A Method Comparison Study of Flow Cytometry and Cytomorphology to Determine the Percentages of Blasts in Patients with Acute Leukemia after Induction and Consolidation Chemotherapy

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Background: Enumeration of blasts in the bone marrow is an essential component in the diagnosis and treatment of acute leukemia. The current gold standard method is based on a morphologic counting of 500 marrow nucleated cells despite its operator dependence and inter-observer variability.

Objectives: To compare the percentages of marrow blasts derived from two different approaches comprising routine morphology-based manual counting and flow cytometric analysis.

Material and Method: Fifty-five marrow samples were collected from 38 acute leukemia patients (36 AML and 19 ALL) after hematologic recovery from chemotherapy. The blast percentages were enumerated manually and by flow cytometer using CD45 and side scatter gates.

Results: A good correlation was found in the overall 55 samples (r = 0.829) and 36 AML samples (r = 0.86). The blast percentages derived from flow cytometer were higher than from morphologic counting in 46 samples (83.6%). Using a cut-off point of < 5% blasts to define complete remission (CR), 48 cases (87%) were classified as morphological CR (83% CR in AML and 95% CR in ALL). By flow cytometry, only 24 cases (44%) were in CR (28% CR in AML and 74% CR in ALL). The results from each method were concordant in determining CR in 27 samples (49%), with a kappa value of 0.07 for overall samples, 0.057 for AML and -0.096 for ALL samples.

Conclusion: A good correlation between the percentages of blasts achieved by either method was demonstrated, particularly in AML samples. Discordant results occurred when <5% blasts were used as a cut-off point to determine CR. Both methods should be complementarily performed to ensure a truly complete response to chemotherapy. The method discrepancy should be further investigated in order to increase the level of confidence in CR status.

Keywords: acute leukemia, flow cytometry, cytomorphology, blast counts, bone marrow, complete remission

J Med Assoc Thai 2010; 93 (Suppl. 1): S157-164
Full text. e-Journal: http://www.mat.or.th/journal

Acute leukemia is the second most common hematologic malignancy in the Thai population and is associated with great morbidity and mortality worldwide(1). Current standard treatment of acute leukemia consists of induction chemotherapy to decrease the burden of leukemic “blasts” to the lowest possible level and post-remission therapy to further consolidate the treatment and prevent recurrence of the abnormal leukemic clone(2,3). Enumeration of blasts in the patients' bone marrow after chemotherapy is a critical step in deciding treatment response and planning further intensification treatment strategies(4,5). Microscopic examination of the bone marrow is the mainstay for the initial diagnosis of acute leukemia as well as for follow-ups of the residual disease after treatment(5,6). The gold standard morphology-based microscopic examination requires a 500 marrow nucleated cell count to determine the percentages of blasts in the marrow specimens(7,8). Other alternative methods include the estimation of the percentages of blasts from bone marrow biopsy specimens and minimal residual disease (MRD) detection by flow cytometry and molecular techniques such as cytogenetics and polymerase chain reaction.
Flow cytometric immunophenotyping has become an essential tool for leukemia diagnosis, classification and monitoring after treatment. The advantage of flow cytometry lies in the ability of the automated flow cytometer instrument to rapidly analyze a large number of marrow cells and generate the computerized marrow profile with specific cell population composition. The presence of blasts can be detected in the blast window or blast gate generated from CD45 and side scatter (SSC) gating techniques. Availability of an expanded range of antibodies and fluorochromes has also led to more accurate analysis of antigenic expression of normal and leukemic cells, leading to enhanced identification of abnormal populations.

This study explored whether flow cytometric analysis could be utilized to determine treatment response better than routine morphology-based manual counting of marrow cells in acute leukemia patients after receiving chemotherapy. The percentages of blasts obtained from CD45/SSC gated population and the manual method were compared to determine if correlations between the two techniques existed. A cutoff point of < 5% blasts was used to define complete remission (CR) after chemotherapy by either method. Since flow cytometer can efficiently analyze millions more marrow cells than manual morphology-based counting, it could potentially offer clinically useful information to aid a proper clinical decision, especially when blast cells are present at a low percentage in the bone marrow or blood.

Material and Method

Leukemia samples

The study was approved by the Ethical Committee for Human Research, Faculty of Medicine Siriraj Hospital. Leukemic samples were obtained from de novo acute leukemia patients who underwent routine hematologic work-ups at the Faculty of Medicine Siriraj Hospital. Fifty-five marrow samples from 38 acute leukemia patients were collected over a 10-month period from July 2008 to April 2009. All patients were treated under standard chemotherapy regimens and samples were collected after the induction phase or consolidation phase of acute myeloid leukemia (AML) or acute lymphoblastic leukemia (ALL) chemotherapy protocol. Hematologic recovery as defined by an absolute neutrophil count greater than 1x10^9/L and a platelet count greater than 100x10^9/L was required before enrollment into the study.

Cytomorphologic analysis

Dry aspirated bone marrow smears were stained with Wright-Giemsa dye according to the standard method. The marrow smears were analyzed morphologically by two independent readers who were not aware of the results of flow cytometry. Differential counts and the percentage of blasts were obtained after a total count of 500 marrow nucleated cells. A morphological complete remission (CR) was defined as having fewer than 5% blasts in the marrow sample.

Flow cytometric analysis

Marrow samples were analyzed in the Flow Cytometry Laboratory, Division of Hematology, Department of Medicine, Siriraj Hospital. Marrow cells were labeled with a standard panel of monoclonal antibodies according to our previously established methods. CD45 and side scatter (SSC) gates were used to distinguish blast cells from normal hematopoietic cells in the bone marrow using FACScalibur flow cytometer instrument (Becton Dickinson, San Jose, CA, USA). The percentage of blasts was derived from the number of cells within the selected blast gates. A CR was defined as having fewer than 5% of total marrow cells occupying in the blast gates.

Statistical analysis and outcome measurement

Paired samples t-test was used to analyze the percentages of blasts derived from each method with standard deviation (SD) and 95% confidence interval (CI) of the mean. Agreement in the percentages of blasts (< 5 or > 5) to determine CR status between manual counting and flow cytometry was evaluated by kappa value.

Results

Clinical and laboratory characteristics of acute leukemia cases

Fifty-five marrow samples were obtained from 38 acute leukemia cases consisting of 17 women and 21 men with the age ranging from 16 to 70 years (median 35 years) (Table 1). The specimens were collected during routine diagnostic work-ups to determine response to chemotherapy. The median hemoglobin concentration was 11.6 g/dL (7.8-15.4 g/dL) with the median white blood cell (WBC) count of 6.5 x 10^9/L (2.1-18.9 x 10^9/L) and the median platelet count of 288 x 10^9/L (111-1,690 x10^9/L). The subtypes of AML were M0 in 1 sample, M1 in 2 samples, M2 in 14 samples, M3 in 1 sample, M4 in 8 samples, M5 in 3 samples, and unclassified in 7 samples. The majority of ALL samples were of B-cell in...
Table 1. Clinical and hematologic characteristics of acute leukemia cases and samples

<table>
<thead>
<tr>
<th>Variables</th>
<th>Median (range)</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients’ characteristics: n = 38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>35 (16-70)</td>
<td></td>
</tr>
<tr>
<td>Male gender</td>
<td>21 (55)</td>
<td></td>
</tr>
<tr>
<td>AML</td>
<td>27 (71)</td>
<td></td>
</tr>
<tr>
<td>ALL</td>
<td>11 (29)</td>
<td></td>
</tr>
<tr>
<td>Samples: n = 55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>11.6 (7.8-15.4)</td>
<td></td>
</tr>
<tr>
<td>WBC (x10⁹/L)</td>
<td>6.5 (2.1-18.9)</td>
<td></td>
</tr>
<tr>
<td>Platelet (x10⁹/L)</td>
<td>288 (111-1,690)</td>
<td></td>
</tr>
<tr>
<td>AML</td>
<td>36 (65.4)</td>
<td></td>
</tr>
<tr>
<td>ALL</td>
<td>19 (34.5)</td>
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</tr>
</tbody>
</table>

origin. Only 3 samples were T-cell ALL.

**Enumeration of blast percentages in marrow specimens**

The manual blast counts ranged from 0.8% to 54.4% (mean 4.02%, n = 55) (Table 2). In AML cases, the manual blast counts ranged from 0.8% to 54.4% (mean 4.94%, n = 36) while ALL cases ranged from 0.8-4.5% (mean 2.28% n = 19). The percentage of blast counts derived from the blast gates estimated by flow cytometer ranged from 1.13% to 62.89% (mean 7.04%). In AML cases, the flow cytometric blast counts ranged from 1.13% to 62.89% (mean 8.66%) while ALL cases ranged from 0.96-15.01% (mean 3.97%). The percentages of blasts derived by flow cytometry were significantly higher than those derived by manual counting methods in overall samples and AML samples (p-value = 0.000). A good positive correlation was found for the percentages of blast counts by the two methods for overall samples and AML samples (p-value = 0.000) and AML (p = 0.86) (p-value = 0.000). In ALL samples, only a weak negative correlation was observed (r = -0.184) (p-value = 0.451). Four samples (Sample No.5, 6, 32, 33) including 3 AML samples and 1 ALL sample showed greater than 10% difference in the percentages of blasts counted by the two methods.

**Determination of CR by flow cytometry and cytomorphology**

The percentages of blasts counted by flow cytometry were higher than the percentages of blasts counted manually in 46 samples (83.6%). Using a cutoff point of <5% blasts to define CR in a marrow sample, it was found that 48 samples (87%) of acute leukemia had a morphological CR (83% morphological CR in AML and 95% morphological CR in ALL) as shown in Table 3. By flow cytometry, only 24 samples (44%) were in CR (28% CR in AML and 74% CR in ALL). Therefore, the results from flow cytometry as compared to morphologic counts were concordant in only 27 samples (49%) and discordant in 28 samples (51%) in determining CR. Disagreed results were found in 6 of 19 ALL samples (31.5%) and 22 of 36 AML samples (61.1%). Measurement of agreement by both methods for CR showed a very low kappa value of 0.07 for overall samples, 0.057 for AML and -0.096 for ALL samples.

A representative flow cytometric diagram is shown in Fig. 1. In this particular AML patient (Sample No.5), a manual count was clearly compatible with CR (blast 1%) whereas flow cytometric analysis distinctly yielded no CR (blast 17.23%). In the next follow-up sample from the same patient, the percentage of blasts by flow cytometry was 2.46% which was slightly lower than 4.0% by a morphologic count. Both numbers, however, were concordant with CR. The blast count could also be lower than the manual count as observed in one case of B-ALL (Sample No.51) whereby a manual blast count was 8.6% (no CR) but the blast count by flow cytometry was only 0.96% (CR).

**Discussion**

It was estimated that at the initial diagnosis of acute leukemia, patients may carry as many as 10^{12} leukemic blast cells in the body. After induction chemotherapy with appropriate anti-leukemic drugs, a good proportion of blast cells may have been killed, but at least 10^9 of them still persist, necessitating additional courses of post-remission chemotherapy for further elimination. The ability to accurately assess the number of residual blasts in the bone marrow after treat-
Table 2. Comparison of the percentages of blasts derived from flow cytometry and cytomorphology

<table>
<thead>
<tr>
<th>n</th>
<th>Flow cytometry</th>
<th>Manual counting</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean  SD</td>
<td>95% CI</td>
<td>Mean  SD</td>
</tr>
<tr>
<td></td>
<td>Lower Upper</td>
<td></td>
<td>Lower Upper</td>
</tr>
<tr>
<td>Total</td>
<td>55 7.03 8.64</td>
<td>4.70 9.37 4.02 7.69</td>
<td>1.94 6.10 0.000</td>
</tr>
<tr>
<td>ALL</td>
<td>19 3.97 5.48</td>
<td>2.45 5.48 2.28 1.83</td>
<td>1.40 3.16 0.077</td>
</tr>
<tr>
<td>AML</td>
<td>36 8.65 10.11</td>
<td>5.23 12.08 4.93 9.33</td>
<td>1.78 8.09 0.000</td>
</tr>
</tbody>
</table>

Table 3. Outcome analysis of flow cytometry and cytomorphology methods

<table>
<thead>
<tr>
<th>Flow cytometry: Blasts</th>
<th>Morphologic counting: Blasts</th>
<th>Total n (%)</th>
<th>Kappa value</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 5% n (%)</td>
<td>≥ 5% n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>22 (40.00) 2 (3.63)</td>
<td>24 (43.63)</td>
<td>0.07</td>
</tr>
<tr>
<td>≥ 5% n (%)</td>
<td>26 (47.27) 5 (9.10)</td>
<td>31 (56.37)</td>
<td>-0.096</td>
</tr>
<tr>
<td>AML</td>
<td>48 (87.27) 7 (12.73)</td>
<td>55 (100.00)</td>
<td></td>
</tr>
<tr>
<td>&lt; 5% n (%)</td>
<td>13 (68.42) 1 (5.26)</td>
<td>14 (73.68)</td>
<td></td>
</tr>
<tr>
<td>≥ 5% n (%)</td>
<td>5 (26.32) 0</td>
<td>5 (26.32)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>18 (94.75) 1 (5.26)</td>
<td>19 (100.00)</td>
<td></td>
</tr>
<tr>
<td>AML</td>
<td>9 (25.00) 1 (2.78)</td>
<td>10 (27.78)</td>
<td>0.057</td>
</tr>
<tr>
<td>&lt; 5% n (%)</td>
<td>21 (58.33) 5 (13.89)</td>
<td>26 (72.22)</td>
<td></td>
</tr>
<tr>
<td>≥ 5% n (%)</td>
<td>30 (83.33) 6 (16.67)</td>
<td>36 (100.00)</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1 Representative flow cytometric dot-plot diagrams from a single patient. The percentage of blasts by flow cytometry in this case (Sample No. 5) was 17.23% as shown by the circled population of cells in the blast window. The manual blast count in this sample was only 1%.

Tablement is essential for the determination of treatment response such as CR or no CR and for subsequent selection of appropriate treatment intensities. Although enumeration of blasts using conventional morphology-based manual marrow cell counting has been in use for many decades, there are certain limitations to this method. The major limitation is its low sensitivity to detect a low or minimal level of leukemic cells since the total number of cells analyzed is usually in the range of only 200-500 cells. The ability to detect blast cells also depends on the experiences of the readers and some blasts may be overlooked leading to a falsely low percentage of blasts in the marrow. On the other hand, some regenerating marrow cells after chemotherapy may be overrated as leukemic cells, leading to a “no CR” diagnosis. Despite inaccuracies inherent in the manual differential counts and its labor-intensive process, this manual method is still used in the current classification scheme and therefore remains the gold standard for determining blast percentages.

Flow cytometric immunophenotyping has a well-established role in the diagnosis and classification of acute leukemia. Although flow cytometer has an advantage in its ability to rapidly assess a large quantity of marrow cells and their lineage-specific subpopulations, its role as a mandatory tool for monitor-
ing of the disease in acute leukemia is still controversial[11,13]. We considered flow cytometry as an attractive tool for more precise determination of the percentages of blasts as ongoing advances in flow cytometric instrumentation permits more reliable detection of rare populations that may represent approximately 0.1-1% of the whole population[9]. Moreover, the flow cytometer is capable of detecting aberrantly expressed antigens of leukemic blasts which could provide additional information enabling the definition of CR status with more confidence[15,17].

In this study, we evaluated whether flow cytometry could be of value in the determination of treatment response, i.e. CR or no CR, after induction and consolidation chemotherapy of patients with acute leukemia. CD45/SSC gates were used to select the blast gates and the percentages of blasts were determined by the instrument. Flow cytometric analysis yielded a higher percentage of blasts than the manual counts in the majority of cases. Several possible factors could be considered to explain these different results. These factors may be a consequence of the different principles of the two tests or from the operator-dependent factors. When using CD45/SSC gating strategy, the operator had to decide the proper location and size of the blast window. Sometimes, the blast populations could not be clearly separated from other nearby cell populations or their locations may overlap with normal cells. In regenerating marrow samples after chemotherapy, the selected blast population by CD45/SSC gates may possibly include regenerating immature normal precursor cells or hematogones[22]. Some AML blasts lacked expression of CD34 and CD117 and can be difficult to distinguish from more mature cells. For example, it may be difficult to distinguish CD34-negative monoblasts from more mature monocytes[23].

Despite the higher percentages of blasts frequently detected by flow cytometer than the manual counts, there was a good correlation between the results from the two methods from the overall 55 samples and in 36 AML samples. Interestingly, the correlation curve which displayed in a quadratic plot of the overall samples and AML samples showed a negative correlation when the manual counts were lower than 3%. However, a positive correlation was found beyond this cut-off point. This result could be considered in two different ways. First, when blast counts were lower than 3%, it was more difficult to identify the blast populations than when the blasts were present at higher percentages. The operator may have overdone the blast gates to increase the chance for positive events which may incidentally include other nearby normal events leading to increased blast counts. On the other hand, the high blast counts by flow cytometry could reflect the true presence of blasts that were not identifiable by “human eyes” using morphological criteria. When the manual counts were higher than 3%, both techniques seemed to correlate well. Addition of immunologic markers such as a combination of CD45/CD34/CD117 for back gating strategy may be useful to improve the accuracy of blast counts by flow cytometry[15,24]. Another alternative way to determine a low level of blasts in the bone marrow specimens is also available such as molecular analysis of leukemic blasts by PCR technique. The sensitivity of PCR to detect a single leukemic cell is comparable to flow cytometry (1 in 10^4 to 10^5)[10]. In cases with unique genetic mutations, utilization of both flow cytometry and PCR analysis has been suggested to enhance the sensitivity and specificity of MRD detection[11,21]. However, not all cases of acute leukemia have specific genetic abnormalities to allow the design of specific PCR primers for the study of the abnormal leukemic genes[25,26].

Conclusion

Flow cytometry has a much higher capacity to analyze a larger number of marrow cells than a morphology-based manual counting method. Although there was a good correlation between the percentages of blasts counted by flow cytometry and manual counting method, discordant results occurred when < 5% blasts were used as a cut-off point to determine CR. A flow cytometric CR is less frequently observed than morphological CR in the majority of acute leukemia cases. The discrepancy should be further investigated in order to increase the level of confidence in the patients’ CR status. For example, a combination of more rigid CD45/SSC gates and additional immunologic markers for back gating strategy should improve the accuracy of blast quantification by flow cytometry. Enumeration of marrow blasts by both methods is recommended to ensure a truly complete response to chemotherapy.

Acknowledgements

The authors wish to thank Dr. Yingyong Chinthammitr, Division of Hematology, Department of Medicine, Faculty of Medicine Siriraj Hospital for his kind help with statistical analysis and Miss Orathai Promsuwicha and Miss Wayuree Songmuang for her excellent technical assistance in flow cytometric analy-
CUA is the recipient of the Faculty Development Awards from Siriraj Chalermprakiat Fund and is the principal investigator of the Leukemia Project currently funded by Mahidol University (2000-2009). SW was a hematology resident who undertook this present study under the supervision of CUA with the research scholarship from the Thai Society of Hematology. The authors reported no potential conflicts of interest.

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การศึกษาเปรียบเทียบจำนวนร้อยละของเซลล์มะเร็งตัวอ่อนในไขกระดูกของผู้ป่วยมะเร็งเม็ดเลือดขาวชนิดเฉียบพลัน ด้วยเทคนิคโฟลซัยโตเมทรีและการประเมินทางสัณฐานวิทยาของเซลล์หลังผู้ป่วยได้รับการรักษาด้วยเคมีบำบัด

สมวงศ์ วงศ์พระจันทร์, จิรายุ เอื้อวรากุล

ภูมิหลัง: การประเมินจำนวนเซลล์มะเร็งตัวอ่อน (blast) ในไขกระดูกเป็นองค์ประกอบที่จำเป็นสำหรับการตรวจวิจัย และรักษาโรคมะเร็งเม็ดเลือดขาวชนิดเฉียบพลัน วิธีมาตรฐานในปัจจุบันในการนับจำนวนเซลล์มะเร็งตัวอ่อนด้วยกล้องจุลทรรศน์ โดยอาศัยการนับเซลล์ในกระจกหั้นหลอด 500 เซลล์ ซึ่งผลที่ได้มีความมั่นคงตามผู้อานวัดอุปสรรค์: เพื่อเปรียบเทียบระหว่างเทคนิคการนับเซลล์มะเร็งตัวอ่อนในไขกระดูกที่ได้จากการวินิจฉัยโดยเคมีบำบัด และวิธีวินิจฉัยโดยทางสัณฐานวิทยา วิสัยทัศน์และวิธีการ: เก็บตัวอย่างไขกระดูก 55 ตัวอย่าง จากผู้ป่วยมะเร็งเม็ดเลือดขาวชนิดเฉียบพลันจำนวน 38 ราย (AML 36 ราย และ ALL 19 ราย) หลังไขกระดูกฟื้นตัวจากการรักษาด้วยเคมีบำบัด ทั้งนี้ร้อยละเซลล์มะเร็งตัวอ่อนจะถูกนับด้วยกล้องจุลทรรศน์ หรือนับด้วยเครื่องโฟลซัยโตมิเตอร์ โดยอาศัยคุณสมบัติการแสดงออกของ CD45 และ side scatter

ผลการศึกษา: พบความสัมพันธ์ที่ดีในกลุ่มตัวอย่างโดยรวม (r = 0.829) และตัวอย่างตรวจ AML 36 ราย (r = 0.86) รายละเซลล์มะเร็งตัวอ่อนที่ได้จากการตรวจโฟลซัยโตมิเตอร์สูงกว่าจากกระบวนการนับตัวอย่างเฉลี่ยใน 46 ตัวอย่างตรวจ (คิดเป็นร้อยละ 83.6) ที่ใช้เกณฑ์อย่างกว้างระยะเวลา 5 ของเซลล์มะเร็งตัวอ่อนในการตัดสินระยะระยะinyin

สรุป: มีความสัมพันธ์ระหว่างผลของเซลล์มะเร็งตัวอ่อนจากทั้งสองวิธี โดยเฉพาะอย่างยิ่งในข้อมูลตรวจ AML แต่ไม่ในตรวจแบบเกณฑ์อย่างกว้างระยะระยะเวลา 5 เป็นเกณฑ์ในการตัดสินระยะระยะิน การใช้วิธีการนี้คงมีประโยชน์และควรนำมาใช้เพื่อให้มีการตัดสินระยะระยะินการรักษาอย่างแท้จริง ทั้งนี้ควรมีการศึกษาเพิ่มเติมโดยทำให้เกิดความมั่นคงโดยชัดเจนในการตัดสินว่าผู้ป่วยเข้าสู่ระยะสงบหรือไม่