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ORIGINAL ARTICLE

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% 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.[1–3] 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.[4–6]

Quantification of clonal rearrangements of immunoglobulin/T-cell receptor (IG/TCR) genes or fusion genes by PCR[7–10] and leukemia-associated immunophenotypes (LAIPs) by multiparameter flow cytometry (MFC)[11–16] 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.[8,17–19] 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.[4,12,13,20–22] 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.[23] Furthermore, this method has recently been well standardized by the Euro Flow Consortium, which provides researchers with a practicable guideline.[24,25]

Although early MRD evaluation in induction period has been introduced to guide the treatment in most major childhood ALL protocols,[11,26–29] the value of MRD in adult ALL is not evaluated so widely as pediatric patients.[30–36] 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.[3] All of them gave informed consent according to the Declaration of Helsinki.

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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 were 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 $>2\times10^9$/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 time points of 1 (57 cases), 2 (25), 3 (52), 4 (58), 5 (54), 6 (38), 7 (58), 8 (56), 9 (51) and 10 (51) months after one 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, $\times10^9$/l (range)	9.5 (1.0–379.0)
$>30\times10^9$/l	20/106 (18.9)
$\leqslant30\times10^9$/l	86/106 (81.1)
Median platelets count, $\times10^9$/l (range)	41.0 (2–467)
$<30\times10^9$/l	42/106 (39.6)
$\geqslant30\times10^9$/l	64/106 (60.4)
Median Hb level, g/l (range)	93.0 (40–778)
<75 g/l	32/106 (30.2)
$\geqslant75$ g/l	74/106 (69.8)
Median BM blasts, % (range)	80.4 (8.6–97.9)
Median number of LAIPs per patient (range)	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 CR[a]	42/106 (39.6)
Cytogenetic normal	28/95 (29.5)
Cytogenetic quantity abnormalities	
Hyperdipoidy	2/95 (2.1)
Hypodipoidy	7/95 (7.4)
Non-translocation structural changes	17/95 (17.9)
Karyotype, fusion gene[b]	
t(9;22), *BCR-ABL*	30/95 (31.6)
t(1;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(1;19), *TCF3-PBX1*	3/95 (3.2)
t(12;21), *TEL-AML1*	3/95 (3.2)
t(9;22) and t(1;19), *BCR-ABL* and *TCF3-PBX1*[c]	1/95 (1.1)
CRLF2 overexpression	5/65 (7.7)
IK6 variant of *IKZF1* gene	15/65 (23.1)

Abbreviations: B-ALL, B-lineage acute lymphoblastic leukemia; BM, bone marrow; CR, complete remission; Hb, hemoglobin; LAIP, leukemia-associated immunophenotype; WBC, white blood cell. [a]Complete remission is achieved after 35 days after initiation of the treatment. [b]Two or one of them are positive. [c]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 NH_4Cl 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 titered reagent cocktail and incubated in the dark at room temperature for 15 min, then about 2.0 ml of buffered NH_4Cl 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 TdT and cytoplasmic (Cy) CD79a and IgM (cμ) 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 1×10^5 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 MRD measurement in 120 B-lineage ALL cases by single-tube panel. Dead cells and debris were excluded by forward scatter (FSC)/SSC and CD45/SSC 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 homogeneous. The 'or' logistic gating strategy was applied to include the highest quantity of MRD when the leukemic blasts were heterogeneous containing $\geqslant2$ 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

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

Seven fixed mAbs	*Alternative mAb*	*Number (%)*
Including CD58-FITC, CD38-perCP-cy5.5, CD34-PE-Cy7, CD10-APC, CD20-APC-eFluor780, CD19-Brilliant Violet 421, CD45-Pacific Orange	CD66C-PE	28 (23.3)
	CD13-PE	4 (3.3)
	CD33-PE	8 (6.7)
	CD13-PE + CD33-PE	12 (10.0)
	CD66C-PE + CD33-PE	13 (10.8)
	CD66C-PE + CD13-PE + CD33-PE	1 (0.8)
	7.1-PE	3 (2.5)
	CD15-PE	1 (0.8)
	CD15-PE + 7.1-PE	5 (4.2)
	None	45 (37.5)

Abbreviations: APC, allophycocyanin; B-ALL, B-lineage acute lymphoblastic leukemia; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; MRD, minimal residual disease; PE, phycoerythrin; perCP, peridinin chlorophyl protein. MAb clone of CD58, CD66C, CD10, CD13, CD33, 7.1 and CD15 are AICD58, KORSA-3544, ALB1, SJ1D1, D3HL60.251, 7.1 and 80H5, respectively, from Immunotech (Marseille, France); MAb clone of CD38 and CD34 are HIT2 and 581, respectively, from Becton Dickinson; MAb clone of CD20 is B9E9 from eBioscience (San Diego, CA, USA); MAb clone of CD19 is HIB19 from Biolegend (San Diego, CA, USA); MAb clone of CD45 is HI30 from Invitrogen (Camarillo, CA, USA).

induction of CR and after one consolidation failed to reach this level (only 5.0×10^5 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 $>1.0 \times 10^6$ WBCs available could reach a theoretical sensitivity of 1.0×10^{-5}. 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 cytometric 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, 1:10000, 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 1:10 000 to 1:200 000. 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 (Yuanqi Bio-pharmaceutical 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.[37,38] 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 $>100 \times 10^9$/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 survivals. Two-sided *P*-values <0.05 were considered statistically significant. Cox model was used for the multivariate analysis of association of potential independent prognostic factors with OS and RFS. A limited backward selection procedure was used to exclude redundant variates. All the above 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 ($1.0–2.0 \times 10^6$) were attainable, the sensitivity of eight-color flow cytometric analysis of LAIPs for MRD detection could reach 10^{-5} 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

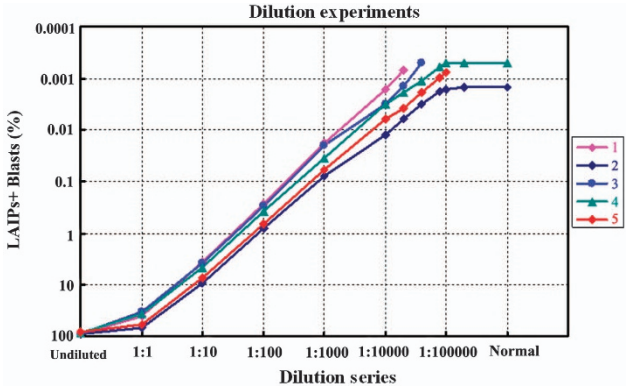

Figure 1. Linear correlation was shown between the percentage of LAIPs+ blasts and different titers of dilution from five B-ALL samples with different LAIPs features. The LAIP combinations of samples from numbers 1 to 5 were shown as: CD10-CD34+/58st/38dim, CD10-CD34+, CD10+CD34+CD33+/CD38dim/45dim, CD10+CD34+CD45dim/CD19st/CD66c+ and CD10+CD34+58st/38dim/CD13+. 'Undiluted' and 'Normal' on the X-axis mean the original B-ALL BM sample and regenerating BM sample, respectively. The minimum MRD quantity by eight-color flow cytometric assay according to samples from numbers 1 to 5 were 0.0007, 0.0015, 0.0005, 0.0005 and 0.0008%, respectively. The data were not shown on the Figure when the number of LAIP+ blasts were <10 within 2×10^6 WBCs (<0.0005%).

sample is shown in Supplementary Figure S2. The quantification of LAIP+ blasts of each dilution in five cases are shown in Supplementary Table S3.

In this work, using eight-color flow cytometry as compared with 3–4-color panels, different mAb combinations could represent several types of aberrant immunophenotypes in one tube. At diagnosis, 120 (96%) and 102 (81.6%) out of 125 patients could reach a sensitivity of 0.01% and 0.001% for MRD measurement, respectively, when compared with normal BM cells by eight-color flow cytometry.[39–42] The distribution of LAIP combinations in B-ALL samples and their background expression in normal BM are shown in Supplementary Table S2.

Frequencies and distribution of LAIPs and BCR-ABL and IK6 variants in B-ALL

The incidence and distribution of LAIP and molecular characteristics in 106 B-ALL patients are shown in Table 3 in detail. For comparison of LAIP and molecular abnormalities, we observed that an expression of CD66c ($P=0.002$), coexpression of CD66c and CD33 ($P=0.001$), dim expression of CD34 ($P=0.021$) and CD38 ($P<0.001$) were associated with a genotype of *BCR-ABL*+ ALL; a coexpression of CD66c and CD33 ($P=0.024$) and strong expression of CD34 ($P=0.028$) were related to *IK6* variant of *IKZF1* gene.

Comparison of MRD quantification by RQ-PCR and flow cytometry in BCR-ABL-positive patients

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

Table 3. Incidence and distribution of LAIP and *BCR-ABL* and *IK6* variants in 106 B-ALL patients

LAIPs	*Number of patients (%)*	*B-ALL subtypes* (n = *106*)				*BA fusion gene* (n = *95*)			*IK6 variant of IKZF1 gene* (n = *65*)		
		Pro-B ALL	*Common-B ALL*	*Pre-B ALL*	*Mature-B ALL*	*BA +* [a] (n = *31*)	*BA −* (n = *64*)	P-*value*	*IK6 +* [b] (n = *15*)	*IK6 −* (n = *50*)	P-*value*
Cross-lineage antigen expression											
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 +	7/106 (6.6)	2	5	0	0	3	3	0.388	1	3	1.000
Asynchronous antigen expression											
CD20 + CD34 +	16/106 (15.1)	4	9	2	1	4	11	0.813	3	7	0.875
CD20 + CD10 +	8/106 (7.5)	1	5	0	2	3	5	1.000	1	4	1.000
Antigen dim/st expression											
CD38dim[c]	66/106 (62.3)	13	48	5	0	29	30	<0.001	12	28	0.170
CD19dim	5/106 (4.7)	2	1	1	1	0	4	0.300	1	1	0.411
CD34dim	13/106 (12.3)	3	10	0	0	7	3	0.021	2	7	1.000
CD10dim	24/106 (22.6)	5	16	3	0	6	14	0.778	4	10	0.847
CD45dim	45/106 (42.5)	5	35	5	0	16	25	0.247	8	20	0.360
CD58st[d]	54/106 (50.9)	11	32	9	2	13	35	0.244	6	28	0.277
CD19st	40/106 (37.7)	12	25	2	1	15	22	0.189	8	17	0.177
CD34st	20/106 (18.9)	6	13	1	0	5	14	0.512	5	5	0.028
CD10st	16/106 (15.1)	0	14	1	1	5	9	0.790	3	7	0.875
Ectopic phenotype											
NG2 + [e]	11/106 (10.4)	10	1	0	0	0	8	0.050	1	1	0.411

Abbreviations: BA, BCR-ABL; B-ALL, B-lineage acute lymphoblastic leukemia; LAIP, leukemia-associated immunophenotype. [a]*BCR-ABL* positive. [b]*IK6* variant of *IKZF1* gene. [c]Dim expression. [d]Strong expression. [e]Human homologue of the rat chondroitin sulfate proteoglycan NG2.

positive by PCR but negative by flow cytometry, of which five samples had detectable leukemic cells between 0.001% and 0.01% by flow cytometric assay. Conversely, in two samples from the same patient, MRD were 10.8% and 0.55% according to flow cytometry but 0.07% and <0.001% by PCR, respectively (as indicated by arrows in Figure 2).

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 positive, low (0.01%⩽MRD<0.1%), intermediate (0.1%⩽MRD<1.0%) and high (MRD⩾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 ($P<0.001$) and estimated 2-year OS rate ($P<0.001$) 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.

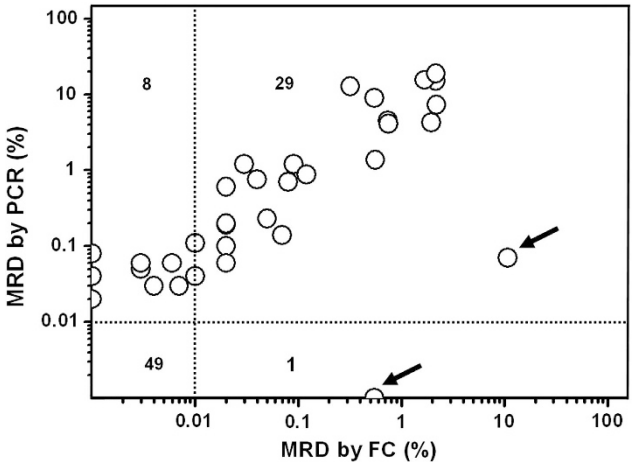

Figure 2. Comparison of MRD quantification by RQ-PCR and flow cytometry (FC) in *BCR-ABL*-positive patients. Circles indicate the percentage of MRD⩾0.01% obtained by the two methods in each sample. Two samples indicated by arrows were obtained from the same patient at different time points.

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 $1.0–2.0\times10^6$, with the exception of one patient with MRD of 0.006% (only 5.0×10^5 WBCs were attainable). Patients

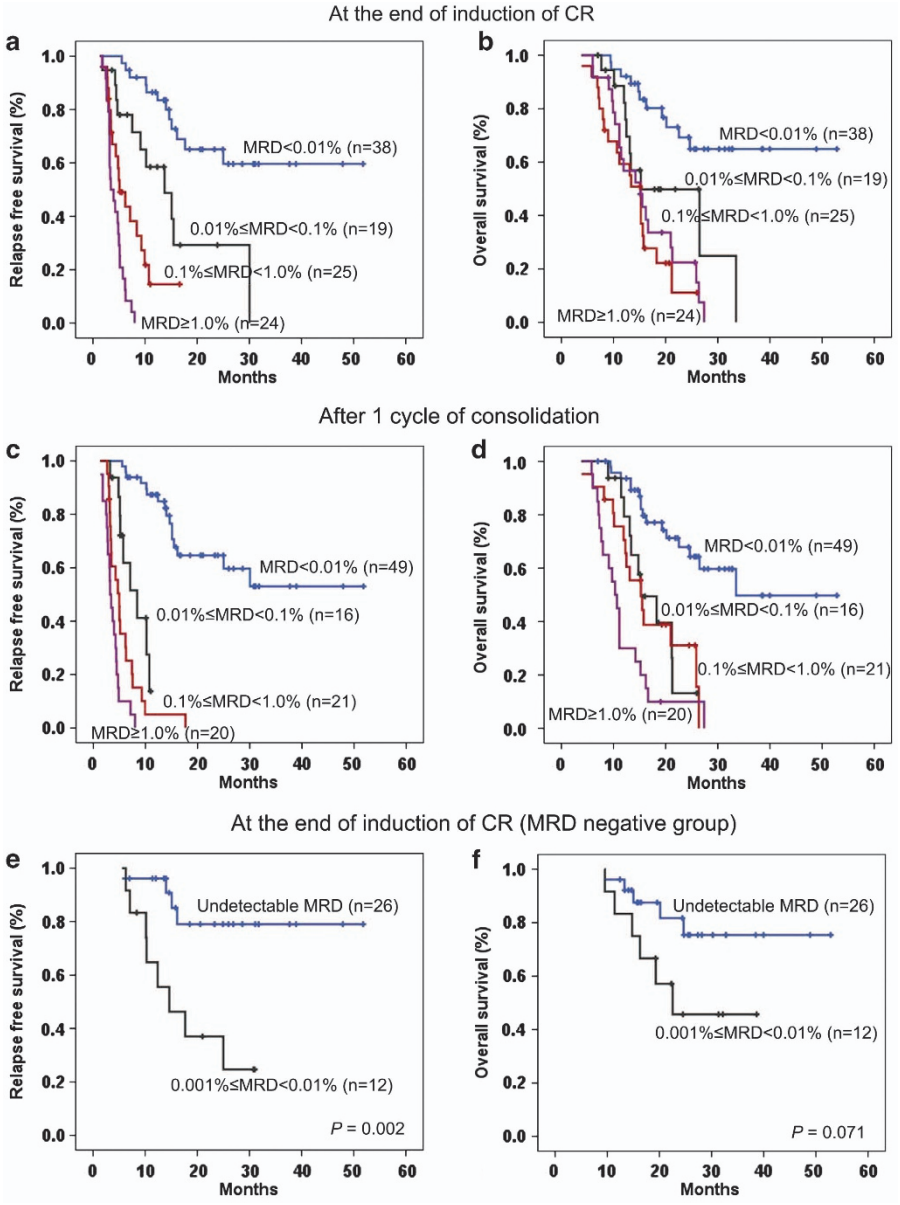

Figure 3. The 2-year RFS and OS in 106 adult patients with B-ALL according to immunophenotypic MRD level at the end of induction of CR and after one consolidation. (**a**) The RFS of the patients with negative and positive MRD status at the end of induction of CR were 65.1 ± 8.7% and 12.3 ± 5.1%, respectively ($P < 0.001$). 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.

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

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.[43,44] 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 (2.0×10^6) could be reached, the sensitivity of methodology of eight-color flow cytometry could be as high as close to 1.0×10^{-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 (1.5×10^{-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

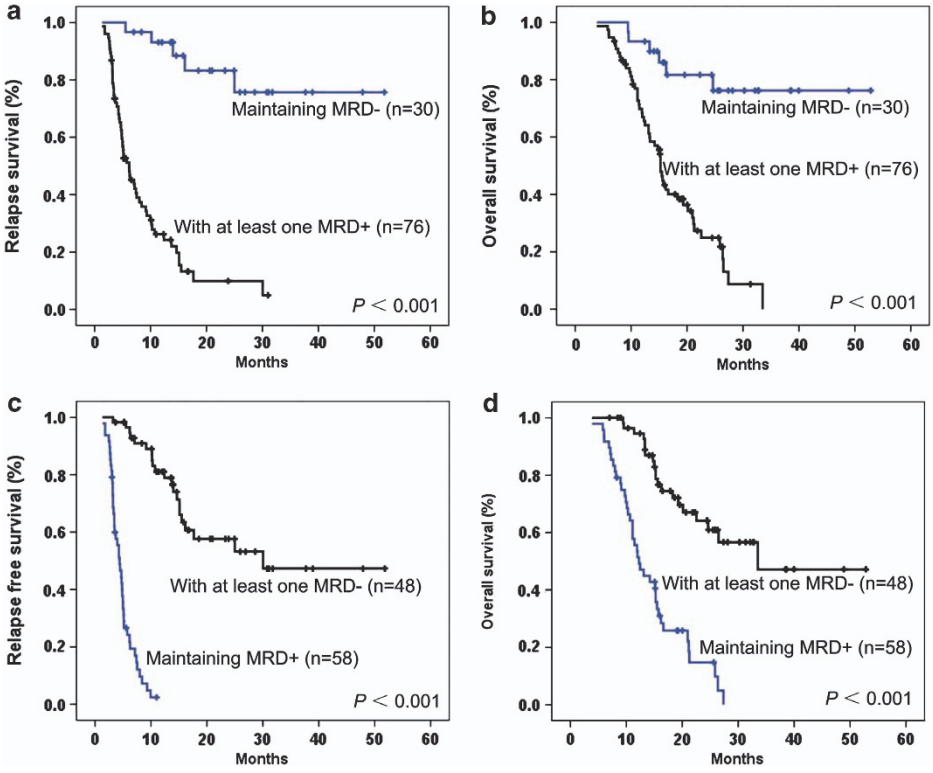

Figure 4. The 2-year RFS and OS in 106 adult patients with B-ALL according to dynamic MRD. (**a**, **b**) The 2-year RFS and estimated 2-year OS rate of the patients with at least a MRD-positive result or sustaining negative during the first year were $9.9\pm4.5\%$ and $83.2\pm7.8\%$ ($P<0.001$), respectively, and $24.9\pm5.9\%$ and $81.7\pm7.5\%$ ($P<0.001$), 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 $57.6\pm7.7\%$ and $2.4\pm2.4\%$ ($P<0.001$), respectively, and $64.2\pm7.2\%$ and $14.8\pm6.1\%$ ($P<0.001$), respectively.

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*	
	P-*value*	P-*value*	*OR (95% CI)*	P-*value*	P-*value*	*OR (95% CI)*
Gender	0.440	NS	—	0.668	NS	—
Age	0.187	NS	—	0.791	NS	—
WBC count	0.003	NS	—	0.280	NS	—
Hb level	0.552	NS	—	0.442	NS	—
Platelets count	0.016	NS	—	0.431	NS	—
BM blasts	0.842	NS	—	0.099	NS	—
Late CR	<0.001	NS	—	0.138	NS	—
B-ALL subtype	0.552	NS	—	0.692	NS	—
Consolidation therapy[a]	0.009	NS	—	0.224	NS	—
BCR-ABL-positive	0.273	NS	—	0.681	NS	—
Number of LAIPs	0.440	NS	—	0.612	NS	—
MRD positive[b] at the end of induction	<0.001	0.002	4.427 (1.750–11.197)	<0.001	NS	—
MRD positive after one consolidation	<0.001	<0.001	9.832 (4.545–21.268)	<0.001	<0.001	5.652 (2.739–11.662)
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. [a]Allogeneic stem cell transplantation or chemotherapy. [b]MRD $\geqslant$ 0.01%.

be comparable if properly standardized and are highly complementary. More recently, Denys *et al*.[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^{-5}–10^{-6}) 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 this patient has complicated chromosomal translocations, as the quantification of *BCR-ABL* fusion gene transcripts of this case was as low as 0.18% at diagnosis while LAIPs+ blasts were 95.9%.

Although there were different cutoff values of MRD,[11,47] we used a more popular cutoff value of <0.01% as the criteria of MRD negative. In this study, we proved that statistical significance could be reached when comparing the 2-year RFS rate ($P<0.001$ and $P<0.001$, respectively) and estimated OS rate ($P<0.001$ and $P<0.001$, respectively) between the patients with MRD-negative and -positive status at the end of induction and after one cycle of consolidation. In a hierarchical analysis of the prognosis of patients with positive MRD result, a decreased RFS and OS was observed in the patients with low, intermediate and high levels of leukemic blasts at two time points, respectively. Furthermore, in dynamic analysis of MRD change and prognosis, we had proved that maintenance of a MRD-negative status was important to the prolonging of the RFS and OS, as the patients with persistent MRD-negative results in the 1 year of treatment had a superior RFS ($P<0.001$) and OS ($P<0.001$) to those without.

Interestingly, in examining the few patients with MRD-negative status (<0.01%) who suffered from short relapse with poor OS, we found that most of such patients had a detectable low level of MRD of 0.001% to <0.01% at the end of induction. Although our results showed that patients with MRD of 0.001 to 0.01% experienced significantly higher cumulative risk of relapse and a tendency of inferior 2-year OS rate than those with undetectable MRD, more patients were needed to be enrolled to testify this supposition.

In multivariate prognostic analysis in this series of patients, we proved that MRD-positive status at the end of induction and after one consolidation were independently associated with an increased risk of relapse; on the other hand, MRD of >0.01% after one consolidation was the only factor related with OS independently. 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.

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 allogenetic stem cell transplantation are still uncertain, with a few exception for tailored tyrosine kinase inhibitor treatment for patients with Ph chromosome.[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 indicated in this study. Patients with <0.01% leukemic cells at the end of remission induction and after one cycle of consolidation are likely to have an excellent treatment outcome, especially for those with undetectable MRD; otherwise, early intervention with high-dose chemotherapy or other strategies should be considered.

In conclusion, our study suggests that MRD status at the end of induction and one cycle of consolidation by eight-color flow

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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