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Original Article

BCR-ABL1 and CD66c exhibit high concordance in minimal residual disease detection of adult B-acute lymphoblastic leukemia

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Abstract: Objective: To investigate the relationship between surface expression of CD66c and the breakpoint cluster region-Abelson (*BCR-ABL1)* fusion gene in B-acute lymphoblastic leukemia (B-ALL) at primary diagnosis, and their concordance during minimal residual disease (MRD) monitoring. Methods: Bone marrow biopsies were collected from newly diagnosed B-ALL patients (n = 43) between September 2011 and September 2014. Karyotyping was used to detect Philadelphia chromosome (Ph), and fluorescence *in situ* hybridization (FISH) and reverse transcription-polymerase chain reaction (RT-PCR) were used to detect *BCR-ABL1* fusion gene. Immunophenotyping was performed by flow cytometry for leukemia. Patients with both CD66c expression and *BCR-ABL1* were further assessed for MRD during treatment. Results: Overall, 26/43 (60.5%) B-ALL patients were positive for *BCR-ABL1* fusion gene expression, and all Ph positive cases (17/43; 39.5%) expressed *BCR-ABL1* and CD66c. CD66c was expressed at significantly higher levels in *BCR-ABL1* positive than negative patients (24/26, 92.3% vs. 11/17, 64.7%; P = 0.042), and furthermore, in all Ph positive cases (17/17, 100% vs. 18/26, 69.2%; P = 0.014). When *BCR-ABL1* was set as the gold standard for the presence or absence of MRD after treatment, both CD66c alone and the MRD panel including CD66c demonstrated high diagnostic performance for the detection of MRD, with values of area under the receptor operation curve (ROC) of 0.881 vs. 0.891 respectively. Conclusions: The stable expression pattern of CD66c has noteworthy clinical value in B-ALL not only in the recognition of abnormal leukemia cells at primary diagnosis but also in monitoring of MRD during the treatment, especially in patients without definitely cytogenetic or molecular abnormal, and thus, warrants further investigation as a routine clinical marker for MRD detection by flow cytometry.

Keywords: B-acute lymphoblastic leukemia (B-ALL), *BCR-ABL1*, CD66c, diagnostic performance, minimal residual disease (MRD)

Introduction

B-acute lymphoblastic leukemia (B-ALL) is an example of a human cancer where the molecular characterization of the disease has led to the development of a small molecule therapy specific for the cancer cells. The therapy is based on the hallmark genetic event, the Philadelphia chromosome (Ph), which is a translocation between chromosomes 9 and 22 involving the breakpoint cluster region and the *ABL* gene (*BCR/ABL1*). The translocation produces a novel fusion gene kinase, *BCR-ABL1*. Thus, detection of the Ph chromosome/*BCR/ABL1* translocation has become standard in the diagnosis and clinical treatment of B-acute lymphoblastic leukemia. In addition, many lineage specific markers are currently in use to diagnose the disease and to monitor for minimal residual disease (MRD) by flow cytometry during and following treatment. However, as the status of these markers changes under treatment, the quest for markers that exhibit stable expression remains even after treatment.

Recent reports have illuminated carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6; CD66c) as one such potential marker. CD66c is a member of the carcinoembryonic antigen family which has been shown to be

Table 1. Clinical and molecular features of adult B-ALL in 43 patients

Parameter	Number of Patients		*P* value
	$CD66c^+$ cases	$CD66c^-$ cases	
Gender (n = 43)			
Male	18	2	0.167
Female	17	6	
BCR/ABL1 (n = 43)			Likelihood Ratio P = 0.042
Positive	24	2	McNemar test, P = 0.022;
Negative	11	6	Kappa = 0.304, P = 0.023
BCR-ABL1 Positive (n = 26)			1
P190	15	1	
P210	9	1	
Ph chromosome (n = 43)			0.014
Positive	17	0	
Negative	18	8	
Clinical classification			0.518
Pro-B-ALL	16	2	
Common-B-ALL	14	4	
Pre-B-ALL	5	2	

aberrantly expressed in a considerable proportion of pediatric B-ALL cases and is more frequently expressed than other myeloid antigens including CD13, CD15, CD33, and CD65 in this form of the disease [1]. Aberrant expression of CD66c has also been correlated with some specific genetic changes in B-ALL, such as *BCR-ABL1*, hyperdiploidy, and *TEL-AML1* fusion gene negativity [1, 2].

Aberrant expression of CD66c on malignant lymphoblasts has, therefore, been exploited for the diagnosis of pediatric ALL, and in our clinical laboratory, as well as some others, for the follow-up of minimal residual disease (MRD) using flow cytometry [1, 3-5]. The status of CD66c expression in adult B-ALL, however, has not yet been well characterized. In the present study, the frequency of expression of the CD66c molecule in adult B-ALL and its potential correlation to the *BCR-ABL1* fusion gene expression, which was detected by fluorescence *in situ* hybridization (FISH) or reverse transcription-polymerase chain reaction (RT-PCR), was investigated. In addition, for the first time, the utility of the myeloid antigen CD66c as an independent immunophenotype marker for the detection of MRD by flow cytometry was evaluated in the adult B-ALL patients where leukemia cells were already positive for both CD66c and *BCR-ABL1* at the primary diagnosis.

Materials and methods

Ethics statement

The present study was approved by the Changhai Hospital Institutional Review Board (Shanghai, China) and signed informed consent was obtained from each patient in accordance with the Declaration of Helsinki.

Patients

The diagnosis of B-ALL was determined according to criteria established by the "WHO Classification of Tumors of Hematopoietic and Lymphoid Tissues". Primary B-ALL patients (n = 43; male, n = 20; female, n = 23) with a median age of 38 yr (range, 18-66 yr), who were referred to the Institute of Hematology, Changhai Hospital, between September 2011 and September 2014, were included in this study. All patients had at least one diagnostic bone marrow aspirate sample submitted for flow-cytometric immunophenotyping, RT-PCR, and cytogenetic analysis. FISH was performed to screen for the *BCR/ABL1* fusion gene in 35 of these patients at the primary diagnosis. The 23 patients who were both positive for the *BCR-ABL1* fusion gene (detected by RT-PCR) and CD66c expression (detected by flow cytometry) at the primary diagnosis were subsequently monitored regularly during the treatment phase. In total, 162 bone marrow samples were included for the correlation analysis of CD66c and *BCR-ABL1* in the MRD detection of leukemia patients.

Flow cytometric immunophenotyping

Flow-cytometric immunophenotyping was performed using a panel of antibodies designed for B-ALL. The acute leukemia screening panel included CD45, CD7, CD19, CD13, CD33, CD34, CD117, HLA-DR, CD10, cytoplasmic myeloperoxidase (cMPO), cytoplasmic CD3 (cCD3), and cytoplasmic CD79a (cCD79a). The B-ALL extended panel included CD66c, CD22, CD20, CD58, CD38, CD123, CD45, cytoplasmic heavy

Table 2. Frequency of aberrant expression of cell surface antigens on leukemia cells

Molecule	Positive cases /43 total	Percent (%)
CD66c	35	81.4
CD13	4	9.3
CD33	5	11.6
CD117	0	0
CD123	26	60.5
CD66c and CD13	4	9.3
CD66c and CD33	5	11.6
CD66c and CD117	0	0
CD66c and CD123	22	51.2

chain of immunoglobulin M (cμ), and cytoplasmic TdT. The MRD panel included CD10, CD66c, CD34, CD19, CD20, CD45, CD38, and CD58 which was performed in a single tube using an 8-color combination. Bone marrow aspirates were collected in tubes with ethylene diamine tetra-acetic acid (EDTA). After incubation with monoclonal antibodies for 15 min at room temperature, erythrocytes were lysed with BD FACS™ lysing solution (BD Biosciences; San Jose, CA, USA) using a standard lyse/wash technique. For the detection of cytoplasmic antigens, fixation and permeabilization steps were performed before staining with antibodies for cMPO, cCD3, cCD79a, μIgM and cTdT. All antibodies were obtained from BD Biosciences or Beckman-Coulter (Indianapolis, IN, USA). Data were acquired and analyzed by flow cytometry on the FACSAria I or II with Diva software (BD Biosciences; San Jose, CA, USA). Surface antigens were considered as positively expressed when > 20% of analyzed events were stained while a cutoff of > 10% was set for cytoplasmic antigens. In MRD detection, cells with "different from normal" immunophenotypes such as $CD19^{+}CD66c^{+}$ were defined as abnormal leukemia cells, and MRD was judged as positive when the ratio of these cells possessed more than 0.01% of bone marrow nucleated cells. Immunological criteria for lineage affiliation and subtype were applied according to the NCCN 2014 recommendations.

Conventional karyotyping and FISH

Chromosome analyses (G-banding) were performed on diagnostic bone marrow samples that were prepared from stimulated bone marrow aspirate cultures using standard techniques. Twenty metaphases were analyzed and reported using the International System for Human Cytogenetic Nomenclature. FISH for *BCR/ABL1* was performed on interphase nuclei using the Vysis LSI BCR-ABL ES, dual-color, translocation, locus-specific probe (Abbott Molecular; Des Plaines, IL, USA). The cutoff that defined a positive result for *BCR/ABL1* was set at 1.0%.

Real-Time quantitative RT-PCR assay

Levels of *BCR-ABL* fusion transcripts were quantified in a multiplex RT-PCR assay that simultaneously detected b2a2, b3a2, and e1a2 transcripts. RNA was extracted from bone marrow samples using Trizol reagent (Life Technologies; Grand Island, NY, USA) according to the manufacturer's instructions. Reverse transcription was performed on total RNA (1 ng) using random hexamers and SuperScript II reverse transcriptase (Life Technologies; Grand Island, NY, USA). The cutoff value was set at 0.001% for MRD evaluation using *BCR-ABL1* determined by RT-PCR.

Statistical analysis

All statistical calculations were performed with SPSS software (SPSS 15.0; Chicago, IL, USA) or GraphPad Prism 5. Pair-wise comparisons between characteristics of patients were performed using McNemar and Kappa tests. The Fisher's exact test and the likelihood ratio test were used for non-paired categorical variables. The receiver operating characteristic (ROC) curve was generated with SPSS software, and the area under the corresponding ROC curve (AUC) was computed, along with the associated standard error (SE) and a 95% confidence interval (CI).

Results

Reliability of detection of Ph chromosome and BCR/ABL1 by FISH or RT-PCR in B-ALL patients

Immunophenotyping was used to confirm a diagnosis of B-ALL in all 43 patients in the study and to further classify cases into the different subtypes of B-ALL according to the criteria established in WHO 2008 and NCCN 2014 (**Table 1**).

The 43 cases of B-ALL were subsequently molecularly characterized by karyotyping, FISH, and RT-PCR to determine the status of *BCR/*

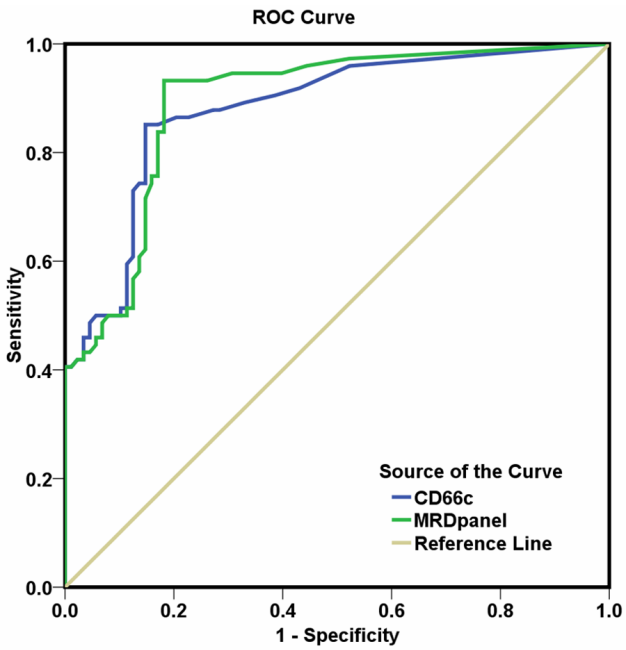

Figure 1. ROC curve of CD66c or MRD panel for prediction of MRD in B-ALL patients (n = 162). The status of *BCR-ABL1* was set as the "gold standard" for the existence or not of MRD, and AUC for CD66c is 0.881 (SE, 0.27; 95% CI, 0.828-0.934; P < 0.001, AUC for MRD panel (where the CD66c was included) is 0.891 (SE, 0.26; 95% CI, 0.841-0.942; P < 0.001).

ABL1 as well as concordance among the methods. All patient material was analyzed by karyotyping for the Ph chromosome; 17/43 cases were found to be Ph positive (17/43; 39.5%; **Table 1**). Sufficient material was available to perform FISH on 36/43 patients, and 23/36 were found to be *BCR/ABL1*-positive (23/36; 63.9%). RT-PCR demonstrated 23 *BCR-ABL1* positive B-ALL cases (23/43; 53.5%). All 17 Ph positive cases were confirmed by FISH and RT-PCR (17/23) to possess the *BCR/ABL1* fusion gene. In only three cases was *BCR-ABL1* detected by FISH (3/23) but not confirmed by RT-PCR, and in only one case was *BCR-ABL1* expression detected by RT-PCR but not confirmed by FISH (1/23). In summary, there was a significant correlation between the presence of the Ph chromosome and expression of the *BCR-ABL1* fusion gene, with relatively few discordant findings in the detection of *BCR/ABL1* by FISH or RT-PCR in B-ALL patients.

Frequency of aberrant expression of CD66c in B-ALL patients

Bone marrow samples obtained at the primary diagnosis were evaluated for the expression of CD66c by flow cytometry in the context of other potential B-ALL cell surface markers as listed in the materials and methods. Comparison with other non-B-specific antigens showed thatCD66c was more frequently expressed than CD13, CD33, CD-117, or CD123 in leukemic cells (35/43; 81.4%; **Table 2**). Cross-lineage expression of CD13 and CD33 were seldom found in B-ALL patients (4/43 and 5/43; 9.3% vs. 11.6%, respectively), but both markers were coexpressed with CD66c in leukemic cells. Coexpression of CD66c with CD123 was more frequent than with CD13 or CD33 (51.2%). CD117, however, was not detected in any of the enrolled adult B-ALL cases (0/43).

CD66c expression is significantly greater in BCR-ABL1 positive patients

Due to limitations of the methodology, a Ph chromosome in some B-ALL patients might be missed because of the lower sensitivity of the karyotyping technique as only 20 metaphases were analyzed. A negative result for the Ph chromosome might also be caused by unsatisfactory sample preparation, such as attenuated bone marrow specimens. One of the major goals of the work was therefore to determine whether CD66c expression may be used to identify leukemic cells and thus, supplement clinical evaluation of patients. Leukemic cells, as determined by CD66c expression, were found in 35/43 (81.4%; **Table 2**) patients whereas *BCR-ABL1* positive cases by FISH or RT-PCR yielded 26/43 (60.5%; **Table 1**). The frequency of detection of leukemic cells by *BCR-ABL1* was therefore lower than detection by CD66c (60.5 vs. 81.4%; P = 0.022).

A second major goal was to potentially use CD66c to distinguish B-ALL patients positive for *BCR-ABL1* from any other subset of patients negative for the translocation in order to potentially facilitate MRD detection and/or treatment. CD66c expression was therefore assessed with respect to gender, B-ALL subtype, and *BCR-ABL1* status (**Table 1**). CD66c expression in adult B-ALL did not correlate with gender (male, n = 20; female, n = 23; P = 0.167). Furthermore, CD66c expression was not exclusive to any subtype classification of B-ALL. However, CD66c was aberrantly expressed in most cases that were *BCR-ABL1* positive

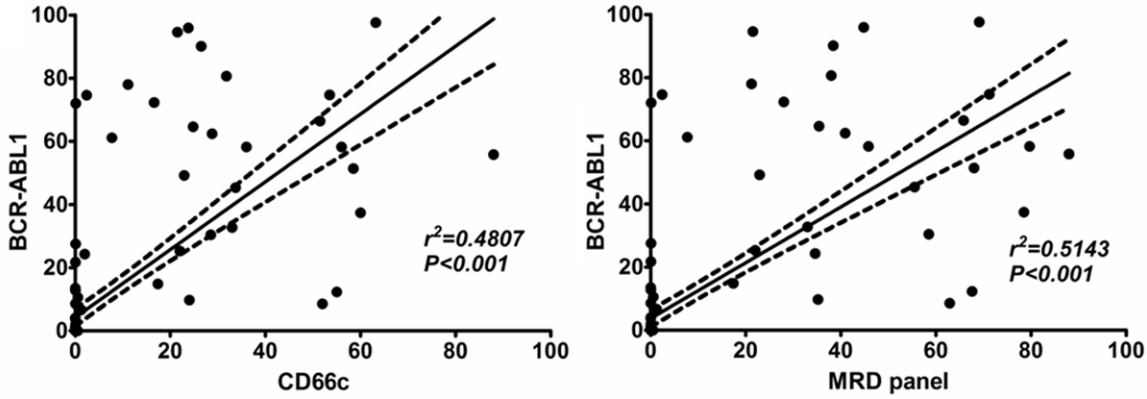

Figure 2. Correlations between the quantitative values of CD66c or MRD panel markers with relative expression of *BCR-ABL1/ABL* in MRD follow-up samples from adult B-ALL patients (n = 162). The data under the cutoff values were set as 0 (0.01% for CD66c or the MRD panel by flow cytomety and 0.001% for BCR-ABL1 by RT-PCR, respectively). Dashed lines correlate with 95% confidential intervals.

(24/26, 92.3%), and expression of CD66c was detected in all B-ALL cases that were Ph positive (17/17, 100%). In addition, significantly higher expression of CD66c was found in *BCR-ABL1* positive cases than in *BCR-ABL1* negative cases (24/26 vs. 11/17, P = 0.042) as well as in Ph positive cases relative to negative cases (17/17 vs. 18/26, P = 0.014). Therefore, the Kappa test demonstrated poor agreement of *BCR-ABL1* and CD66c expression in adult B-ALL patients (Kappa = 0.304, P = 0.023). Further analysis of the *BCR-ABL1* positive cases revealed no difference in expression of CD66c between P190 and P210 *BCR-ABL1* subtypes (P = 1).

MRD diagnostic performance of CD66c

As detection of *BCR-ABL1* by RT-PCR has been thought to be the most sensitive method for MRD monitoring in leukemia patients with a positive *BCR-ABL1* fusion gene, the status of *BCR-ABL1* was set as the standard for the presence or absence of MRD, and the performance of CD66c was evaluated as a marker for MRD in B-ALL patients. Bone marrow was collected from the 23 patients with both positive *BCR-ABL1* and CD66c at primary diagnosis and regularly assessed during treatment for MRD by RT-PCR for *BCR-ABL1* and flow cytometry for CD66c. In total, there were 162 paired MRD samples. A high diagnostic value of CD66c in the detection of MRD was observed, 0.828-0.934, based on the area under the ROC curve (AUC) which was 0.881, SE, 0.27 at 95% CI (**Figure 1**, blue line). In addition, the MRD panel, where CD66c was included, also demonstrated a similar diagnostic value, 0.841-0.942, based on the AUC which was 0.891, SE, 0.26 at 95% CI (**Figure 1**, green line). Both MRD detection methods, CD66c alone or in the MRD panel, differentiated patients with positive MRD effectively ($P < 0.0001$).

When the positive cutoff value was set to ≥ 0.01%, the detection performance of CD66c or the MRD panel for the presence of MRD was as follows: sensitivity, 85.3% vs. 93.3%; specificity, 86.2% vs. 83.9%; accuracy, 85.8% vs. 88.2%; positive predictive value, 84.2% vs. 83.3%; negative predictive value, 87.2% vs. 93.6%; false positive rate, 13.8 % vs. 16.1%; and false-negative rate, 14.7% vs. 6.67%, Youden index 0.715 vs. 0.772, respectively. A paired Kappa test further demonstrated that there was no difference in the positive results between CD66c or the MRD panel with *BCR-ABL1* ($P = 1$ vs $P = 0.064$) and there was a high correlation between the results of CD66c or the MRD panel with *BCR-ABL1* (Kappa = 0.715, vs. 0.766, $P = 0.000$).

The quantitation of MRD might provide an additional relevant clinical parameter rather than a qualitative result alone. The concordance of the absolute values between the percentage of CD66c or MRD panel markers and the relative ratio of *BCR-ABL1/ABL* in both primary and follow-up samples was therefore analyzed. All numbers that were under the cutoff values were set as 0 in order to perform these comparisons more objectively because of variability

in sensitivities between flow cytometry and RT-PCR: 0.01% for CD66c and the MRD panel; 0.001% for *BCR-ABL1*. A good correlation was also observed between the quantitative detection of CD66c and *BCR-ABL1* (r^2 = 0.4807, $P < 0.001$), as well as the MRD panel and *BCR-ABL1* (r^2 = 0.5143, $P < 0.001$), respectively (**Figure 2**).

Discussion

Leukemia diagnosis and subsequent MRD monitoring with testing for morphology, immunology, cytogenetic, and molecular status (MICM) is currently recommended by many guidelines and clinical trials. Each method possesses unique characteristics, and the corresponding test results can complement each other. In this study, CD66c was investigated as an additional surface marker that may not only be specific for the identification of leukemia cells but also stable for MRD monitoring during treatment. In adult B-ALL cases where the molecule was aberrantly expressed at the primary diagnosis, CD66c demonstrated a high diagnostic performance for the detection of MRD in *BCR-ABL1* positive cases during treatment in our cohort.

Owing to many uncontrollable factors, karyotype analysis always has lower detection sensitivity but demonstrated high specificity in our study. Our data, together with those reported by others [6, 7], indicated that the cytogenetic technique had a high positive predictive value if performed by experienced technical personnel, and therefore, might be used to effectively guide clinical treatment options, such that this method should not be replaced by FISH. FISH should be used as a complementary method for the detection of more subtle abnormalities, such as inv(16), t(11q23) and t(8; 21), in all patients with newly diagnosed AML, as these abnormalities are difficult to diagnose in most cases by conventional cytogenetics alone. Furthermore, molecular cytogenetics should also be considered in cases with insufficient yields of metaphase cells, poor chromosome morphology, or both [6-8].

Because of its high sensitivity and specificity, RT-PCR has been widely recognized as the best method to detect the abnormal fusion gene. However, the main restriction is that only about 30% to 40% of precursor B-ALL patients have specific PCR-detectable chromosomal aberrations [9, 10]. In this study, one *BCR-ABL1* positive case detected by RT-PCR could not be confirmed by FISH. In addition, there were three positive cases identified by FISH that were negative by RT-PCR. All of these patients were clinically confirmed, as clinical efficacy was obtained with the tyrosine kinase inhibitor specific for the BCR-ABL1 fusion kinase. There are many reasons why both FISH and RT-PCR methods may miss detecting the *BCR-ABL1* fusion gene, including differences in fusion gene cleavage sites, primer design, specimen processing, interpretation of the results, and different operators. These factors cannot be completely avoided or overcome in practice. Thus, it is suggested that all these strategies should be conducted as far as is possible so as to improve the detection rate of abnormal genetic alterations.

Aberrantly expressed surface molecules on leukemia cells are important not only for the diagnosis and classification of different types of leukemia, but also for MRD evaluation [10-13]. Currently, use of multi-parameter flow cytometry for surface markers is widely recommended to evaluate MRD in leukemia patients because of its multiple advantages, such as high speed, simple operation, quantitative capabilities, higher sensitivity, and most importantly, the high prognostic impact of flow cytometric MRD detection in myeloid leukemia [10, 11]. As has been widely recognized, immunophenotype shift will undoubtedly occur during the treatment of leukemia, which can lead to an underestimation of MRD [14, 15]. Simultaneous detection of multiple abnormal expression molecules in one patient has been currently recommended, so as to reduce the false negative rate and improve the sensitivity of MRD [12-15]. However, this work is time-consuming, and will inevitably increase the economic burden of patients. To try to identify a group of markers that are stably expressed in leukemia cells from onset to recurrence for MRD detection is a more attractive approach.

Cross-lineage antigens in B-ALL cells have also been widely reported. The most commonly recognized cross-lineage markers include CD13, CD33, CD117, CD65, CD66c, CD15, and NG2 [1, 2, 4]. In our adult case cohort, an even higher expression frequency of CD66c was found on leukemia cells than previously reported for

pediatric B-ALL [1] (81.4% vs. 43%, respectively), which might indicate a more important role for CD66c in adult leukemia cells. Our data also show that CD66c is more frequently expressed in B-ALL than the myeloid antigens CD13, CD33 and CD117, but was still comparable to CD123, a potential marker for leukemic stem cells [16, 17]. CD66c expression has been previously examined in both pediatric and adult B-ALL patients, but not found to correlate specifically with BCR/ABL1 rearrangement [1, 4]. In our cohort, the expression of CD66c was also not strictly limited to cases harboring the *BCR/ABL1* fusion gene. Although there was no statistical discrimination of CD66c expression in patients of different gender, different B-ALL clinical or BCR-ABL1 subtypes, significantly higher expression of CD66c was found in patients with the *BCR-ABL1* fusion gene or the Ph chromosome. Our data, together with the tight correlations between CD66c and BCR/ABL1 or other genotypes, and the qualitatively stable expression from diagnosis to relapse [1, 4], renders CD66c an important target of research both for the mechanisms underlying aberrant expression and MRD monitoring.

Until now, however, no study has focused on the actual clinical performance of CD66c in B-ALL MRD. In the MRD samples obtained from cases with both CD66c and *BCR-ABL1*, CD66c alone was found to have high sensitivity and specificity for the detection of MRD, and the monitoring results were comparable to the complete MRD panel in which CD66c was included. The quantitative value of leukemia cells positive for CD66c and CD19 in the MRD samples correlated well with the data determined by the MRD panel, and both results were consistent with the relative ratio of *BCR-ABL1/ABL*, which is currently the most recommended and sensitive strategy for MRD monitoring in *BCR-ABL1* positive patients. Our results also further confirmed that expression of CD66c on leukemia cells was stable in B-ALL patients undergoing different chemotherapeutic regimens or stem cell transplantation. As not all leukemia cells were positive for CD66c at the primary diagnosis or during relapse, there is a risk for MRD monitoring in these patients with CD66c as a single marker. These findings, together with the high frequency of CD66c positive cases in both adult and pediatric B-ALL cases, strongly support the inclusion of CD66c into an MRD panel for MRD detection in patients with CD66c at the primary diagnosis.

Several potential caveats remain however. Firstly, a relatively small number of patients (43) were retrospectively included under the criteria that analysis for all adult patients had to include flow cytometry, RT-PCR, and cytogenetic analysis at the primary diagnosis. One reason for the higher ratio of CD66c expression in adult relative to pediatric B-ALL patients (81.4% vs. 43%) is that some adult patients with incomplete laboratory data were excluded [1]. Furthermore, some of the aspirate bone marrow samples were too dilute or yielded insufficient metaphases, and perhaps contributed to a somewhat lower degree in sensitivity of karyotype analysis in this study. Secondly, MRD might escape detection even in the highly sensitive RT-PCR assay for *BCR-ABL1*, which would inevitably affect the assessment of CD66c MRD diagnostic performance. In fact, in clinical practice, patients were observed where CD66c predicted leukemia relapse earlier than *BCR-ABL1* (data not shown). In these circumstances, the sensitivity of CD66c in MRD decision-making might be underestimated, while false positivity might be overestimated. Finally, the correlation coefficients between the quantitative results of CD66c, the MRD panel, and *BCR-ABL1* might be partially influenced by the variability in sensitivities of these three methods in monitoring MRD (0.01% for CD66c and the MRD panel; 0.001% for *BCR-ABL1*), where values under the different cutoffs were set as zero for all of the methods.

In summary, to our knowledge, our data is the first to provide conclusive clinical evidence for the excellent diagnostic performance of CD66c in MRD monitoring during the follow-up of treated B-ALL patients. This high stability expression pattern of CD66c is especially important for MRD monitoring in B-ALL patients where other molecular markers, such as *BCR-ABL1* and *TEL-AML1,* are negative at primary diagnosis. CD66c, as determined by flow cytometry, warrants further investigation as a potential clinical marker in MRD detection in a multicenter prospective clinical study.

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Disclosure of conflict of interest

None.

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