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급성백혈병의 Lineage Assignment에 있어서 면역표현형 표지자의 적정 개수

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Defining an Optimal Number of Immunophenotypic Markers for Lineage Assignment of Acute Leukemias Based on the EGIL Scoring System

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Background : The lineage assignment in acute leukemias is critical in therapeutic decisions. Immunophenotyping by flow cytometry plays the main role in the lineage assignment; however, few studies have been done to determine the optimal set of markers. In this regard, we tried to find out the optimal first-line set of markers with a minimal compromise in its diagnostic sensitivity.

Materials and Methods : We retrospectively analyzed 321 cases of acute leukemias whose diagnoses were based on the EGIL (European Group for Immunological Classification of Acute Leukemia) scores. At our institution, flow cytometic analyses included 15 first-line markers and 4 additional second-line markers as needed, along with immunohistochemical stains. We performed simulational studies for the expected EGIL scores involving every possible combination of markers and analyzing the overall diagnostic sensitivities in each combination.

Results : The cytoplasmic antigens including MPO stain and CD79a stain contributed greatly to the lineage assignment. For a sensitivity over 95%, there needed a combination of MPO stain with other 5 flow markers (CD33, CD13, CD14, CD15 and CD117) for myeloid lineage; CD79a stain with 3 flow markers [CD19, CD10, and CD20 (or TdT)] for B-lymphoid lineage; and 4 flow markers (CD2, CD3, CD5, and CD7) for T-lymphoid lineage.

Conclusions : To maintain diagnostic sensitivities over 95% for each lineage, at least 14 markers (including MPO stain and CD79a stain) were needed; while 16 markers were needed for a sensitivity of 100%. When combined with other important markers for specific aims such as CD45, the minimum number of markers needed for the accurate diagnosis of acute leukemias would be more than about 18 to 20. *(Korean J Lab Med 2006;26:393-9)*

Key Words : *Acute leukemia; Immunophenotype; Optimal marker set; Simulation study*

INTRODUCTION

Acute leukemia is a heterogeneous group of diseases characterized by uncontrolled rapid proliferation of clonal hematopoietic progenitor cells[1]. This disease entity is subdivided into myeloid or lymphoid according to the cellular lineage of origin and differentiation[2, 3]. For each subtype of disease, different treatment protocols and patients' prognostic stratification are needed. In addition, since the disease course is acute and aggressive, the diagnoses should be established in a timely manner[4, 5]. Therefore, initial diagnostic workup is very critical in acute leukemia.

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However, the lineage assignment in acute leukemia is complicated in certain cases, which fall into the subtypes such as undifferentiated acute leukemia (UAL), biphenotypic acute leukemia (BAL) and bilineal acute leukemia according to the new WHO classification[3]. Moreover, there are a significant proportion of cases of AML or ALL with aberrant antigen expressions, which needs to be differentiated from BAL. In this regard, the European Group for Immunological Classification of Acute Leukemia (EGIL) has proposed a scoring system for assigning the cell lineage in acute leukemia[6, 7]. When the score for a lineage is greater than two points, the leukemic cells were considered to be committed to that lineage; when the scores for more than two different lineages are greater than two points, the diagnosis falls into BAL.

Immunophenotyping using flow cytometry has been adopted in many laboratories for lineage assignment of acute leukemias because it offers a powerful tool for precise definition of cell populations in bone marrow or peripheral blood specimens[4, 8]. However, it seems that including all the 26 markers in the EGIL scoring system for initial work-up would be too costly and labor-intensive. Furthermore, the cytoplasmic and nuclear antigens need a painstaking process of cell permeabilization so that the monoclonal antibodies can reach the intracellular antigens[9]. For this reason, most laboratory centers are concerned about how many and which markers should be included for the diagnosis of acute leukemias, but few studies have been done to resolve this issue[10, 11].

In this study, we tried to figure out the best and minimum possible combination of markers that are both cost-effective and sensitive. For this purpose, we retrospectively reviewed the cases of acute leukemias at our institution and analyzed the data by simulation studies.

MATERIALS AND METHODS

1. Subjects

We included a total of 321 patients who had been diagnosed as having acute leukemia based on the findings from flow cytometric analyses and immunohistochemical stains along with cytogenetic and molecular studies from April 2003 to January 2006 at our institution. The final diagnosis was established according to the WHO classification employing the EGIL scoring system for lineage assignment. The cases consisted of 191 AML (59.9%, including 4 cases of megakaryoblastic leukemia), 87 precursor B-cell acute lymphoblastic leukemia (B-ALL, 27.1%), 14 precursor T-cell acute lymphoblastic leukemia (T-ALL, 4.4%), 18 BAL (5.6%), 9 UAL (2.8%), and 2 bilineal acute leukemia (6.2%). The four acute megakaryoblastic leukemia cases with CD61 positive results whose EGIL score did not exceed 2 points were excluded in the simulation analysis; two bilineal cases were also excluded. Fourteen of the 18 BAL cases were myloid/B-lymphoid (M/B-BAL), 3 cases were myloid/T-lymphoid (M/T-BAL), and 1 case was B-lymphoid/T-lymphoid (B/T-BAL).

2. Flow cytometry

The immunophenotype was determined on bone marrow or peripheral blood cells by flow cytometry by the direct immunofluorescence method using monoclonal antibodies against cellular antigens; double labeling was carried out by using monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC) and phycoerythrin (PE). The analysis was performed on the FACSort flow cytometer (Becton-Dickinson, CA, USA), gating the blast population on CD45/side-scatter. A marker was considered positive if expressed in more than 20% of the gated cells in excess of negative control.

We used a first-line panel of markers, and added a second-line panel when the lineage was not clear; the majority of these cases were ALL with expression of myeloid antigens whose myeloid score were between 0.5 and 2 points. The first-line panel consisted of myeloperoxidase (MPO), CD13, CD33 and CD14 for myeloid lineage; CD10, CD19, CD20, CD2, CD5, CD7 and terminal deoxynucleotidyl transferase (TdT) for lymphoid lineage; and CD34, CD45, CD61 and HLA-DR. The second-line panel consisted of CD15, CD64, CD65 and CD117 when additional myeloid markers were needed and CD3, CD4, CD8, CD16/56 and cytoplasmic μ (cMu) when additional lymphoid markers were needed. All of these monoclonal antibodies were purchased from the Becton-Dickinson Company except for the Mouse Anti-human MPO/RPE antibody (DakoCytomation, Glostrup, Denmark) and the Anti-TdT-FITC antibody (GenTrak, Plymouth Meeting, PA). CD2, CD3, CD4, CD5, CD7, CD10, CD15, CD34, CD45, CD61, CD65 and TdT were FITC-labeled; and MPO, CD8, CD13, CD14, CD33,

CD19, CD20, CD64, CD16/56, CD117 and HLA-DR were PE-labeled. CD45 was labeled with PerCP.

3. Immunohistochemical stain

Routine immunohistochemical stains were done in the majority of cases: MPO (stMPO), CD3 (stCD3), CD20 (stCD20), TdT (stTdT) and CD79a (stCD79a) stains were performed on paraffin-embedded bone marrow section slides. Cytochemical stains for peroxidase, alpha-naphthyl butyrate esterase (ANBE), Sudan black B (SBB), and periodic acid-Schiff (PAS) were done by using reagents of the Sigma Chemical Company (St. Louis, MO, USA). Reagents and monoclonal antibodies of immunostains were purchased from DakoCytomation except for those of stCD3, which were purchased from Novocastra (Newcastle, UK). Noncontributory results of inadequate specimen qualities were excluded.

4. Simulation studies and data analyses

The cases of AML, M/T-BAL and M/B-BAL were defined as myeloid positive group (204 cases): B-ALL, M/B-BAL and B/T-BAL as B-lymphoid positive group (102 cases); and T-ALL, M/T-BAL and B/T-BAL as T-lymphoid positive group (18 cases). The positivity of individual markers acquired from the flow cytometric analyses and immunohistochemical stains in each case was recorded on the data sheet of the Microsoft Excel program. These data were analyzed by obtaining the expected EGIL score when each possible combination of markers was applied and by estimating the overall sensitivities of each combination; the sensitivity means the percentage of cases above 2 points according to the EGIL scoring system in each combination. The combination was classified according to the number of markers; two-marker combination, three-marker combination, and so on. We compared them and searched the best combination of markers that yielded the highest sensitivity for each given number of markers. Target sensitivities were set as high as 95% (in laboratory aspects) and 100% (in clinical aspects). All analyses were done by aid of the Macros and the Functions implemented in the Microsoft Excel program.

Table 1. The distribution of scores for each lineage according to the types of leukemias

Lineage	Score	AML	B-ALL	T-ALL	M/B-BAL
M score	0	-	32/87 (36.8%)	6/14 (42.9%)	-
	0.5-2	-	55/87 (63.2%)	8/14 (57.1%)	-
	2.5-4	119/187 (66.3%)	-	-	13/14 (92.9%)
	>4	63/187 (33.7%)	-	-	1/14 (7.1%)
B score	0	128/187 (68.4%)	-	9/14 (64.3%)*	-
	0.5-2	59/187 (31.5%)	-	5/14 (35.7%)*	-
	2.5-4	-	34/87 (39.1%)	-	10/14 (71.4%)
	>4	-	53/87 (60.9%)	-	4/14 (28.6%)
T score	0	147/187 (78.6%)*	80/87 (92.0%)*	-	14/14 (100%)*
	0.5-2	40/187 (21.4%)*	7/87 (8.0%)*	-	0
	2.5~4	-	-	3/14 (21.4%)	-
	>4	-	-	11/14 (78.6%)	-

* CD10 and TdT were excluded.

RESULTS

1. Distribution of scores

The distribution of scores for each lineage according to the types of acute leukemias was summarized in Table 1. The percentages of aberrant expression of B-lymphoid and T-lymphoid markers in AML cases were 31.5% and 21.4%, respectively; the expression of myeloid antigens in B-ALL and T-ALL was observed in 63.2% and 57.1%, respectively. CD10 and TdT, which are both B-lymphoid and T-lymphoid markers, were excluded in assessing the aberrant expression of T-lymphoid antigens in B-ALL, and vice versa (Table 1).

The concordance rates of flow cytometric markers and immunohistochemical stains are shown in Table 2. The concordance rate was relatively high between TdT and stTdT (95.9%). For MPO, immunohistochemical stain was more sensitive than flow cytometry. For CD3, CD20 and TdT, there were no significant differences in sensitivities between the two methods. For identifying the monocytoid lineage, ANBE was more sensitive than CD14, except for 4 cases that were CD14-positive but ANBE-negative.

2. Myeloid lineage

We evaluated the first-line markers and second-line markers separately; the second-line panel was assessed in 140 cases to establish the exact scores for myeloid lineage; among them, 66 cases (35.3% of AML cases) were diagnosed as AML and 11 (78.6% of M/B-BAL cases) were diagnosed as M/B-BAL.

Among the first-line markers, MPO was shown to be the most important marker for myeloid lineage, MPO stain being more sensitive than flow cytometric MPO (Table 3). CD33 and CD13 were the second most contributory markers, with similar significances. CD14 showed a minor contribution to lineage assignment of myeloid cells. The sensitivity increased gradually as the number of markers increased, and the full first-line flow cytometric markers (MPO, CD13, CD33 and CD14) could give a sensitivity of 77.0%, whereas the combination of MPO stain raised the sensitivity up to 86.9%.

Among the second-line markers, CD15 and CD117 moderately contributed to the improvement of sensitivity, followed by CD65. The maximum sensitivity could be achieved by adding these three markers. Sensitivity over 95% required the combination of MPO stain and other 5 flow cytometric markers (CD33, CD13, CD14, CD15 and CD117). MPO stain and flow cytometric MPO plus other 4 markers (CD33, CD13, CD14 and CD15) yielded a similar sensitivity (>95%). The former combination would be more suitable because CD117 can be assessed additionally and the detection of surface markers is technically easier than that of cytoplasmic markers. Even when all the flow cytometric markers were combined, the maximum sensitivity reached was 94.1%. The maximum sensitivity of 100% could be achieved by combining stMPO plus all available flow markers except for CD64; CD64 exhibited no contribution to the myeloid lineage assignment in this study.

Table 2. The concordance rate between flow cytometric markers and immunocytochemical stains

	+/+	+/-	-/+	-/-	NC	Concordance rate
MPO/stMPO	107	2	23	82	101	88.3%
CD3/stCD3	3	3	2	21	286	82.8%
CD20/stCD20	35	8	3	45	224	87.9%
TdT/stTdT	81	2	2	12	218	95.9%
CD14/ANBE	11	4	19	111	170	84.1%

Abbreviations: NC, not comparable; MPO, myeloperoxidase; stMPO, MPO stain; stCD3, CD3 stain; stCD20, CD20 stain; TdT, terminal deoxynucleotidyl transferase; stTdT, TdT stain; ANBE, alpha-naphthyl butyrate esterase.

Table 3. The best combinations and sensitivities for myeloid markers

Combination	Number of flow markers	Best combination	Positive	Sensitivity (%)
Flow marker only	2	MPO+CD33	152/204	74.5
	3	MPO+CD33+CD13(or CD14)	152/204	74.5
	4	MPO+CD33+CD13+CD14	157/204	77.0
	4+1	MPO+CD33+CD13+CD14+CD117	178/204	87.3
	4+2	MPO+CD33+CD13+CD14+CD117+CD15	187/204	91.7
	4+3	MPO+CD33+CD13+CD14+CD117+CD15+CD65	192/204	94.1
	4+4	MPO+CD33+CD13+CD14+CD117+CD15+CD65+CD64	192/204	94.1
With stMPO	1	stMPO+CD33	122/145	84.1
	2	stMPO+CD33+CD13(or CD14)	122/145	84.1
	3	stMPO+CD33+CD13+CD14	123/145	84.8
	3+1	stMPO+CD33+CD13+CD14+CD15	135/145	93.1
	3+2	stMPO+CD33+CD13+CD14+CD15+CD117	139/145	95.9
	3+3	stMPO+CD33+CD13+CD14+CD15+CD117+CD65	142/145	97.9
	3+4	stMPO+CD33+CD13+CD14+CD15+CD117+CD65+CD64	142/145	97.9
MPO with stMPO	2	stMPO+MPO+CD33	125/145	86.2
	3	stMPO+MPO+CD33+CD13(or CD14)	125/145	86.2
	4	stMPO+MPO+CD33+CD13+CD14	126/145	86.9
	4+1	stMPO+MPO+CD33+CD13+CD14+CD15	138/145	95.2
	4+2	stMPO+MPO+CD33+CD13+CD14+CD15+CD117	142/145	97.9
	4+3	stMPO+MPO+CD33+CD13+CD14+CD15+CD117+CD65	145/145	100
	4+4	stMPO+MPO+CD33+CD13+CD14+CD15+CD117+CD65+CD64	145/145	100

Abbreviations: See Table 2.

Table 4. The best combinations and sensitivities for B-lymphoid markers

Combination	Number of flow markers	Best combination	Positive	Sensitivity (%)
Flow marker only	3	CD19+CD10+TdT	74/102	72.5
	4	CD19+CD20+CD10+TdT	88/102	86.3
With stCD79a	1	stCD79a+CD19	73/78	93.6
	2	stCD79a+CD19+CD10 (or CD20)	73/78	93.6
		stCD79a+CD19(or CD10 or CD20)+TdT	73/78	93.6
	3	stCD79a+CD19+CD10+ CD20(or TdT)	77/78	98.7
	4	stCD79a+CD19+CD10+ CD20+TdT	78/78	100

Abbreviation: stCD79a, CD79a stain.

3. B-Lymphoid lineage

Total 102 cases were defined as B-lymphoid group, and CD79a was assessed in 78 cases only by immunohistochemical stains. When only three surface flow cytometric markers and TdT were combined, the maximum sensitivity did not reach 90% (Table 4). If CD79a stain was added, CD79a stain with only one flow cytometric marker yielded 93.6% of sensitivity, with CD19 being most contributory. For a sensitivity over 95%, at least 3 flow cytometric markers [CD19+CD10+CD20 (or TdT)] combined with stCD79a were needed; and 4 markers (CD19+CD10+CD20+TdT) with stCD79a give a sensitivity of 100%. Cytoplasmic μ was assessed in 9 cases, and 8 cases were also positive for CD79a stain. The remaining one case did not have CD79a stain result; instead, positive cMu yielded a B-lymphoid score above 2. Due to the limited number of cases, the value of cMu could not be fully assessed in this study. However, it seemed that more than one cytoplasmic antigen were needed to maintain a high sensitivity in the lineage assignment of B-lymphoid cells.

4. T-lymphoid lineage

Due to the small number of cases, we could not perform full simulation analyses. Among the first-line markers, markers assigned to the T-lymphoid lineage in the EGIL scoring system were CD2, CD5, CD7, CD10 and TdT; surface CD3 was assessed in 14 cases. The sensitivity did not exceed 90% even though all the 5 first-line markers were combined, but it increased up to 100% by adding CD3 (Table 5). When we analyzed only the 14 cases with CD3 data, CD3 was shown to be the most contributory marker, followed by CD7. We also performed the CD3 stain in 8 cases, but the CD3 stain did not contributed to the overall sensitivities no better than the flow cytometric analyses of surface CD3. The target sensitivity (>95 %) could be achieved by combining four flow cytometic markers, CD2, CD3, CD5, and CD7.

Table 5. The best combinations and sensitivities for T-lymphoid markers

Combination	Number of flow markers	Best combination	Positive	Sensitivity (%)
1st-line markers	3	CD2+CD5+CD7	13/18	72.2
	4	CD2+CD5+CD7+CD10	16/18	88.9
	5	CD2+CD5+CD7+CD10+TdT	16/18	88.9
	5+1	CD2+CD5+CD7+CD10+ TdT+CD3	18/18	100
1st-line markers with CD3	2	CD3+CD7	12/14	85.7
	3	CD3+CD7+CD2(or CD5 or CD10 or TdT)	12/14	85.7
		CD3+CD2+CD5(or TdT)	12/14	85.7
		CD3+CD10+TdT	12/14	85.7
	4	CD3+CD7+CD2+CD5	14/14	100
	5	CD3+CD7+CD2+CD5+ CD10(or TdT)	14/14	100
	6	CD3+CD7+CD2+CD5+ CD10+TdT	14/14	100

DISCUSSION

The role of immunophenotyping has four major aspects in the diagnosis of acute leukemias[11]: i) lineage assignment that is fundamental for therapeutic decisions, ii) detection of BAL, iii) application of current immunophenotypic classifications, and iv) determination of aberrant antigen expression patterns useful for the follow-up of patients and detection of minimal residual disease. This study was primarily focused on the first two aims. The target sensitivities were set as high as 95% and 100% because acute leukemia is very acute and aggressive in its nature, and thus accurate diagnosis is critical to the patients' survival.

For the myeloid lineage, we included all 8 markers in the EGIL scoring system. To achieve the target sensitivity over 95%, 6 markers (stMPO, CD33, CD13, CD14, CD15 and CD117) were needed in this study. The number of markers needed for the myeloid lineage was relatively larger when compared to that for B- or T-lymphoid lineages,

which is thought to be due to the presence of certain subsets of AML such as minimally differentiated AML that express a paucity of myeloid antigens. The differential diagnoses of these subtypes of AML need to be established by the observation of negative expression of the most of the myeloid antigens. Moreover, there were cases of AML whose EGIL score did not exceed 2 (for example, CD61 positive cases).

Among the B-lymphoid markers in the EGIL scoring system, CD22, CD24, and cytoplasmic IgM were not usually included, and CD79a was assessed only by immunohistochemical stain in this study. So we could present simulation data from limited combinations of markers on B-lymphoid lineage; however, very high sensitivities could be achieved, nonetheless. This was due to the presence of two highly lineage-specific markers (CD79a and CD19) for the B-lymphoid lineage; simply combining these two markers yielded a diagnostic sensitivity of 93.6%. Our results showed that the combination of 4 markers, stCD79a, CD19, CD10, and CD20 (or TdT) would be optimal. We did not perform a full-marker assessment for the T-lymphoid lineage, either; CD1a, anti-TCR and cytoplasmic CD3 (surface CD3 was optionally tested) were not included. Although the data analyses had some limitations due to the small number of cases, our results showed that the combination of 4 surface flow markers (CD2, CD3, CD5, and CD7) was acceptable. Both for B- and T-lymphoid lineages, further studies involving a larger number of cases and more markers including those not assessed in this study are needed to confirm our conclusions.

Some markers are important not for lineage assignment, but for other purposes. CD45 is a pan-leukocytic antigen that displays different patterns of expression in different subpopulations of normal leukocytes and malignant cells [12]. The step of primary gating for CD45 antigen expression along with side scatter (CD45/SSC) provides a clear discrimination of cells of interest (in this case, leukemic blasts), and thereby enables the determination of the immunophenotype specific to the leukemic cells minimizing the contamination of the innocent hematopoietic cells[11, 13]. Thus the CD45/SSC gating is considered to be an essential step in the flow cytometric analysis of acute leukemias. CD34 and/or HLA-DR are markers indicating immaturity, and these are useful for the diagnosis of "undifferentiated" stem-cell acute leukemia that expresses only these two markers. Furthermore, they can serve as useful follow-up markers because they may remain on blasts at later stages of maturation providing an abnormal pattern that can be a landmark for follow-up [11]. These three markers (CD45, CD34, and HLA-DR) were also considered to be essential by most experts from the Clinical Cytometry Society (CCS) in ISAC 2000 Congress[10].

Lastly, certain markers are critical for the determination of subtype of acute leukemias: CD41 or CD61 for the identification of acute megakaryoblastic leukemia; CD71 or glycophorin A for acute erythroid leukemia; and CD56 for acute leukemia of the natural killer (NK) cell lineage, for example[6, 12, 14]. These markers are needed based on other lines of evidence such as morphologic or clinical findings.

Collectively, the minimum number of markers for the lineage assignment to achieve a sensitivity over 95% was 14 (including 2 immunohistochemical stains), while 16 was needed for a sensitivity of 100%. Including other essential markers for other purposes than lineage assignment, at least 18 for a sensitivity of 95% and 20 for a sensitivity of 100% was required for appropriate diagnosis of acute leukemias in our marker panels. This conclusion is in accordance with the consensus from the CCS meeting after a questionnaire survey in a group of experts, which estimated 13-15 as the number of indispensable markers and 20-24 for the appropriate number of markers needed for complete diagnosis[10]. More studies on a large number of cases using more panels of markers would be required for the establishment of optimal number of markers, as well as the standardization of immunophenotyping techniques.

요 약

서론 : 급성백혈병에 있어서 계통의 지정(lineage assignment)은 치료의 방향을 결정하는 데 있어 필수적이다. 유세포분석검사는 이러한 계통의 지정에 있어서 주된 역할을 하고 있지만, 세포 표지자들의 적절한 개수와 종류에 관한 연구는 거의 없는 실정이다. 이에 저자들은 적절한 진단적 민감도(검사적인 측면에서 95%, 임상적인 측면에서 100%)를 유지할 수 있는 최소한의 세포 표지자들의 조합을 알아보고자 하였다.

방법 : EGIL (European Group for Immunological Classification of Acute Leukemia) scoring system을 바탕으로 본원에서 진단된 총 319명의 급성백혈병 환자들을 대상으로 하였다. 모든 환자들에 대하여 15개의 기본 표지자들과 함께 필요에 따라

4개의 추가 표지자들을 사용하여 유세포분석검사를 시행하였고, 면역조직화학염색 검사를 병행하였다. 가상으로 각각의 표지자들의 조합을 해본 후, 각각의 조합에 대해서 예측되는 EGIL 점수를 토대로 진단적 민감도를 산출해 보았다.

결과 : 세포질내 항원인 MPO 및 CD79a의 면역염색검사가 계통 지정에 있어서 가장 중요한 역할을 하였다. 95% 이상의 민감도를 유지하기 위해서, 골수계에서는 MPO 염색과 함께 5개의 유세포분석검사 표지자(CD33, CD13, CD14, CD15, CD117), B-림프구계에서는 CD79a 염색과 함께 3개의 유세포분석검사 표지자[CD19, CD10, CD20 (혹은 TdT)], T-림프구계에서는 4개의 유세포분석검사 표지자(CD2, CD3, CD5, CD7)가 필요하였다.

결론 : 각각의 계통에 대해서 95% 이상의 진단적 민감도를 유지하기 위해서는 최소한 14가지 이상, 그리고 100%의 민감도를 위해서는 16가지 이상의 표지자가 필요하였다. 이와 함께, CD45 등과 같이 다른 특수한 목적에 중요한 표지자들을 포함하면, 급성백혈병의 정확한 진단을 위해서는 최소한 18-20개 이상의 표지자가 필요하리라 판단된다.

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