Performance of a novel 8-color flow cytometry panel in the detection of minimal residual disease assessment of chronic lymphocytic leukemia

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Research Article

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Abstract

Background

The status of minimal residual disease (MRD) has been established as an important prognostic indicator in chronic lymphocytic leukemia (CLL).

Methods

Owing to the requirements of high accuracy, reproducibility and comparability of MRD, this study investigated the performance of a flow cytometric approach (CD45-ROR1 panel) in the MRD detection of CLL patients, with European Research Initiative on CLL (ERIC) 8-color panel as the “gold standard”.

Results

The sensitivity, specificity and concordance rate of CD45-ROR1 panel in the MRD assessment of CLL were 100% (87/87), 88.5% (23/26) and 97.3% (110/113), respectively. Two of the 3 non-consistent samples were further verified by the next-generation sequencing. In addition, the MRD results obtained from the CD45-ROR1 panel were positively associated with the ERIC 8-color results for MRD assessment (R = 0.98, p < 0.0001). MRD detection at low levels (≤ 1.0%) demonstrated a smaller difference between the two methods (bias, -0.11; 95% CI, -0.90-0.68) as compared with that at high levels (>0.1%). For the reproducibility assessment, the bias was smaller at three datapoints in the CD45-ROR1 panel as compared with that of the ERIC 8-color panel. Moreover, MRD level detected using the CD45-ROR1 panel for the same samples between different laboratories showed a strong statistical correlation (R = 0.99, p < 0.0001) with a trivial inter-laboratory variation (bias, 0.135; 95% CI, -0.439-0.709). Interestingly, the MRD level detected in the lymph nodes samples were significantly higher than that of the peripheral blood and bone marrow samples (p = 0.029).

Conclusions

Collectively, this study demonstrates that the CD45-ROR1 panel is a reliable method for the MRD assessment of CLL, with higher sensitivity, reproducibility, and reliability.

Introduction

Chronic lymphocytic leukemia (CLL) is a neoplasm characterized by the monoclonal proliferation of mature B-cells within the peripheral blood (PB), bone marrow (BM) and secondary lymphoid tissues, and becomes the most common leukemia type in Western countries (1). Although the advanced therapeutic approaches have improved the response rates (2), residual leukemic cells is often present in CLL patients which significantly affect the progression-free survival (PFS) and overall survival (OS) (3–6). Thus, it is of
importance for the detection of minimal residual disease (MRD). The MRD is more and more being used as a surrogate endpoint to evaluate the therapy efficacy of CLL (7, 8).

Multiparametric Flow cytometry (MFC), which has advantages in typical immunophenotype in CLL, is increasingly adopted in the diagnosis, classification and monitoring of hematologic neoplasms and becomes a cornerstone for the detection of MRD in CLL during and after treatment (9, 10). MFC identifies CLL cells from benign B cells based on the “difference from normal” and “leukemia-associated immunophenotype” approaches. The co-expression of CD19 and CD5 in combination with dim expression of light chain assessed by MFC is popularly used for the MRD detection in CLL (11, 12). However, this method is challenged by acquiring a quantitative result and has a low sensitivity. In order to improve the reliability and reduce the inter-operator variability, approaches for MRD monitoring by MFC have been developed over years (13). In detail, the European Research Initiative on CLL (ERIC) previously established a standardized MFC method to detect MRD using specific 4-color antibody combinations and codified gating strategies (12). Subsequently, the introduction of multiparameter MFC has allowed a more comprehensive MRD evaluation, with six or more fluorochromes in parallel. Accordingly, the 6-color (2 tubes) and 8-color (1 tube) MFC panels were developed by the ERIC to overcome the disadvantage of ERIC 4-color (4 tubes) panel for which the sensitivity is too low for the samples with infertile cells. Noticeably, the sensitivity of ERIC 8-color panel can reach $10^{-5}$ and this panel shows high concordances with high-throughput sequencing–based approaches at the $10^{-4}$ level (14, 15). Regrettably, FC technology is still challenged by the standardized techniques to increase the accuracy and comparability of MRD results, especially in evaluating treatment superiority of novel therapeutics.

With increasing interests and demands for residual CLL cell detection, we designed an 8-color panel (CD45-ROR1 panel) through combining CD45 and ROR1. ROR1 is the B-cell tumor-associated antigen (16) and is selected in our panel because of its high percentage expression in CLL cells as compared with the non-CLL cells, aiming to reduce the inter-operator variability and enhance the precision. Herein, we assessed the performance and applicability of CD45-ROR1 panel in the MRD detection of CLL, with the ERIC 8-color panel as the “gold standard”.

**Patients And Methods**

**Patients and samples**

A total of 163 samples from 96 CLL patients were used for this study, including 58 bone marrow (BM) specimens, 94 peripheral blood (PB) specimens, 2 pleural effusions or ascites specimens and 9 lymph nodes (LN) specimens. In detail, 113 samples were submitted for FC detection using both CD45-ROR1 panel and the ERIC 8-color panel; 2 samples were applied for next-generation sequencing (NGS) to validate the MRD level detected by FC assays; 10 samples were used for repeated examination at different time points to assess the reproducibility of CD45-ROR1 panel and ERIC 8-color panel in parallel; 10 samples were submitted to CD45-ROR1 panel detection in different laboratories to determine the inter-laboratory variation; 51 paired PB and BM samples, together with 9 paired PB, BM and LN samples were
used to compare the MRD level in different sample types. The diagnosis of CLL was according to the National Cancer Institute Working Group (NCI-WG) criteria(17). All of the patients signed the informed consents, and this study was approved the hospital ethics committee.

**Immunologic detection of MRD by MFC**

Specimens including BM or PB were incubated with labeled antibodies with different combinations (CD45-ROR1 panel or ERIC 8-color panel) for 15 min at room temperature in the dark. After that, FACSLyse was used to lyse the red blood cells, and the cells were washed with phosphate-buffered saline (PBS), centrifuged and then re-suspended in 500 µL PBS. The data were acquired using the Navios flow cytometer (Beckman Coulter, USA). Our CD45-ROR1 panel includes CD81, CD20, CD45, CD5, CD19, CD79b, CD43 and ROR1, the 8-color antibody combination, while the ERIC 8-color MRD panel includes CD81, CD20, CD22, CD5, CD19, CD79b, CD43 and CD3. The specific fluorochromes, clones and reagent volumes were listed in Table 1. The limit of detection (LOD) and the limit of quantification (LOQ) have been established at 20/total leukocytes and 50/total leukocytes, respectively. To achieve a limit of detection of 0.005%-0.01%, we aimed to acquire the events ranged from 200,000 to 1,000,000 leukocytes for each sample. MRD was calculated by dividing the number of CLL events by the number of leukocyte events.

<table>
<thead>
<tr>
<th>Fluorochrome</th>
<th>CD45-ROR1 panel</th>
<th>ERIC 8-color panel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marker</td>
<td>Clone</td>
<td>Reagent volumes (mL)</td>
</tr>
<tr>
<td>FITC</td>
<td>CD81</td>
<td>JS-81</td>
</tr>
<tr>
<td>PE</td>
<td>CD43</td>
<td>1G10</td>
</tr>
<tr>
<td>PerCPcy5.5</td>
<td>CD79b</td>
<td>CB3-1</td>
</tr>
<tr>
<td>PE-Cy7</td>
<td>CD5</td>
<td>UCHT2</td>
</tr>
<tr>
<td>APC</td>
<td>CD19</td>
<td>SJ25C1</td>
</tr>
<tr>
<td>APC-Cy7 (APC-A750)</td>
<td>CD20</td>
<td>L27</td>
</tr>
<tr>
<td>BV421</td>
<td>ROR1</td>
<td>2A2</td>
</tr>
<tr>
<td>K0 (BV510)</td>
<td>CD45</td>
<td>J33</td>
</tr>
<tr>
<td>ECD</td>
<td>/</td>
<td>/</td>
</tr>
</tbody>
</table>
Next-generation Sequencing (Ngs)

Genomic DNA was extracted from the BM or PB mononuclear cells at the time of diagnosis and follow-up using Magnetic Universal Genomic DNA Kit (TIANGEN, Beijing, China). Immunoglobulin heavy (IGH) complementarity determining regions (CDR3) were amplified and sequenced using an IGH multiplex consensus PCR and Illumina NextSeq 500 platform (Illumina, San Diego, CA, USA). The IGH sequences obtained from sequencing in diagnostic samples were analyzed with the IMGT V-QUEST tool (http://www.imgt.org) to assess the IGH gene rearrangements and determine the cancer-derived sequences that were then used in the following MRD monitoring. For MRD quantitation, genomic DNA was added with a known quantity of reference IGH sequence and amplified using multiplexed degenerate primers sets for IGH V region and a single J primer. MRD positive was defined as the presence of cancer-derived clone identified in the diagnostic samples.

Statistical analysis

Data analysis was performed using SPSS statistical analysis software (version 23.0) and GraphPad PRISM (version 6.01). Comparison analysis between the two methods was performed using Pearson correlation. Bland–Altman plots, mean difference and 95% limit of agreement (± 1.96 SD), were calculated between groups. $p < 0.05$ was considered statistically significant.

Results

Performance analysis of the novel 8-color panel

In order to identify residual CLL cells from normal leukocytes efficiently, the combination of 8 antibodies, including CD45, CD5, CD19, CD20, CD43, CD81, CD79b and ROR1 was assembled based on the immunophenotype of CLL populations (Fig. 1). In our study, 89 of the 96 CLL cases had a typical immunophenotypic features, with co-expression of CD5, CD19, CD20 (dim), and CD43 while lacking of the expression of CD81 and CD79b. Of the cases with an atypical phenotype, ROR1 was positive in 5 of 7 CLL cases which had residual CLL cells and restricted light chain expression, providing an opportunity for MRD detection in atypical CLL cases. With regard to initial gating strategy, light scatter (FSC-Height vs. FSC-Area, SSC-Area vs. FSC-Area) was used to exclude doublets, and CD45 was required for leukocyte gating and enumeration. In addition, CD43/CD81 was helpful in excluding contaminating T events. For BM samples, CD5/CD81 was used to differentiate CLL cells from plasma cells and normal B-progenitors. These features in antigen combination allowed the CD45-ROR1 panel to be used for MRD analysis.

Comparison Of The Novel 8-color Panel And Eric 8-color Panel For Mrd Detection

A subset of 113 samples were submitted to both of the CD45-ROR1 panel and the ERIC 8-color panel for MRD assessment. Three samples negative as detected using ERIC 8-color panel showed positive MRD+
(<0.01%) in CD45-ROR1 panel, with the sensitivity, specificity and concordance rate of 100% (87/87), 88.5% (23/26) and 97.3% (110/113), respectively (Table 2). The proportion of CLL cells detected using CD45-ROR1 panel ranged from 0–98.0%, with median of 1.77%, while the proportion of CLL cells detected using the ERIC 8-color panel ranged from 0–96.0%, with a median of 1.32%. Also, we found a significant correlation between the proportion of CLL cells detected using CD45-ROR1 panel and ERIC 8-color panel ($R = 0.98, p < 0.0001, 95\% CI, 0.98–0.99$) (Fig. 2).

### Table 2
Concordance of MRD status detected by CD45-ROR1 panel with ERIC 8-color panel

<table>
<thead>
<tr>
<th>CD45-ROR1 panel</th>
<th>ERIC 8-color panel</th>
<th>MRD + &lt; 0.01%</th>
<th>MRD + 0.01-1%</th>
<th>MRD + ≥ 1%</th>
<th>Und-MRD</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRD + &lt; 0.01%</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>MRD + 0.01-1%</td>
<td>0</td>
<td>21</td>
<td>4</td>
<td>0</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>MRD + ≥ 1%</td>
<td>0</td>
<td>0</td>
<td>59</td>
<td>0</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>Und-MRD</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>23</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>4</td>
<td>23</td>
<td>63</td>
<td>23</td>
<td>113</td>
<td></td>
</tr>
</tbody>
</table>

MRD, measurable residual disease; Und-MRD, undetectable MRD.

In addition, we analysed the differences between CD45-ROR1 panel and ERIC 8-color panel. MRD results at all levels detected by the two panels demonstrated a small difference (bias, -1.62; 95\%CI, -12.61-9.37), as well as at the MRD level $\leq$ 1% (bias, -0.11; 95\%CI, -0.90-0.68) and >1% (bias, -3.0; 95\%CI, -17.73-11.74) (Fig. 3). Bland-Altman method comparison showed that the two panels had a smaller difference in the detection of MRD at a low level $\leq$ 1.0% than at high level (>1%) in this dataset (Fig. 3). Overall, CD45-ROR1 panel showed a good comparability to ERIC 8-color panel for MRD quantification.

As three samples showed MRD positive as detected using the CD45-ROR1 panel (<0.01%) while negative as detected by ERIC 8-color panel, NGS-based approaches was further adopted to detect residual CLL cells of 2 of the 3 cases. As expected, both samples showed MRD positive at low level as detected by the NGS (Table 3), indicating that the quantification of residual disease below the 0.01% threshold by the CD45-ROR1 panel was credible.
Table 3
Discordances recorded: the MRD levels of 2 CLL patients were detected by ERIC 8-color panel, CD45-ROR1 panel and NGS.

<table>
<thead>
<tr>
<th>MRD (%)</th>
<th>ERIC 8-color panel</th>
<th>CD45-ROR1 panel</th>
<th>NGS</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample 1</td>
<td>Und-MRD</td>
<td>0.01</td>
<td>0.00169</td>
</tr>
<tr>
<td>Sample 2</td>
<td>Und-MRD</td>
<td>0.006</td>
<td>0.0429</td>
</tr>
</tbody>
</table>

MRD, measurable residual disease; Und-MRD, undetectable MRD.

Reproducibility Assessment Of The New 8-color Panel And Eric 8-color Panel For Mrd Detection

Samples collected from 10 patients at different timepoints (within 24 hours, 48 hours and 72 hours) were submitted to the MRD detection using CD45-ROR1 panel and ERIC 8-color panel to evaluate the reproducibility of both panels. As shown in Fig. 4, the CD45-ROR1 panel showed a more powerful agreement than the ERIC 8-color panel. The bias was smaller at three datapoints in the CD45-ROR1 panel (24 hours vs. 48 hours: bias, 0.0024; 95%CI, -0.158-0.163; 24 hours vs. 72 hours: bias, 0.025 95%CI, -0.308-0.358), as compared with that of the ERIC 8-color panel (24 hours vs. 48 hours: bias, -0.018; 95%CI, -0.654-0.619; 24 hours vs 72 hours: bias, -0.079; 95%CI, -0.829-0.670) (Fig. 4).

To assess the stability of CD45-ROR1 panel resulted from different preparation methods, the same samples were prepared and analysed in different laboratories. The data of 10 samples were analysed and recorded in both laboratories by a blinded fashion. The difference between the MRD levels obtained from the two laboratories was not significantly (paired T-test, \( p = 0.18 \)) and the statistical correlation was strong (\( R = 0.99, p < 0.0001, 95\%CI, 0.959–0.999 \)) (Fig. 5). In addition, the inter-laboratory variation was small (bias, 0.135; 95%CI, -0.439-0.709), indicating a good reproducibility of the CD45-ROR1 panel which was not affected by preparation methods.

Mrd Detection Using The New 8-color Panel In Different Sample Types

Also, we assessed the effect of CD45-ROR1 panel on the MRD assessment using different sample types in paired PB, BM and LN samples. Among the 51 paired PB and BM samples, the consistent rate was 82.4% (42/51) (Table 4). 14/51 (27.5%) cases showed undetectable MRD and 28/51 (54.9%) showed positive MRD results in both PB and BM samples. Discordant MRD results were observed in 9 paired samples, among which 8 samples showed positive MRD in BM while negative MRD in PB, while 1 case
showed positive MRD in PB and negative MRD in BM. Among the 22 paired cases with positive but low level MRD (<1% in PB or BM), the proportions of CLL cells in the BM were significantly higher than that in the PB [median CLL cells (range): 0.08% (0.00–0.76%) and 0.30% (0.00–21.00%), respectively, \( p = 0.046 \)]. Among the 22 samples with undetectable MRD in the PB, MRD was positive in 8 cases of the BM. In addition, a low proportion of CLL cells (≤ 0.01%) was observed in 2 samples and the remaining 6 samples had a proportion of CLL cells > 0.01% in the BM samples. The positive rate of MRD in BM samples was higher than that in PB samples, indicating the necessity for BM examination when the MRD was negative on PB cells.

<table>
<thead>
<tr>
<th>Table 4</th>
</tr>
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<tbody>
<tr>
<td>Concordance of MRD status detected by CD45-ROR1 panel in PB and BM</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>BM MRD</th>
<th>PB MRD</th>
<th>MRD + ≤ 0.01%</th>
<th>MRD + 0.01-1%</th>
<th>MRD + ≥ 1%</th>
<th>Und-MRD</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRD + ≤ 0.01%</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>MRD + 0.01-1%</td>
<td>0</td>
<td>3</td>
<td>9</td>
<td>1</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>MRD + ≥ 1%</td>
<td>0</td>
<td>0</td>
<td>15</td>
<td>0</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Und-MRD</td>
<td>2</td>
<td>5</td>
<td>1</td>
<td>14</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>2</td>
<td>9</td>
<td>25</td>
<td>15</td>
<td>51</td>
<td></td>
</tr>
</tbody>
</table>

Data are number of patients with simultaneous PB and BM MRD data.

PB, peripheral blood; BM, bone marrow; MRD, measurable residual disease; Und-MRD, undetectable MRD.

Among the 9 paired PB, BM and LN samples, the consistent rate was 77.8% (7/9). Only 2 cases had discordant MRD results in three compartments with undetectable MRD in PB while positive MRD in BM (0.15% and 0.27%, respectively) and LN (42.6% and 44.5%, respectively). The MRD positive rate detected using the LN samples was significantly higher than that of the PB and BM samples (\( p = 0.029 \)). These data indicated a higher sensitivity for the MRD detection using the LN samples as compared with the PB and BM samples.

**Discussion**

MRD measurement is widely used to evaluate response to therapy, guide clinical decision and predict the OS and PFS of CLL, and it is proposed as a cornerstone for the relapse risk stratification (18, 19). CLL treatment has evolved from traditional chemotherapy into combination therapeutics with novel agents, increasing the CR rate of CLL. However, CR doesn’t represent disease eradication, thus the quantification of MRD with more sensitive approaches, gained importance (20–22). In the present study, we evaluated the performance of a novel 8-color MFC panel, the CD45-ROR1 panel, for the MRD detection of CLL, with
ERIC 8-color panel as “gold standard”. Through combining CD45, tumor specific antigen ROR1, and core markers of the ERIC methodology, the CD45-ROR1 panel focuses on improving the standardization, reproducibility, and reliability of MRD quantification.

With the advent of multiparameter cytometers, more and more antibodies can be put into one single tube, contributing to the more accurate identification of residual CLL cells. It is difficult to identify the MRD when it is at low levels using traditional MFC approach of CD19/CD5 co-expression together with light chain restriction (14, 23). Accordingly, an 8-color MFC assay was developed, which makes 8 antibodies in one tube come into reality (24). Of note, in this study, CD22 was excluded from the panel because it may be equivalent to CD20, the best discriminator between CLL cells and normal B cells (15, 25). CD43/CD81 can help to exclude contaminated CD3\(^+\)CD19\(^+\) events, thus CD3 was dropped from our panel as the ERIC has proposed (15). It has been demonstrated that the 6-marker core marker panel identified by the ERIC may be insensitive for some patients with atypical phenotypes, which are characterized by reduced expression of CD5 and CD43, and/or bright expression of CD20 and CD79b. Also, it has been reported that CD20 is negative in normal mature B lymphocytes in patients treated with anti-CD20 monoclonal antibodies (26), which challenges the MRD detection. Fortunately, ROR1 could assist in resolving these problems. ROR1 is one member of the Receptor Tyrosine Kinases (RTK) family which expresses in vast majority of CLL cases, but negatively expresses in the adult normal cells (27). In addition, ROR1 positivity was not affected by different treatment (28). Moreover, ROR1 expression is higher in the residual leukemic cells than that of the mature B cells (29, 30), further confirming that this receptor may play an important role in the MRD analysis of patients with typical or atypical phenotype. These properties make ROR1 an attractive candidate for MRD monitoring in combination with other core molecules proposed by the ERIC. Furthermore, light scatter (FSC-Height vs. FSC-Area, SSC-Area vs. FSC-Area) was used to exclude doublets in our MFC assay (CD45-ROR1 panel) according to the gating strategy published by the International Clinical Cytometry Society (ICCS) and the European Society for Clinical Cell Analysis (ESCCA) for the assessment of MRD in multiple myeloma (MM) (31).

With ERIC 8-color panel as the “gold standard”, the CD45-ROR1 panel showed high sensitivity, specificity and concordance rate, with 100% (87/87), 88.5% (23/26) and 97.3% (110/113), respectively. Also, it showed a close correlation to the CD45-ROR1 8-color panel (R = 0.98, p < 0.0001). Interestingly, 3 cases presented positive MRD as detected by CD45-ROR1 panel while negative MRD detected by ERIC 8-color panel. In order to validate the MRD results, NGS, a highly sensitive method (32), was used to assess the MRD status of 2 of the 3 samples, and the results showed that both of them were MRD positive. This result further verified the reliability and higher sensitivity of CD45-ROR1 panel in the MRD monitoring of CLL.

In the aspect of reproducibility, the data showed that the CD45-ROR1 panel produced smaller variation than the ERIC 8-color panel at different timepoints. In general, MRD level in CLL is defined as the percentage of leukemic cells in total leukocyte population. However, several pre-analytical factors affect the number of events in the leukocyte gate, such as insufficient erythrocyte lysis, inter-operator variability, or long time of samples placement. Based on our results, it seems that using CD45 for leukocyte gating
can increase the reproducibility of MRD evaluation in CLL patients. In addition, we found that the MRD level in CLL patients detected using the CD45-ROR1 panel showed no significant difference between different laboratories, indicating a high reproducibility of CD45-ROR1 panel.

Sample selection of the CLL patients remains an important issue as CLL is a multicompartmental disease. Besides BM and PB, residual disease can also exist in the liver, spleen and LN tissues when relapse (8). Herein, we assessed the application of CD45-ROR1 panel in the MRD detection of CLL using BM, PB and LN samples. In agreement with previous studies (22, 33), we found that the positive rate of MRD detected in BM samples was higher than that in PB samples. This result further verified that BM aspiration is not necessary if MRD is positive in PB samples for the qualitative assessment. In contrast, BM aspiration is required when MRD is negative in PB samples, as recommended by the guidelines. In clinical trials, both PB and BM are recommended for MRD assessment with the aim of eradicating the disease (34). In addition, we found that the MRD levels detected in LN samples were higher than that of the PB and BM samples, indicating that LN assay should be suggested when patients present with low or undetectable BM and PB MRD.

In conclusion, this study demonstrated that the CD45-ROR1 panel is a higher sensitivity MFC panel as compared to the ERIC 8-color panel in MRD detection. Noticeably, it increases the standardization, reproducibility, and reliability of the MRD measurement. It is essential to carry out more researches to further explore the potential benefit of this technology in guiding novel therapies and predicting prognosis.

**Abbreviations**

MRD: minimal residual disease; CLL: chronic lymphocytic leukemia; ERIC: European Research Initiative on CLL; PB: peripheral blood; BM: bone marrow; PFS: progression-free survival; OS: overall survival; MFC: Multiparametric Flow cytometry; LN: lymph nodes; NCI-WG: National Cancer Institute Working Group; PBS: phosphate-buffered saline; LOD: limit of detection; LOQ: limit of quantification; NGS: next-generation sequencing; IGH: Immunoglobulin heavy; RTK: Receptor Tyrosine Kinases; ICCS: International Clinical Cytometry Society; ESCCA: European Society for Clinical Cell Analysis; MM: multiple myeloma.

**Declarations**

**Acknowledgments**

Not applicable.

**Authors’ contributions**

XC, SSZ and YJW designed the project and wrote the manuscript. XC, SSZ, YJW, YS, NHZ analyzed the data. ZG, CQ and HMJ performed experiments. LYZ, HYZ and JYL provided critical comments and
suggestions and made the figures. All authors reviewed the manuscript and approved the submitted version of the manuscript.

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**Availability of data and materials**

All data generated or analyzed during this study are included in this article.

**Ethics approval and consent to participate**

This research was approved by the Ethics Committee of Jiangsu Province Hospital and all patients provided written informed consent to have their samples included in the study.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**References**


**Figures**
Figure 1

Example for identification of CLL MRD in PB using CD45-ROR1 panel. Light scatter (FS INT vs. FS PEAK, FS INT vs. SS INT) was used to exclude doublets, nonviable cells and debris, followed by gating in CD45+ cells to include all leukocytes with excluding erythrocytes and cell debris. All B cells were identified by CD19+, and the distinction of CLL cells from normal B cells was achieved based on the different expression of CD5, CD43, CD81, CD20, CD79b and ROR1. Please note that using CD43/CD81 could eliminate contaminating CD3+CD19+ events. The CLL cells are identified as events present in gates P, AH and W. MRD quantification was calculated by dividing the number of CLL events by the number of leukocyte events).
Correlation of CD45-ROR1 panel and ERIC 8-color panel in MRD detection in 113 CLL patients (correlation coefficient of $R=0.98$, $p<0.0001$).

**Figure 2**

Correlation of CD45-ROR1 panel and ERIC 8-color panel in MRD detection in 113 CLL patients (correlation coefficient of $R=0.98$, $p<0.0001$).

**Figure 3**

A) MRD at all levels

- Bias: -1.62, 95% CI: -12.61 - 9.37

B) MRD level ≤ 1%

- Bias: -0.11, 95% CI: -0.90 - 0.68

C) MRD level > 1%

- Bias: -3.0, 95% CI: -17.73 - 11.74
Difference of MRD detection using CD45-ROR1 panel and ERIC 8-color panel. Bland-Altman method comparison was used to calculate the difference for MRD detection at all levels (n=113), ≤1.0% level (n=54) and >1% level (n=59), respectively. (A) MRD at all levels: bias, -1.62 (95% CI, -12.61-9.37). (B) MRD at levels ≤1.0%: bias, -0.11 (95% CI, -0.90-0.68). (C) MRD at levels >1%: bias, -3.0 (95% CI, -17.73-11.74).

Figure 4

Assessment of the reproducibility of CD45-ROR1 panel and ERIC 8-color panel at three timepoints (within 24 hours, 48 hours and 72 hours). Bland-Altman method was used to calculated the difference at three timepoints in 10 samples. (A) CD45-ROR1 panel (24 vs 48 hours): bias, 0.0024 (95% CI, -0.158 -0.163). (B) ERIC 8-color panel (24 vs 48 hours): bias, -0.018 (95% CI, -0.654-0.619). (C) CD45-ROR1 assay (24 vs 72 hours): bias, 0.025 (95% CI, -0.308-0.358). (D) ERIC 8-color (24 vs 48 hours): bias, -0.079 (95% CI, -0.829-0.670).
Figure 5

Correlation and variation of MRD results between 2 laboratories using the CD45-ROR1 panel. (A) The Pearson correlation analysis was used to assess the correlation of MRD level between the 2 laboratories (R=0.99, p<0.0001). (B) The inter-laboratory variation of MRD detection using the CD45-ROR1 panel (bias, 0.135, 95%CI: -0.439-0.709).