Prognostic significance of monitoring leukemia-associated immunophenotypes by eight-color flow cytometry in adult B-acute lymphoblastic leukemia

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Minimal residual disease (MRD) is of the most important factor for predicting prognosis and guiding treatment of acute lymphoblastic leukemia (ALL). In this study, we investigated the prognostic significance of leukemia-associated immunophenotypes (LAIPs) as assessment of index of MRD in 125 adult B-lineage ALL (B-ALL) patients by eight-color flow cytometry. The LAIPs could be identified in 96.6% and 81.6% of patients with the sensitivity of $10^{-4}$ and $10^{-5}$, respectively. MRD-negative status could clearly predict a favorable 2-year relapse-free survival (RFS) and overall survival (OS) at the end of induction of complete remission and one cycle of consolidation treatment. Moreover, we identified a group of cases with MRD of 0.001% to <0.01%, which showed significantly higher 2-year relapse rate than those with undetectable one. In multivariate analysis, MRD status was associated with RFS or OS independently. Furthermore, MRD assessed by LAIPs and RQ-PCR assay for patients with BCR-ABL fusion gene yielded concordant results in 89.7% of cases. In conclusion, MRD evaluated by eight-color flow cytometry could provide an important tool to assess treatment response and prognosis precisely in adult B-ALL.

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INTRODUCTION

It is well known that acute lymphoblastic leukemia (ALL) is a group of heterogeneous diseases in terms of chromosome translocations or molecular genetic abnormalities, which have an important role in the leukemogenesis and risk stratification. However, a great proportion of ALL patients lack these typical genetic abnormalities, and more importantly, minimal residual disease (MRD) has an essential role in predicting relapse and even overall survival (OS). Currently, MRD assessment is increasingly applied in clinical practice for monitoring ALL that may provide physicians enough information to intervene the planned treatment of a patient earlier with intensification of chemotherapy or allogeneic stem cell transplantation.

Quantification of clonal rearrangements of immunoglobulin/T-cell receptor (IG/TCR) genes or fusion genes by PCR and leukemia-associated immunophenotypes (LAIPs) by multiparameter flow cytometry (MFC) are the most commonly used methods for MRD assessment. PCR assay has been highly standardized by several study groups and considered as the gold standard for MRD monitoring in most European trials. Recently, with the development of multi-color flow cytometry and new markers, MFC method for MRD evaluation based on LAIPs is increasingly used in the management of ALL with high applicability, sensitivity and specificity and has been regarded as an important counterpart of PCR detection. Eight-color flow cytometric assay in assessing MRD allows to explore the expression of more cellular antigens in one combination associating a larger number of monoclonal antibodies. In addition, as compared with the classic one (3–4 color assay), it can dramatically save samples and reagents and can also offer the possibility of increasing accuracy in population identification. Furthermore, this method has recently been well standardized by the Euro Flow Consortium, which provides researchers with a practicable guideline.

Although early MRD evaluation in induction period has been introduced to guide the treatment in most major childhood ALL protocols, the value of MRD in adult ALL is not evaluated so widely as pediatric patients. In this study, we attempted to utilize a sensitive and reliable assay for monitoring MRD by eight-color flow cytometry. Furthermore, we attempted to address the prognostic value of MRD status using LAIPs in adult B-ALL at different time points, such as the end of induction of complete remission (CR) and one consolidation therapy.

MATERIALS AND METHODS

Patients and samples
A total of 125 patients with de novo B-ALL were enrolled in this study from October 2008 to August 2011 in our center. The diagnostic and immunological classification of ALL was established according to the WHO 2008 criteria. All the patients were treated in a schedule of Shanghai Institute of Hematology-based regimen. All of them gave informed consent according to the Declaration of Helsinki.
At diagnosis, 120 out of 125 patients (96%) had at least one suitable LAIPs with 0.01% sensitivity for MRD measurement by eight-color flow cytometry. With the exclusion of 14 patients from the above 120 cases who could not achieve a CR, finally a cohort of 106 adult B-ALL patients was formed for further prognostic analysis. MRD evaluation was performed in 712 follow-up samples from the 106 cases that were obtained at the end of induction of CR and after one cycle of consolidation and then were followed up every 1–2 months if the white blood cells (WBCs) was >2 × 10^9/l within the first year. All of the 106 patients underwent MRD detection at the end of induction of CR and one cycle of consolidation.

Then the patients were followed up if the samples were available at the detection at the end of induction of CR and one cycle of consolidation. After CR was achieved, 33 cases were consolidated with stem cell transplantation and other 73 patients were treated with chemotherapy consolidation. The detailed baseline clinical, immunophenotypic and cytogenetic characteristics of 106 patients are shown in Table 1.

### Table 1. Baseline clinical characteristics of 106 B-ALL patients enrolled for survival analysis

| Characteristics                        | Number of cases/total number (%) |
|----------------------------------------|----------------------------------|
| **Gender**                             |                                  |
| Male                                   | 53/106 (50.0)                    |
| Female                                 | 53/106 (50.0)                    |
| **Median age, in years (range)**       | 34 (18–60)                       |
| **Median WBC count, × 10^9/l (range)** | 9.5 (1.0–379.0)                  |
| >30 × 10^9/l                            | 20/106 (18.9)                    |
| ≤30 × 10^9/l                            | 86/106 (81.1)                    |
| **Median platelets count, × 10^9/l (range)** | 41.0 (2–467)                    |
| <30 × 10^9/l                            | 42/106 (39.6)                    |
| ≥30 × 10^9/l                            | 64/106 (60.4)                    |
| **Median Hb level, g/l (range)**       | 93.0 (40–778)                    |
| >75 g/l                                | 32/106 (30.2)                    |
| ≥75 g/l                                | 74/106 (69.8)                    |
| **Median BM blasts, % (range)**        | 80.4 (8–97.9)                    |
| **Median number of LAIPs per patient** | 4 (1–8)                          |
| **B-ALL subtypes**                     |                                  |
| pro-B ALL                              | 23/106 (21.7)                    |
| pre-B ALL                              | 13/106 (12.3)                    |
| common-B ALL                           | 66/106 (62.3)                    |
| mature-B ALL                           | 4/106 (3.8)                      |
| Late CRa                                | 42/106 (39.6)                    |
| **Cytogenetic normal**                 | 28/95 (29.5)                     |
| **Cytogenetic quantity abnormalities** |                                  |
| Hyperdiploidy                          | 2/95 (2.1)                       |
| Hypodiploidy                           | 7/95 (7.4)                       |
| Non-translocation structural changes   | 17/95 (17.9)                     |
| **Karyotype, fusion gene**             |                                  |
| t(9;22), BCR-ABL                      | 30/95 (31.6)                     |
| t(11;11), MLL-EPS15                   | 1/95 (1.1)                       |
| t(4;11), MLL-AFF1                     | 2/95 (2.1)                       |
| t(11;19), MLL-MLLT1                   | 1/95 (1.1)                       |
| t(11;19), TCF3-PBX1                   | 3/95 (3.2)                       |
| t(12;21), TEL-AML1                    | 3/95 (3.2)                       |
| t(9;22) and t(11;19), BCR-ABL and TCF3-PBX1 | 1/95 (1.1)                     |
| **CRLF2 overexpression**               | 5/65 (7.7)                       |
| **IK6 variant of IKZF1 gene**          | 15/65 (23.1)                     |

**Table 2. The mAb combinations utilized for MRD follow-up in 120 B-ALL patients**

| Seven fixed mAbs | Alternative mAb | Number (%) |
|------------------|-----------------|------------|
| CD58-FITC        | CD66C-PE        | 28 (23.3)  |
| CD38-perCp-cy5.5 | CD13-PE         | 4 (3.3)    |
| CD34-PE-Cy7, CD10-APC | CD33-PE   | 8 (6.7)    |
| CD20-APC-eFlour780 | CD13-PE + CD33-PE | 12 (10.0) |
| CD19-Brilliant Violet-421 | CD66C-PE + CD33-PE | 13 (10.8) |
| CD45-Pacific Orange | CD66C-PE + CD13-PE | 1 (0.8)    |
| 7.1-PE           | CD15-PE         | 3 (2.5)    |
| 7.1-PE           | CD15-PE + 7.1-PE | 2 (1.5)    |
| None             |                 | 45 (37.5)  |

**Abbreviations:** APC, allophycocyanin; B-ALL, B-lineage acute lymphoblastic leukemia; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; MAb, monoclonal antibody; WBC, white blood cell; LAIP, leukemia-associated immunophenotype; WBC, white blood cell. *Complete remission is achieved after 35 days after initiation of the treatment. **Two or one of them are positive. *This patient has two kinds of abnormalities.

Immunophenotypic investigation of MRD by LAIPs

Fresh heparinized whole-bone marrow (BM) samples were processed on a standard NH4Cl whole-blood lysing technique for immunophenotyping at diagnosis and MRD monitoring during follow-up. Briefly, the BM sample containing up to 3 × 10^6 WBCs were incubated with a titrated reagent cocktail and incubated in the dark at room temperature for 15 min, then about 2.0 ml of buffered NH4Cl containing 0.25% ultrapure formaldehyde (Polysciences, Warrington, PA, USA) was added and incubated at room temperature in the dark for 15 min followed by a single wash with phosphate-buffered saline containing 0.3% bovine serum albumin. If > 200 μl of BM were needed for collecting up to 3 × 10^6 WBCs, the lysing procedure followed by a single wash would be performed before staining process. For samples where TDG and cytoplasmic (Cy) CD79a and IgM (μ) were assessed, the BM were processed using the Fix-and-Perm kit according to the manufacturer’s guidelines. The information of monoclonal antibodies (mAbs) and reagents used at diagnosis are shown in Supplementary Table S1. At least 1 × 10^6 blast cells identified by a low expression of CD45 and low side scatter (SSC) properties were obtained, and antigenic expression on blast population was systematically analyzed by eight-color flow cytometry (LSR-II, Becton Dickinson, San José, CA, USA) at diagnosis. Subtypes of B-ALL were classified into four groups as pro-B, common-B, pre-B and mature-B ALL. LAIP was identified as a cell population that could be separated completely from its counterpart at specific stage of maturation in either normal or regenerating marrow by the patterns of antigenic expression. Four main types of aberrant phenotypes in B-ALL were defined at diagnosis for LAIPs as follows: (1) cross-lineage antigen expression, (2) asynchronous antigen expression, (3) antigen dim/strong expression, and (4) ectopic phenotypes.

Although the median number of LAIPs for each case were 4 (1–7), only the aberrant antigens expressed on majority (>90%) of leukemic blasts in certain case were chosen for MRD detection. Table 2 shows the mAb combinations utilized for WBC, B-lineage ALL cases by single-tube panel. Dead cells and debris were excluded by forward scatter (FSC)/SSC and CD45/SCC dot plots. Doublets were excluded on FSC-A/FSC-H dot plots. All B-lineage cells were identified by expression of CD19 with low-to-intermediate SSC. To identify LAIPs as specific as possible, we used the ‘and’ logistic gating strategy by FACSDiva software (Becton Dickinson, San Jose, CA, USA) to define final MRD population to display co-existence of multiple aberrant antigen expression if leukemic population was homogenous. The ‘or’ logistic gating strategy was applied to include the highest quantity of MRD when the leukemic blasts were heterogeneous containing ≥2 subpopulations with absolutely different aberrant phenotypes. MRD was defined as an accumulation of 10 clustered events showing lymphoid-scattering properties and LAIP characteristics. When identified, MRD was quantified as a percentage of the total WBCs. To reach a theoretical maximum sensitivity of 0.001%, we needed 2.0 × 10^6 WBCs for each sample. Within 712 total follow-up samples, 14 and 6 at the end of
induction of CR and after one consolidation failed to reach this level (only 5.0 × 10^6 WBCs were attainable), respectively. In these 20 samples, 19 contained MRD > 0.01% and one sample had MRD of 0.006%. For the remaining 201 and 491 samples, at least 1.0 × 10^6 and 2.0 × 10^6 cells could be obtained, respectively. Consequently, 97.2% (692/712) of follow-up BM samples with > 1.0 × 10^6 WBCs available could reach a theoretical sensitivity of 1.0 × 10^-3. We have shown in Supplementary Figure S1 the process of LAIPs identified at diagnosis and MRD assessed at different treatment time points of a B-ALL case in detail.

MRD was considered negative when leukemic cells were < 0.01%, and, for the patients with positive MRD results, three groups of low (0.01% ≤ MRD < 0.1%), intermediate (0.1% ≤ MRD < 1.0%) and high (MRD ≥ 1.0%) levels were classified.

Sensitivity evaluation of eight-color flow cytometry assay
To examine the sensitivity of eight-color flow cytometric assay in MRD detection in B-ALL, dilution experiments were performed by adding LAIPs-positive (LAIPs+) cells obtained from B-ALL patient to regenerating BM sample from age-matched patients of AML with undetectable MRD after chemotherapy, in which the dilution factor ranged from 1:1, 1:10, 1:100, 1:1000, 1:20,000, 1:40,000, 1:80,000, 1:100,000 to 1:200,000 by volume. The BM samples from five B-ALL patients with main types of LAIPs, including major cross-lineage antigen expression and antigen dim/st expression, underwent this test. At least 2.0 × 10^6 WBCs were acquired in dilution series of 1:10000 to 1:200000. The same analytical strategies as that for leukemic blasts was applied in each dilution.

Normal BM cells from 20 non-hematological malignant patients and 30 regenerating BM samples after chemotherapy were used as control to establish normal antigenic expression patterns and evaluate the background expression of LAIP combinations.

Real-time quantitative (RQ)-PCR amplification of BCR-ABL fusion gene
Total RNA was extracted from mononuclear cells using TRizol reagent (Life Technologies, Grand Island, NY, USA), and RQ-PCR was performed using 1 μg RNA with the BCR-ABL p210/p190 KIT (Yuanni Bio-pharmacetical CO., LTD, Shanghai, China) according to the manufacturer’s recommendations in triplicate on ABI 7500 Real Time PCR system (Life Technologies). The standardization of RQ-PCR assay was performed as in previous studies. The quantity of BCR-ABL transcript was calculated as a ratio of BCR-ABL copy number relative to ABL copies.

Statistical analysis
CR was defined by <5% blast cells in a regenerated BM aspirate, lack of extramedullary leukemia and peripheral blood platelet and neutrophil counts of > 100 × 10^9/L and 1.5 × 10^9/L, respectively. OS was calculated from the date of disease diagnosis to death (failure) or alive at last follow-up (censored). Relapse-free survival (RFS) was defined as the time of achieving CR to treatment failure such as relapse, death or alive in CR at last follow-up (censored). The final visiting was censored on October 2012 with a median follow-up time of 18 (4–53) months, and 65 cases (61.3%) have already relapsed. Kaplan–Meier analysis was used to calculate the distribution of OS and RFS. Log rank comparison was performed to compare the difference of OS and RFS. Cox model was used for the multivariate analysis of association of potential variants in B-ALL. Statistical procedures were performed with the SPSS statistical software package, version 16.0 (IBM, Newyork, NY, USA).

RESULTS
Sensitivity of eight-color flow cytometric assay in B-ALL
Linear correlation was shown between the percentage of LAIP+ cells and different titers of dilution from five BM samples of B-ALL diluted into regenerating BM. As shown in Figure 1, if enough WBCs (1.0–2.0 × 10^6) were attainable, the sensitivity of eight-color flow cytometric analysis of LAIPs for MRD detection could reach 10^-3 with good linearity in four of the five samples; the other one could not reach this level because of the background expression in normal BM. The detailed analytical matrix by MFC for one sample is shown in Supplementary Figure S2. The quantification of LAIP+ blasts of each dilution in five cases are shown in Supplementary Table S3.

As shown in Figure 2, we assessed MRD in 87 samples obtained from 28 B-ALL patients with BCR-ABL fusion gene after CR by RQ-PCR and by flow cytometric assessment of LAIPs simultaneously. Two methods showed concordant results in 78 out of 87 (89.7%) samples studied when 0.01% was used as threshold to define MRD positivity, among which 29 were MRD positive and 49 were MRD negative according to the criteria by the two methods. The proportion of leukemic cells in the 29 MRD-positive (≥ 0.01%) samples ranged from 0.01% to 10.8% of nucleated cells (median, 0.08%) by flow cytometry and from 0.04% to 19.0% (median, 0.7%) of mononuclear cells by PCR. In other eight samples, MRD was

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were MRD positive, low (0.01% \(p\) (64.2%) who remained MRD positive. Among the patients who achieved MRD negative, in contrast to 68 patients gated at the end of induction therapy of CR, and 38 patients induction of CR. Association between MRD level and prognosis at the end of immunophenotypic study of MRD was investigated at the end of induction therapy of CR, and 38 patients (35.8%) achieved MRD negative, in contrast to 68 patients (64.2%) who remained MRD positive. Among the patients who were MRD low, positive (0.01% \(\leq\) MRD < 0.1%), intermediate (0.1% \(\leq\) MRD < 1.0%) and high (MRD \(\geq\) 1.0%) levels of MRD were presented in 19 (27.9%), 25 (36.8%) and 24 (35.3%) patients, respectively. Statistical significance was reached when comparing the 2-year RFS rate between the patients with intermediate and with high MRD level was observed after one consolidation \((p = 0.056)\), but all the patients with positive MRD experienced relapse, which are displayed in Figures 3c and d in detail.

**Figure 2.** Comparison of MRD quantification by RQ-PCR and flow cytometry (FC) in BCR-ABL-positive patients. Circles indicate the percentage of MRD \(\geq 0.01\%\) obtained by the two methods in each sample. Two samples indicated by arrows were obtained from the same patient at different time points.

| LAIPs | Number of patients (%) | B-ALL subtypes \((n = 106)\) | BA fusion gene \((n = 95)\) | IK6 variant of IKZF1 gene \((n = 65)\) |
|---|---|---|---|---|
| | Pre-B ALL | Common-B ALL | Pre-B ALL | Mature-B ALL | | |
| CD13 | 26/106 (24.5) | 6 | 18 | 2 | 0 | \(8\) | 14 | 0.670 | 2 | 12 | 0.601 |
| CD33 | 42/106 (39.6) | 10 | 32 | 0 | 0 | 17 | 22 | 0.057 | 8 | 20 | 0.360 |
| CD15 or CD65 | 16/106 (15.1) | 10 | 4 | 2 | 0 | 2 | 12 | 0.202 | 2 | 3 | 0.325 |
| CD66c | 53/106 (50.0) | 6 | 43 | 3 | 1 | 23 | 26 | 0.002 | 12 | 24 | 0.059 |
| CD13 + CD33 | 19/106 (17.9) | 5 | 14 | 0 | 0 | 6 | 11 | 0.796 | 1 | 10 | 0.415 |
| CD13 + CD66c | 11/106 (10.4) | 3 | 8 | 0 | 0 | 4 | 5 | 0.674 | 2 | 5 | 1.000 |
| CD33 + CD66c | 25/106 (23.6) | 3 | 22 | 0 | 0 | 14 | 9 | 0.001 | 7 | 9 | 0.024 |
| CD13 + CD33 + CD66c | 17/106 (6.6) | 2 | 5 | 0 | 0 | 3 | 3 | 0.388 | 1 | 3 | 1.000 |

Univariate analysis of relationship between MRD level and survival

Association between MRD level and prognosis at the end of induction of CR. Immunophenotypic study of MRD was investigated at the end of induction therapy of CR, and 38 patients (35.8%) achieved MRD negative, in contrast to 68 patients (64.2%) who remained MRD positive. Among the patients who were MRD low, positive (0.01% \(\leq\) MRD < 0.1%), intermediate (0.1% \(\leq\) MRD < 1.0%) and high (MRD \(\geq\) 1.0%) levels of MRD were presented in 19 (27.9%), 25 (36.8%) and 24 (35.3%) patients, respectively. Statistical significance was reached when comparing the 2-year RFS rate between the patients with MRD negative and MRD positive. Similarly, 2-year RFS was statistically different among the three groups of patients at different MRD levels (low vs intermediate, \(p = 0.014\); intermediate vs high, \(p = 0.004\)). However, no statistical significance was reached in terms of OS (low vs intermediate, \(p = 0.070\); intermediate vs high, \(p = 0.411\)). The detailed data are shown in Figures 3a and b.

Association between MRD level and prognosis after one cycle of consolidation.

After one course of consolidation was completed, 49 patients (46.2%) showed MRD negative while 57 patients (53.8%) were MRD positive. And low, intermediate and high levels of MRD were distributed in 16 (28.1%), 21 (36.8%) and 20 (35.1%) patients, respectively. Identically, there was statistical significance of 2-year RFS rate (\(p < 0.001\)) and estimated 2-year OS rate (\(p < 0.001\)) between the MRD negative and MRD positive groups. An improved 2-year OS rate between the patients with intermediate and with high MRD level was observed after one consolidation \((p = 0.056)\), but all the patients with positive MRD experienced relapse, which are displayed in Figures 3c and d in detail.

**The potential prognostic value of MRD between 0.001% and 0.01% in the MRD-negative group.** Of the 38 patients who achieved MRD negative (<0.01%) at the end of induction of CR, 12 had an MRD of 0.001% to <0.01%, and for another 26 patients, LAIPs were undetectable. Most of the patients \((n = 37)\) could reach enough WBCs of 1.0–2.0 \(\times 10^9\), with the exception of one patient with MRD of 0.006% (only 5.0 \(\times 10^9\) WBCs were attainable). Patients
with this very low but detectable levels of leukemic blasts experienced an inferior 2-year RFS ($P = 0.002$) and OS ($P = 0.071$) rate to those with undetectable MRD (shown in Figures 3e and f). No further analysis for different MRD levels in the negative group was performed after one consolidation therapy, as LAIPs were not detectable in nearly all the patients (48/49, 98.0%).

**Association between dynamic MRD change and prognosis.** Among the 106 patients who achieved CR, 76 patients (71.7%) presented at least one MRD-positive result during the follow-up within the first year of treatment, and 30 patients (28.3%) sustained in MRD-negative status. Statistical significances were observed in these two groups of patients when the 2-year RFS

Figure 3. The 2-year RFS and OS in 106 adult patients with B-ALL according to immunophenotypic MRD level at the end of induction of CR and after one consolidation. (a) The RFS of the patients with negative and positive MRD status at the end of induction of CR were 65.1 ± 8.7% and 12.3 ± 5.1%, respectively ($P < 0.001$). In parallel, the RFS of the patients with low and intermediate burden of MRD were 29.3 ± 13.4% and 14.5 ± 8.6%; however, the patients with high burden of MRD all relapsed (low vs intermediate, $P = 0.014$, and intermediate vs high, $P = 0.004$). (b) The estimated 2-year OS of the patients with negative and positive MRD status at the end of induction of CR were 69.2 ± 8.3% and 25.0 ± 6.2%, respectively ($P < 0.001$). No statistical significance was observed in the three groups with a 2-year estimated OS rate of 49.8 ± 12.7, 11.0 ± 9.0 and 22.4 ± 9.3%, respectively (low vs intermediate, $P = 0.070$; and intermediate vs high, $P = 0.411$). (c) The 2-year RFS of the patients with negative MRD status after one consolidation were 64.6 ± 7.8%, and no patients with positive MRD status were relapse-free ($P < 0.001$). (d) The estimated 2-year OS of the patients with negative and positive MRD status after one consolidation were 67.9 ± 7.5% and 18.9 ± 6.2%, respectively ($P < 0.001$). There was no significance between the three groups in MRD-positive status: Low, 13.2 ± 11.7%; Intermediate, 31.1 ± 11.3%; and High, 10.0 ± 6.7% (low vs intermediate, $P = 0.859$; and intermediate vs high, $P = 0.056$). (e) The 2-year RFS of the patients with MRD 0.001% to < 0.01% and those with undetectable MRD at the end of induction of CR ($n = 38$) were 37.0 ± 14.6% and 79.1 ± 9.5%, respectively ($P = 0.002$). (f) Patients with undetectable MRD had a better 2-year OS than those with MRD 0.001% to < 0.01% at the end of induction of CR (81.7 ± 8.5% and 45.7 ± 15.5%, respectively, $P = 0.071$), but no statistical significance was observed.
rate \((P < 0.001)\) and OS rate \((P < 0.001)\) were compared as seen in Figures 4a and b. In a similar way, 58 patients \((54.7\%)\) obtained at least one MRD-negative result in contrast to 48 \((45.3\%)\) patients never reached during the treatment of the first year. Figures 4c and d shows the statistical difference of 2-year RFS \((P < 0.001)\) and OS rate \((P < 0.001)\) between these two groups.

Multivariate analysis of MRD status and clinical factors with survival

In order to explore whether the status of MRD was an independent prognostic factor for RFS and OS among adult patients with ALL, a multivariate analysis was performed with the variants, including clinical factors such as gender, age, WBC count, Hb level, platelet count, consolidation therapy, blasts percentage in BM and time to achieving CR, BCR-ABL-positive, number of LAIPs, B-ALL subtype and levels of MRD at the end of induction of CR and completion of one consolidation therapy. Univariate analysis indicated that WBC count, platelet count, late CR, consolidation therapy and MRD status at CR1 and after consolidation had association with RFS \((P < 0.05)\); MRD status at two time points had association with OS \((P < 0.05)\). Multivariate analysis showed that positive MRD status (MRD > 0.01%) after induction \((P = 0.002)\) and one consolidation \((P < 0.001)\) were associated with an increased risk of relapse independently. However, only MRD-positive status after one consolidation \((P < 0.001)\) suggested an inferior OS independently. The detailed data are shown in Table 4.

DISCUSSION

MRD status quantified by flow cytometric analysis has been integrated as an essential part of the algorithm of the treatment guidelines in evaluating the early treatment response and predicting outcome in pediatric patients.3,44 However, the correlation between MRD and prognosis in adult ALL needs further exploration. Therefore, we performed this study to establish the role of MRD status in predicting the prognosis of adult B-ALL by eight-color flow cytometric method.

In our study, linear correlation analysis showed the stability and sensitivity of this method in quantification of the leukemic cells in different dilution levels. If enough WBCs \((2.0 \times 10^6)\) could be reached, the sensitivity of methodology of eight-color flow cytometry could be as high as close to \(1.0 \times 10^{-5}\). As shown in Figure 1, the background clusters were \(<0.001\%\) in regeneration BM according to the aberrant phenotypic features of B-ALL samples except for one patient \((1.5 \times 10^{-5})\). These very low levels of noisy signal ensured the high specificity of methodology of eight-color MFC. Also, we observed that eight-color evaluation procedure could identify LAIPs in most of the patients by single-tube panel with a high sensitivity \((96.0\%\) for \(10^{-4}\) and \(81.6\%\) for \(10^{-5}\), respectively) in comparison to normal BM. As shown in one clinical application sample in Supplementary Figure S1, LAIPs could be monitored precisely during treatment at 0.005% level even with shifted immunophenotypes by eight-color flow cytometry.

Four main types of LAIPs were identified at diagnosis (Table 3). We also observed that coexpression of CD66c and CD33 was associated with BCR-ABL fusion gene and IK6 variant of IKZF1 gene, which suggested potential genetic associations between these two molecular abnormalities, as previously reported from our group by Chen et al.2

Many studies proved that MRD evaluated by PCR targeting to IG/TCR rearrangement and MFC for LAIPs yielded concordant results in the vast majority of patients.7,45,46 A recent study by Garand et al.20 proved that QPCR and MFC can therefore

![Figure 4](https://example.com/figure4.png)

**Figure 4.** The 2-year RFS and OS in 106 adult patients with B-ALL according to dynamic MRD. (a, b) The 2-year RFS and estimated 2-year OS rate of the patients with at least a MRD-positive result or sustaining negative during the first year were \(9.9 \pm 4.5\%\) and \(83.2 \pm 7.8\%\) \((P < 0.001)\), respectively, and \(24.9 \pm 5.9\%\) and \(81.7 \pm 7.5\%\) \((P < 0.001)\), respectively. (c, d) The 2-year RFS and estimated 2-year OS rate of the patients with at least a MRD-negative result or never achieving this status during the first year were \(57.6 \pm 7.7\%\) and \(2.4 \pm 2.4\%\) \((P < 0.001)\), respectively, and \(64.2 \pm 7.2\%\) and \(14.8 \pm 6.1\%\) \((P < 0.001)\), respectively.
be comparable if properly standardized and are highly complementary. More recently, Denys et al.\textsuperscript{12} reported that six-color MFC could significantly improve the concordance with PCR-based MRD data (88 versus 96%) and particularly improve the specificity of the MRD analysis as compared with 4-color MFC. In our study, we attempted to test the reliability and accuracy of MFC assay in MRD measurement in comparison to RQ-PCR method targeting BCR-ABL fusion gene transcripts. PCR evaluation of fusion gene transcripts reported an about 10-fold higher sensitivity (10\textsuperscript{-5}–10\textsuperscript{-9}) than MFC, but when a cutoff level of 0.01% was used to define MRD positivity, our results showed remarkable concordance between both the methods in MRD status. In the present study, mononuclear cell by Ficoll centrifugation and whole BM by lysing procedure were performed during sample preparation of PCR and MFC, which may lead to systematically higher MRD data of RQ-PCR as compared with the flow cytometry. Interestingly, as shown in Figure 2, two samples from the same patient indicated by arrows showed higher MRD level by flow cytometry (10.8% and 0.55%, respectively) as compared with RQ-PCR (0.07% and 0.001%, respectively), which could be explained by the fact that the leukemic cells may contain more than one genetic abnormality or they occur in different stages of maturation. LAIPs showed higher MRD level by flow cytometry (10.8% and 0.55%, respectively) as compared with RQ-PCR (0.07% and 0.001%, respectively) as evidenced by the wide application of tailored tyrosine kinase inhibitor treatment.

In the treatment of adult ALL, no standard guideline has been established until now, and the patients who should receive more intensive chemotherapy or early allogeneic stem cell transplantation are still uncertain, with a few exception for tailored tyrosine kinase inhibitor treatment for patients with Ph chromosome.\textsuperscript{1,48,49} Due to the poor prognosis of adult ALL when compared with pediatric counterparts, a care of more patient-specific way should be considered. MRD assessing by flow cytometric analysis may provide an important parameter for guiding the next treatment as well as the intensity of chemotherapy or early allogeneic stem cell transplantation. The prognostic value of BCR-ABL fusion gene in adult ALL was not obvious in our analysis, which might be caused by the wide application of tailored tyrosine kinase inhibitor treatment.

### Table 4. Univariate and multivariate analysis for clinical and MRD variables of RFS and OS

| Variables                      | RFS                          | OS                          |
|--------------------------------|------------------------------|-----------------------------|
|                                | Univariate analysis | Multivariate analysis | Univariate analysis | Multivariate analysis |
| Gender                         | 0.440 NS                   | 0.668 NS                    | 0.668 NS           | — |
| Age                            | 0.187 NS                   | 0.791 NS                    | 0.791 NS           | — |
| WBC count                      | 0.003 NS                   | 0.280 NS                    | 0.280 NS           | — |
| Hb level                       | 0.552 NS                   | 0.442 NS                    | 0.442 NS           | — |
| Platelets count                | 0.016 NS                   | 0.431 NS                    | 0.431 NS           | — |
| BM blasts                      | 0.842 NS                   | 0.099 NS                    | 0.099 NS           | — |
| Late CR                        | 0.001 NS                   | 0.138 NS                    | 0.138 NS           | — |
| B-ALL subtype                  | 0.552 NS                   | 0.692 NS                    | 0.692 NS           | — |
| Consolidation therapy\textsuperscript{a} | 0.009 NS               | 0.224 NS                    | 0.224 NS           | — |
| BCR-ABL-positive               | 0.273 NS                   | 0.681 NS                    | 0.681 NS           | — |
| Number of LAIPs                | 0.440 NS                   | 0.612 NS                    | 0.612 NS           | — |
| MRD positive\textsuperscript{b} at the end of induction | 0.001 0.002 4.427 (1.750–11.197) | <0.001 NS      | <0.001 NS         | 5.652 (2.739–11.662) |
| MRD positive after one consolidation | 0.001 <0.001 9.832 (4.545–21.268) | <0.001 <0.001 | 9.832 (4.545–21.268) |
| Undetectable MRD at the end of induction | 0.001 NS                   |                        | 0.001 NS           | — |

**Abbreviations:** B-ALL, B-lineage acute lymphoblastic leukemia; BM, bone marrow; CI, confidence interval; CR, complete remission; Hb, hemoglobin; LAIP, leukemia-associated immunophenotype; MRD, minimal residual disease; NS, not significant; OR, odds ratio; OS, overall survival; RFS, relapse-free survival; WBC, white blood cell. *Allogeneic stem cell transplantation or chemotherapy. \textsuperscript{a}MRD ≥ 0.01%.
cytometry could be potentially taken as a routine index in the evaluation of the treatment response for adult patients with B-ALL. Standardization of MRD assessing method in order to compare the treatment outcome of adult ALL using different treatment protocols is warranted by further multi-centered study.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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