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

Somwong Wongprajun MD*,
Chirayu U Auewarakul MD, PhD*

** Division of Hematology, Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand*

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*

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Acute leukemia is the second most common hematologic malignancy in the Thai population and is associated with great morbidity and mortality world-wide[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

Correspondence to: Auewarakul C, Division of Hematology, Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand. Phone: 0-2419-4418, Fax: 0-2411-2012. E-mail: sicaw@mahidol.ac.th

(PCR)[9-11].

Flow cytometric immunophenotyping has become an essential tool for leukemia diagnosis, classification and monitoring after treatment[11,12]. 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[13]. The presence of blasts can be detected in the blast window or blast gate generated from CD45 and side scatter (SSC) gating techniques[14]. 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[15,16].

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 cut-off 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[11,12].

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 1×10^9/L and a platelet count greater than 100×10^9/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[4,7]. 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[5].

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[17,18]. 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)[14,19]. 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[20].

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 6.5×10^9/L ($2.1\text{-}18.9 \times 10^9$/L) and the median platelet count of 288×10^9/L (111-1,690 $\times 10^9$/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

Variables	Median (range)	n (%)
Patients' characteristics: n = 38		
Age (years)	35 (16-70)	
Male gender		21 (55)
AML		27 (71)
ALL		11 (29)
Samples: n = 55		
Hemoglobin (g/dL)	11.6 (7.8-15.4)	
WBC (x10^9/L)	6.5 (2.1-18.9)	
Platelet (x10^9/L)	288 (111-1,690)	
AML		36 (65.4)
ALL		19 (34.5)

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 (r = 0.829) (p-value = 0.000) and AML (r = 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 cut-off 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[21]. 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

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

Table 3. Outcome analysis of flow cytometry and cytomorphology methods

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

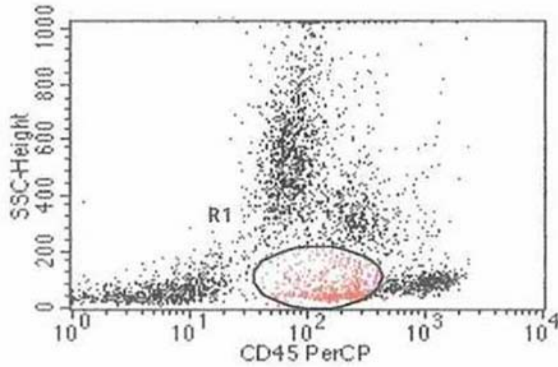

Fig. 1 Representative flow cytometric dot-plot diagrams from a single patient. The percentage of blasts by flow cytometry in this case (Sample No. 5) was 17.23% as shown by the circled population of cells in the blast window. The manual blast count in this sample was only 1%.

ment is essential for the determination of treatment response such as CR or no CR and for subsequent selection of appropriate treatment intensities[10,11]. 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[5].

Flow cytometric immunophenotyping has a well-established role in the diagnosis and classification of acute leukemia[13,19]. 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.

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การศึกษาเปรียบเทียบจำนวนร้อยละของเซลล์มะเร็งตัวอ่อนในไขกระดูกของผู้ป่วยมะเร็งเม็ดเลือดขาวชนิด เฉียบพลัน ด้วยเทคนิคโฟลซัยโตเมทรีและการประเมินทางสัณฐานวิทยาของเซลล์ หลังผู้ป่วยได้รับการรักษาด้วยเคมีบำบัด

สมวงศ์ วงศ์พระจันทร์, จิรายุ เอื้อวรากุล

ภูมิหลัง*: การประเมินจำนวนเซลล์มะเร็งตัวอ่อน (blast) ในไขกระดูกเป็นองค์ประกอบที่จำเป็นสำหรับการตรวจวินิจฉัยและรักษาโรคมะเร็งเม็ดเลือดขาวชนิดเฉียบพลัน วิธีมาตรฐานในปัจจุบันใช้การนับจำนวนเซลล์มะเร็งตัวอ่อนด้วยกล้องจุลทรรศน์ โดยอาศัยการนับเซลล์ไขกระดูกทั้งหมด 500 เซลล์ ซึ่งผลที่ได้อาจมีความผันแปรตามผู้อ่าน*

วัตถุประสงค์*: เพื่อเปรียบเทียบร้อยละของเซลล์มะเร็งตัวอ่อนในไขกระดูกที่ได้จากวิธีที่แตกต่างกันสองวิธี ได้แก่วิธีตรวจนับเซลล์จากการดูสัณฐานวิทยาของเซลล์มะเร็ง และวิธีโฟลซัยโตเมทรี*

วัสดุและวิธีการ*: เก็บตัวอย่างไขกระดูก 55 ตัวอย่าง จากผู้ป่วยโรคมะเร็งเม็ดเลือดขาวชนิดเฉียบพลันจำนวน 38 ราย (AML 36 ราย และ ALL 19 ราย) หลังไขกระดูกฟื้นตัวจากการรักษาด้วยเคมีบำบัด ทั้งนี้ร้อยละของเซลล์มะเร็งตัวอ่อนจะถูกนับด้วยกล้องจุลทรรศน์ หรือนับด้วยเครื่องโฟลซัยโตมิเตอร์โดยอาศัยคุณสมบัติการแสดงออกของ CD45 และ side scatter*

ผลการศึกษา*: พบความสัมพันธ์ที่ดีในกลุ่มตัวอย่างโดยรวม (r = 0.829) และตัวอย่างตรวจ AML 36 ราย (r = 0.86) ร้อยละของเซลล์มะเร็งตัวอ่อนที่ได้จากเครื่องโฟลซัยโตมิเตอร์สูงกว่าจากการนับด้วยกล้องจุลทรรศน์ใน 46 ตัวอย่างตรวจ (คิดเป็นร้อยละ 83.6) ถ้าใช้เกณฑ์น้อยกว่าร้อยละ 5 ของเซลล์มะเร็งตัวอ่อนในการตัดสินระยะสงบ พบว่า 48 ราย ถูกจัดว่าเข้าสู่ระยะสงบด้วยการประเมินทางสัณฐานวิทยา ซึ่งคิดเป็น ร้อยละ 87 (ร้อยละ 83 ใน AML และร้อยละ 95 ใน ALL) แต่จากการนับโดยเครื่องโฟลซัยโตมิเตอร์พบว่าเพียง 24 ราย (ร้อยละ 44) เท่านั้นที่อยู่ในระยะสงบ (ร้อยละ 28 ใน AML และร้อยละ 74 ใน ALL) ผลลัพธ์จากแต่ละวิธีมีความสัมพันธ์สอดคล้องในการพิจารณาระยะสงบของกลุ่มตัวอย่างจำนวน 27 ตัวอย่าง (เท่ากับร้อยละ 49 ของทั้งหมด) โดยมีค่า kappa เท่ากับ 0.07 สำหรับตัวอย่างโดยรวม 0.057 สำหรับ AML และ -0.096 สำหรับตัวอย่าง ALL*

สรุป*: มีความสัมพันธ์ระหว่างร้อยละของเซลล์มะเร็งตัวอ่อนจากทั้งสองวิธี โดยเฉพาะอย่างยิ่งในตัวอย่างตรวจ AML ผลที่ไม่สอดคล้องกันเกิดขึ้นเมื่อใช้จำนวนเซลล์มะเร็งตัวอ่อนน้อยกว่าร้อยละ 5 เป็นเกณฑ์ในการตัดสินระยะสงบ การใช้วิธีการทั้งสองรูปแบบมีประโยชน์ และควรใช้ร่วมกันเพื่อให้แน่ใจว่ามีการตอบสนองต่อการรักษาอย่างแท้จริง ทั้งนี้ควรมีการศึกษาเพิ่มเติมต่อไปเพื่อให้เกิดความมั่นใจยิ่งขึ้นในการตัดสินว่าผู้ป่วยเข้าสู่ระยะสงบหรือไม่*