specimens by 4-color flow cytometry. *Blood*. 2001;98(8):2498–507.
 67. Rhein P, Mitlohner R, Basso G, Gaipa G, Dworzak MN, Kirschner-Schwabe R, et al. CD11b is a therapy resistance- and minimal residual disease-specific marker in precursor B-cell acute lymphoblastic leukemia. *Blood*. 2010;115(18):3763–71.
 68. DiGiuseppe JA, Fuller SG, Borowitz MJ. Overexpression of CD49f in precursor B-cell acute lymphoblastic leukemia: Potential usefulness in minimal residual disease detection. *Cytom Part B Clin Cytom*. 2009;76(2):150–5.
 69. Muzzafar T, Medeiros LJ, Wang SA, Brahmandam A, Thomas DA, Jorgensen JL. Aberrant underexpression of CD81 in precursor B-cell acute lymphoblastic leukemia utility in detection of minimal residual disease by flow cytometry. *Am J Clin Pathol*. 2009;132(5):692–8.
 70. Neale G, Coustan-Smith E, Stow P, Pan Q, Chen X, Pui C, et al. Comparative analysis of flow cytometry and polymerase chain reaction for the detection of minimal residual disease in childhood acute lymphoblastic leukemia. *Leukemia*. 2004;18(5):934–8.
 71. Van der Velden V, Szczepanski T, Wijkhuijs J, Hart P, Hoogeveen P, Hop W, et al. Age-related patterns of immunoglobulin and T-cell receptor gene rearrangements in precursor-B-ALL: implications for detection of minimal residual disease. *Leukemia*. 2003;17(9):1834–44.
 72. Malec M, Van der Velden V, Björklund E, Wijkhuijs J, Söderhäll S, Mazur J, et al. Analysis of minimal residual disease in childhood acute lymphoblastic leukemia: comparison between RQ-PCR analysis of Ig/TcR gene rearrangements and multicolor flow cytometric immunophenotyping. *Leukemia*. 2004;18(10):1630–6.
 73. Van der Velden V, Panzer-Grümayer E, Cazzaniga G, Flohr T, Sutton R, Schrauder A, et al. Optimization of PCR-based minimal residual disease diagnostics for childhood acute lymphoblastic leukemia in a multi-center setting. *Leukemia*. 2007;21(4):706–13.
 74. Aricò M, Valsecchi MG, Rizzari C, Barisone E, Biondi A, Casale F, et al. Long-term results of the AIEOP-ALL-95 trial for childhood acute lymphoblastic leukemia: insight on the prognostic value of DNA index in the framework of berlin-frankfurt-muenster-based chemotherapy. *J Clin Oncol*. 2008;26(2):283–9.
 75. Irving J, Jesson J, Virgo P, Case M, Minto L, Eyre L, et al. Establishment and validation of a standard protocol for the detection of minimal residual disease in B lineage childhood acute lymphoblastic leukemia by flow cytometry in a multi-center setting. *Haematologica*. 2009;94(6):870–4.
 76. Klein O, Schmidt C, Knights A, Davis ID, Chen W, Cebon J. Melanoma vaccines: developments over the past 10 years. *Expert Rev Vaccines*. 2011;10(6):853–73.
 77. Van Dongen J, Macintyre E, Gabert J, Delabesse E, Rossi V, Saglio G, et al. Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease. *Leukemia* (08876924). 1999;13(12):1901–28.
 78. Luria D, Rosenthal E, Steinberg D, Kodman Y, Safanaiev M, Amariglio N, et al. Prospective comparison of two flow cytometry methodologies for monitoring minimal residual disease in a multicenter treatment protocol of childhood acute lymphoblastic leukemia. *Cytom Part B Clin Cytom*. 2010;78(6):365–71.
 79. Robillard N, Cavé H, Méchinaud F, Guidal C, Garnache-Ottou F, Rohrlach PS, et al. Four-color flow cytometry bypasses limitations of IG/TCR polymerase chain reaction for minimal residual disease detection in certain subsets of children with acute lymphoblastic leukemia. *Haematologica*. 2005;90(11):1516–23.
 80. Gaipa G, Cazzaniga G, Valsecchi MG, Panzer-Grümayer R, Buldini B, Silvestri D, et al. Time point-dependent concordance of flow cytometry and RQ-PCR in minimal residual disease detection in childhood acute lymphoblastic leukemia. *Haematologica*. 2012; Haematologica. 2011.060426
 81. Van der Velden V, Wijkhuijs J, Van Dongen J. Non-specific amplification of patient-specific Ig/TCR gene rearrangements depends on the time point during therapy: implications for minimal residual disease monitoring. *Leukemia*. 2008;22(3):641–4.
 82. Campana D, Neale G, Coustan-Smith E, Pui C. Detection of minimal residual disease in acute lymphoblastic leukemia: the St Jude experience. *Leukemia*. 2001;15(2):278–9.
 83. Poopak B, Saki N, Purfatholah AA, Najmabadi H, Mortazavi Y, Arzani MT, et al. Pattern of immunoglobulin and T-cell receptor- δ/γ gene rearrangements in Iranian children with B-precursor acute lymphoblastic leukemia. *Hematology*. 2013.
 84. Germano GD, Del Giudice L, Palatron S, Giarin E, Cazzaniga G, Biondi A, et al. Clonality profile in relapsed precursor-B-ALL children by genescan and sequencing analyses. Consequences on minimal residual disease monitoring. *Leukemia*. 2003;17(8):1573–82.
 85. Faham M, Zheng J, Moorhead M, Carlton VE, Stow P, Coustan-Smith E, et al. Deep-sequencing approach for minimal residual disease detection in acute lymphoblastic leukemia. *Blood*. 2012;120(26):5173–80.
 86. Boyd SD, Marshall EL, Merker JD, Maniar JM, Zhang LN, Sahaf B, et al. Measurement and clinical monitoring of human lymphocyte clonality by massively parallel VDJ pyrosequencing. *Sci Transl Med*. 2009;1(12):12ra23.
 87. Orfao A, Schmitz G, Brando B, Ruiz-Arguelles A, Basso G, Braylan R, et al. Clinically useful information provided by the flow cytometric immunophenotyping of hematological malignancies: current status and future directions. *Clin Chem*. 1999;45(10):1709.