# Immunophenotyping in the Diagnosis and Classification of Acute Leukemia: "Dharmais" Cancer Hospital Experience

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*ABSTRACT*

*Immunophenotyping is recommended in the diagnosis and classification of leukemia. In this report, we present data on the role of immunophenotyping in the diagnosis of acute leukemia in association with cytomorphological assessment and the cytochemistry stain Sudan Black B (SBB) in the Clinical Pathology Laboratory of Dharmais National Cancer Center Hospital (DNCH) from January 2005 until December 2007.*

*According to phenotype, we found 13 cases of T-lineage ALL, 82 cases of B-lineage ALL and 110 cases of myeloid lineage. Coexpression of antigens from other lineage was found in 38% of T-ALL, 28% of B- ALL, and 37% AML. The most frequently lymphoid antigen coexpressed in AML was CD19, followed by CD7.*

*CD33 was positive in 94.6% cases of AML, and CD13 in 87.8%. CD19 was positive in 96% B-ALL, CD10 in 68%, followed by CD20 (50%) and CD22 (33%). CD7 was positive in 92% T-ALL, whereas CD3 was positive in 85%.*

*This study confirmed that immunophenotyping was especially useful in determining the diagnosis of lineage, to distinguish between ALL and AML, especially in cases with negative SBB as in M0 and M5a, to differentiate between B-lineage ALL and T-ALL, and to diagnose cases of biphenotypic/ mixed lineage leukemia.*

***Keywords:*** *Acute leukemia, immunophenotyping, myeloid lineage, B-ALL, T-ALL*

ABSTRAK

Penentuan imunofenotipe merupakan salah satu pemeriksaan yang disyaratkan dalam diagnosis dan klasifikasi leukemia. Dalam tulisan ini, kami melaporkan data peranan penentuan imunofenotipe dalam diagnosis leukemia akut dikaitkan dengan pemeriksaan sitomorfologi dan pemeriksaan sitokimia Sudan Black B (SBB) yang dilakukan di Laboratorium Patologi Klinik Rumah Sakit Kanker "Dharmais" (RSKD) sejak Januari 2005 sampai Desember 2007.

Menurut imunofenotipe, kami menemukan 13 kasus leukemia limfoblastik akut (LLA) galur T, 82 kasus LLA galur B, dan 110 kasus leukemia mieloblastik akut (LMA). Ko-ekspresi antigen dari galur lain ditemukan pada 38% kasus LLA-T, 28% kasus LLA-B, dan 37% LMA. Antigen limfoid yang paling sering ditemukan ekspresinya pada LMA adalah CD19 diikuti CD7.

CD33 ditemukan pada 94,6% kasus LMA dan CD13 pada 87,8% kasus LMA. CD19 ditemukan pada 96% LLA-B, CD10 pada 68% kasus, diikuti CD20 (50%) dan CD22 (33%). CD7 ditemukan pada 92% LLA-T, dan CD3 positif pada 85% kasus LLA-T.

Disimpulkan bahwa penentuan imunofenotipe sangat bermanfaat dalam penentuan diagnosis galur, membedakan antara LLA dan LMA, khususnya pada kasus dengan SBB negatif seperti pada kasus LMA-M0 dan M5a, membedakan LLA-B dan LLA-T, serta mendiagnosis kasus leukemia bifenotipe/leukemia galur campuran.

**Kata kunci:** Leukemia akut, imunofenotipe, galur myeloid, LLA-B, LLA-T.

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## INTRODUCTION

Rapid and precise diagnosis of leukemia is critical so that appropriate treatment can be initiated without delay. The most widely accepted and applied classification of leukemias is based on morphologic and cytochemistry, proposed by the French-American British (FAB) group.[1] However, the fact that some patients showed better or worse outcome than others in

the same FAB sub-group, led to the search for cellular and molecular characteristics of the leukemic cells, which might better define the prognosis, helps in the choice of therapy and predict response to treatment.

Acute leukemia displays characteristic patterns of surface antigen expression (CD antigens), which facilitate their identification and proper classification and hence play an important role in instituting proper treatment plans.[2] The discovery of monoclonal antibodies (MoAbs) that define cell surface antigens have led to important insights into leukocyte differentiation and the cellular origin of leukemia. WHO included immunophenotype and cytogenetic in addition to cytomorphologic and cytochemistry in the recent WHO criteria for diagnosis and classification of leukemia. Nowadays, immunophenotyping for acute leukemia cases has become more important in the determination of the lineage of leukemia, and tends to become universal when facilities are readily available.[2,3]

Dharmais National Cancer Center Hospital (DNCH) is among one of the first centers in Indonesia to apply immunophenotyping by multiparameter flowcytometric analysis in the diagnosis and classification of leukemia. Although leukemia phenotyping in DNCH has been available since 1997, its popularity has just increased in recent years. In this report, we present our data on the application of immunophenotyping in the diagnosis and classification of acute leukemia in association with cytomorphologic and the cytochemistry stain Sudan Black B (SBB) in the Clinical Pathology Laboratory of DNCH, Jakarta, Indonesia, during January 2005 – December 2007.

## MATERIAL AND METHODS

This was a retrospective study. The specimens include bone marrow specimens submitted to the laboratory for cytomorphological assessment and bone marrow and peripheral blood specimens submitted for immunophenotypic analysis. Bone marrow and peripheral blood were anticoagulated with K3EDTA.

The subjects were divided into 2 groups according to age: children (age < 18 years) and adult (age ≥ 18 years).

The results of cytomorphological assessment, cytochemistry and immunophenotyping were compared and analyzed descriptively. We used Wright stain for cytomorphological assessment, and Sudan Black B for cytochemistry. The criteria for acute leukemia was blast cell 20% or greater in bone marrow.[4]

Immunophenotyping was performed on FACS Calibur flowcytometer, with myeloid panel (CD33, CD13, CD14), lymphoid panel (CD3, CD5, CD7, CD10, CD19, CD20, CD22) and CD34 and HLA-DR, while CD45 was used as pan-leucocyte marker. If necessary, k and l light chain was added. All markers are surface markers. The reagents were manufactured by Becton Dickinson (BD). CD2 was applied in 2005 until January 2006.

Analysis of the immunophenotype was done using Cell Quest software from BD.

## RESULT

During January 2005 until December 2007, there were total 1346 requests for cytomorphological assessment of bone marrow, of which 499 cases were acute leukemia. Cases of acute leukemia included 300 newly diagnosed cases, 185 evaluation of therapy and 14 relapse cases. Of the 300 new cases, 92 were children, 191 were adults and there were 10 subjects whose age was not recorded.

Classification by cytomorphology and cytochemistry according to French American British (FAB) criteria were as follow: in children group 72 (78.3%) out of 92 suffered from acute lymphoblastic leukemia (ALL) and 20 (21.7%) had acute myeloblastic leukemia (AML). ALL-L1 subtype was the most frequently found (52 children, 72%), followed by ALL-L2 (15 children, 27%). AML-M5a subtype was the most frequent AML subtype in children (6 cases, 30%).

In adult group, AML was more frequently found (151 cases, 76.3%) compared to ALL (47 cases, 23.7%). The most frequent subtype was AML-M2 (44 cases, 29.1%), followed by M4 (28 cases, 18.5%), M5a (26 cases, 17.2%), M1 (25 cases, 16.6%), M3 (14 cases, 9.3%), M5b (8 cases, 5.3%) and M6 (4 cases, 2.6%) (Table 1).

**Table 1: New cases of acute leukemia according to age group and FAB subtype in Clinical Pathology Department of DNCH during January 2005 – December 2007**

| Type | Children | | Adult | | Age unknown | |
|---|---|---|---|---|---|---|
| ***AML*** | | | | | | |
| M0 | 2 | 10% | 2 | 1.3% | 0 | 0% |
| M1 | 4 | 20% | 25 | 16.6% | 2 | 40% |
| M2 | 4 | 20% | 44 | 29.1% | 0 | 0% |
| M3 | 0 | 0% | 14 | 9.3% | 1 | 20% |
| M4 | 2 | 10% | 28 | 18.5% | 1 | 20% |
| M5a | 6 | 30% | 26 | 17.2% | 1 | 20% |
| M5b | 0 | 0% | 8 | 5.3% | 0 | 0% |
| M6 | 2 | 10% | 4 | 2.6% | 0 | 0% |
| M7 | 0 | 0% | 0 | 0.0% | 0 | 0% |
| Subtotal | 20 | | 151 | | 5 | |
| % | 21.7% | | 76.3% | | 50.0% | |
| ***ALL*** | | | | | | |
| L1 | 52 | 72% | 27 | 57.4% | 5 | 100% |
| L2 | 19 | 27% | 20 | 42.6% | 0 | 0% |
| L3 | 1 | 1% | 0 | 0.0% | 0 | 0% |
| Subtotal | 72 | | 47 | | 5 | |
| % | 78.3% | | 23.7% | | 50.0% | |
| **TOTAL** | 92 | | 198 | | 10 | |

We also found 4 cases of AML-M0 which was morphologically indistinguishable from ALL-L2 and was SBB negative, in which the diagnosis was based on immunophenotyping.

Out of 406 requests for immunophenotyping, 205 was leukemia cases. The specimens submitted for immunophenotypic analysis comprised of bone marrow aspirates (182 cases, 88.8%), peripheral blood (22 cases, 10.7%) and lymph node juice (1 case, 0.5%).

According to phenotype, we found 13 cases of T-lineage ALL, 82 cases of B-lineage ALL and 110 cases of myeloid lineage. Three (23%) of T-lineage ALL cases showed coexpression of one of the B-lineage antigen, i.e. CD10 (2 cases) and CD19 (1 case), while 2 (15%) cases showed coexpression of myeloid antigen (CD33 or CD13). Coexpression of antigens from other lineage was also found in 23 cases (28%) of B-lineage ALL, i.e. the myeloid antigen(s) CD33 (10 cases), CD13 (5 cases), CD 33 and CD13 (6 cases), and T-lineage antigen CD5 (1 case). One case of B-lineage ALL showed coexpression of T-lineage antigen (CD7) and myeloid lineage (CD13).

Lymphoid antigens were more frequently coexpressed in myeloid lineage leukemia (41 cases, 37%). The most frequently lymphoid antigen coexpressed was CD19 (21 cases), followed by CD7 (9 cases). One case of AML expressed CD10 and CD19, and 2 cases showed coexpression of CD7 and CD19.

**Table 2: Immunophenotypic profile of new acute leukemia cases in Clinical Pathology Laboratory of DNCH during 2005-2007**

| T-lineage | 8 | Myeloid lineage | 69 |
|---|---|---|---|
| with co-exp CD10 | 2 | With co-exp CD19 | 21 |
| with co-exp CD19 | 1 | With co-exp CD19 + CD10 | 1 |
| with co-exp CD33 | 1 | With co-exp CD20 | 1 |
| with co-exp CD13 | 1 | With co-exp CD20 + CD22 | 1 |
| TOTAL T-LINEAGE | 13 | With co-exp CD7 | 9 |
| B-lineage | 59 | With co-exp CD7 + CD19 | 2 |
| with co-exp CD33 | 10 | With co-exp CD14 | 6 |
| with co-exp CD13 | 5 | TOTAL MYELOID | 110 |
| with co-exp CD33 + CD13 | 6 | | |
| with co-exp CD5 | 1 | | |
| with co-exp CD7 + CD13 | 1 | | |
| TOTAL B-LINEAGE | 82 | | |

There were several cases in which FAB classification did not match or the diagnosis cannot be established without immunophenotype analysis, as in 4 cases of AML-M0 found in this study. There were 2 cases with negative SBB, but proven to be AML by immunophenotyping. Two other cases were classified as M5a according to their morphology, but showed B-lineage immunophenotype.

**Table 3: Positive expression rates of lineage-associated and non-lineage specific antigens in acute leukemia cases in Clinical Pathology Laboratory of DNCH during 2005-2007**

| Type of leukemia | Antigen | % positive |
|---|---|---|
| *AML* | CD13 | 87.8% |
| | CD33 | 94.6% |
| | CD14 | 5.5% |
| | CD34 | 42.9% |
| | HLA-DR | 66.0% |
| *B-ALL* | CD10 | 68% |
| | CD19 | 96% |
| | CD20 | 50% |
| | CD22 | 33% |
| | CD34 | 56% |
| | HLA-DR | 74% |
| *T-ALL* | CD2* | 60% |
| | CD3 | 85% |
| | CD5 | 54% |
| | CD7 | 92% |
| | CD34 | 15% |
| | HLA-DR | 31% |

* CD2 was positive in 3 out of 5 T-ALL cases diagnosed during 2005 – January 2006

Evaluation of the usefulness of lineage-specific and non-lineage specific markers based on positive expression rate was summarized in Table 3.

## DISCUSSION

In the recent WHO classification, lineage determination and maturation degree is very critical. In the evaluation of acute leukemia, the first thing to do is to distinguish between myeloblastic, lymphoblastic leukemia and myelodisplastic syndrome (MDS), because this will be critical to the choice of therapy. In many cases, cytomorphology and cytochemical stain are sufficient for diagnosis of acute leukemia. Unfortunately some cases can not be diagnosed only by those two modalities, especially if the leukemic cells are negative for SBB or myeloperoxidase (MPO) stain, or showed less than 3% positivity with MPO, such as cases of poorly differentiated myeloid leukemia (M0-AML), megakaryoblastic leukemia (M7-AML) and in some cases of monoblastic leukemia (M5a-AML), and those with primitive erythroid cells as the predominant leukemic cells. In such cases, immunophenotyping is essential.[5]

The most important alteration to the FAB classification by the WHO group, aside from the definition of blast count as 20% or greater for the diagnosis of acute leukemia, is the difference in the classification of

lymphoid neoplasms.[6] The WHO group assigned lymphoid neoplasms into three major categories, related to their lineage or specific subset, i.e. B-cell neoplasms, T-cell and NK cell neoplasms, and Hodgkin lymphoma.[4]

In ALL cases, identification of the immunophenotype has become a major part of diagnosis, including diagnosis of lineage, stage of maturation and specific subset, such as in cases of NK cell leukemia. Three groups can be distinguished: pre–B-cell ALL, mature B-cell ALL, and T-cell ALL. Pre–B-cell ALL blasts are positive for TdT, HLA-dr antigens, CD19, and CD79a. Different stages of maturation have been defined as pro–B-cell ALL, common ALL (cALL), and pre–B-cell ALL. Whereas pre-pre–B-cell ALL blasts are positive for CD19, CD79a, or CD22 but no other B-cell differentiation antigens, common ALL (early pre–B-cell ALL) is characterized by expression of CD10 (common ALL antigen), and pre–B-cell ALL by expression of cytoplasmic immunoglobulins with or without CD10. Mature B-cell ALL blasts are positive for surface immunoglobulins (sIg, usually IgM), are clonal for κ or λ light chains, and are negative for TdT. Similar to B-cell lineage ALL, T-cell ALL can be stratified further into subtypes based on different stages of intrathymic differentiation. Surface CD3 is the most lineage-specific marker for T-cell differentiation and is typically positive in mature T-cell ALL. Mature T-cell ALL is also positive for either CD4 or CD8 but not for both. Blasts in pre–T-cell ALL are negative for surface CD3 but may still express cytoplasmic CD3. Pre–T-cell ALL is negative for both CD4 and CD8.[6]

In this study, we used a panel of 15 antibodies, including the primary panel recommended by the US-Canadian Consensus Group for the Diagnosis and Classification of Acute Leukemia,[3] plus CD3 for T-lineage and CD20 and CD22 for B-lineage.

In this study, we found that the frequency of B-ALL was 86.3%, with the remaining 13.7% being T-ALL. This finding was not different from the report of Khawaja, et al.[7] who reported frequency of T-ALL of 17.22%. The frequency of B-ALL in this study was slightly higher than the average frequency from the literature, which was approximately 80%.[8, 9, 10] CD56 as marker for NK cell was not included in the panel, thus diagnosis for NK cell leukemia might have been missed.

Coexpression of myeloid markers on ALL leukemic cells was found in 23% of all ALL cases. Initial studies showed that expression of myeloid antigen in childhood ALL might be a predictor of poor response to chemotherapy, but this had not been the case in adult ALL.[8, 11]

Immunophenotyping is not only helpful for diagnosis but is of independent significance for prognosis, and may

**Table 4: Panels of monoclonal (or polyclonal) antibodies recommended by the British Committee for Standards in Haematology (BCSH) and by the US-Canadian Consensus Group for the Diagnosis and Classification of Acute Leukemia[3]**

| | | *BCSH* | *Consensus group* |
|---|---|---|---|
| Primary panel | B lymphoid | CD19, cCD22, cCD79a, CD10 | CD10, CD19, anti-kappa, anti -lambda |
| | T lymphoid | cCD3, CD2 | CD2, CD5, CD7 |
| | Myeloid | CD13, CD117, anti-cMPO | CD13, CD14, CD33 |
| | Not lineage specific | Nuclear TdT | CD34, HLA-DR |
| Secondary panel | B lymphoid | μ, SmIg (anti-kappa & anti-lambda),CD138 | CD20, Sm/cCD22 |
| | T lymphoid | CD7 | CD1a, Sm/cCD3, CD4, CD8 |
| | Myeloid | CD33, CD41, CD42, CD61, anti-glycophorin A | CD15, CD16, CD41, CD42b, CD61, CD64, CD71, CD117, anti-cMPO, anti-glycophorin A |
| | Not lineage specific | CD45 | CD38, nuclear TdT |
| | Non-hemopoietic | MAb for the detection of small round cell tumors of childhood | |
| Optional | B lymphoid | CD15 (a myeloid marker often expressed on *MLL*-rearranged B lymphoblasts) and 7.1/NG2 (also for *MLL* -rearranged ALL) | |
| | T lymphoid | Anti-TCRαβ, anti-TCRγδ | |
| | Myeloid | Anti-lysozyme, CD14, CD36, anti-PML (MAb PL1-M3), HLA-DR (for negativity in M3 AML) | |

C, cytoplasmic; CD, cluster of differentiation, MAb, monoclonal antibody; MPO, myeloperoxidase; Sm, surface membrane; TCR, T-cell receptor; TdT, terminal deoxynucleotidyl transferase.

be useful for risk stratification in AML patients.[12] Leukemic cells in AML had been shown to be very heterogenous in immunophenotype. AML blast cells (10-45%) often express lymphoid antigens, with CD2, CD7 and CD19 as the most frequently found markers.[11] The co-expression of lymphoid antigen in AML might have a prognostic value. CD19 expression was found in up to 34% cases of de novo AML. M2 subtype with cytogenetic abnormality of t(8;21) is also associated with coexpression of CD19. Thalhammer-Scherre et al reported that 22.4% AML coexpressed lymphoid antigens, with the most commonly expressed antigens being CD56 and CD7.[13] In AML cases in this study, coexpression of lymphoid antigen were found in 34% of AML cases; the most frequent lymphoid antigen expressed being CD19 and CD7. Coexpression of lymphoid antigens in AML was lower in our studies than reported in other studies, probably because of the difference in antibody panel.[14,15]

Slobinas and Matuzeviene proposed coexpression of CD7 and CD34 in AML as an independent prognostic factor in adult AML; coexpression of CD7 and CD34 was associated with significantly poorer complete remission rate, disease-free survival and overall survival.[16] Chang et al reported that coexpression of CD34 and HLA-DR in AML was associated with lower rate of complete remission.[17] The antigen CD34 is usually found on hematopoietic stem cell. Repp et al also reported that CD34 negative AML was associated with higher complete remission rate.[12] In this study we found 42.9% AML cases positive for CD34, and 66.0% positive for HLA-DR.

In this study we found 4 cases of minimally differentiated AML (AML-M0), which were morphologically similar to ALL-L2, with negative SBB. There were also 2 cases of AML-M5a with negative SBB. Even some cases of ALL maybe misdiagnosed as AML if based only on morphology and cytochemistry. Although most cases of leukemia can be diagnosed accurately based on morphology, immunophenotyping is important, especially in cases with negative SBB and ALL cases.[3]

Application of immunophenotyping has uncovered cases in which leukemia cases display characteristics of both myeloid and lymphoid cells. In such instances, a single neoplastic cell may coexpress features of distinct lineages (biphenotypic) or two distinct subpopulations of leukemic cells may express either myeloid or lymphoid features separately (bilineal).[18] In this study, 8 cases showed coexpression of both myeloid (CD13+, CD33+) and B-lineage (CD10+, CD19+ or CD20+, CD22+). Yet, those cases were not diagnosed as biphenotypic leukemia, because we had not applied all the markers necessary to fulfil the diagnostic criteria for biphenotypic leukemia according to the EGIL criteria for the diagnosis of biphenotypic leukemia (Table 5).

**Table 5: Scoring system for the diagnosis of biphenotypic acute leukemias*[5]**

| Score | B-lymphoid | T-lymphoid | Myeloid |
|---|---|---|---|
| 2 | CD79a | CD3 | MPO** |
| | cCD22 | anti-TCR$\alpha/\beta$ | |
| | cIgM | anti-TCR$\gamma/\delta$ | |
| 1 | CD19 | CD2 | CD117 |
| | CD20 | CD5 | CD13 |
| | CD10 | CD8 | CD33 |
| | | CD10 | CD65 |
| 0.5 | TdT | TdT | CD14 |
| | CD24 | CD7 | CD15 |
| | | CD1a | CD64 |

* Biphenotypic acute leukemia is defined when scores for the myeloid and one of the lymphoid lineages are >2 points.

** Demonstrated by the MoAB anti-MPO or cytochemistry. Each marker scores the corresponding point.

CD33 was the most useful marker for diagnosis of AML, with positive expression in 94.6% cases, followed by CD13 (87.8%). The positive rate was similar to those reported by Khalidi et. al., but higher than the results reported in other studies.[14,19,20] CD19 was positive in 96% B-ALL, CD10 in 68%, followed by CD20 (50%) and CD22 (33%). Our result was similar to those reported by Choi et. al.[20]

CD7 was positive in 92% T-ALL, whereas CD3 was positive in 85%. Aside from cytoplasmic CD3, CD5 and CD7 were the most sensitive antigens in cases of T-ALL.[2]

The other potential role for immunophenotyping in acute leukemia is for the detection of minimal residual disease (Table 7).[5] Detection of minimal residual disease has prognostic value in both ALL and AML, particularly the evaluation of early treatment response, as it allows identification of true low risk and high risk patients.[21] Morphology, cytogenetics and fluorescence in situ hybridization (FISH) are relatively insensitive techniques to detect residual disease. By using a large panel of monoclonal antibodies, an 'aberrant' immunophenotype at diagnosis can be found in 85% of cases. This will be overexpression of an antigen, coexpression of antigens normally associated with different stages of maturation which does not occur in novel hemopoiesis, absence of myeloid antigen expression or expression of non-myeloid antigens. This had been showed in some series that detection of cells with these phenotypes at a sensitivity of 1 in $10^4$ or 1 in $10^5$ is possible and can be predictive of relapse. That is, the persistent expression of the aberrant phenotype found at diagnosis is associated with a higher relapse risk.[22]

**Table 7: Current and possible future role of immunophenotyping in the diagnosis and management of hematological neoplasms[5]**

| Established role of major clinical significance | Potential role |
|---|---|
| Diagnosis of ALL, M0 AML, M6 AML, M7 AML and biphenotypic acute leukemias | Identification of poor prognosis subtypes of acute leukemias, e.g. |
| Demonstration of clonality in suspected B-cell lymphoproliferative disorders | ALL with 11q23 rearrangement |
| Differential diagnosis of B and T lineage lymphoproliferative disorders and recognition of specific subtypes, e.g. hairy cell leukemia | Detection of minimal residual disease in acute leukemias and lymphoproliferative disorders Identification of M3 and |
| Quantification of stem cells in peripheral blood and bone marrow harvest | M3 variant AML |

## CONCLUSION

This was a retrospective study to evaluate the phenotype of acute leukemia cases submitted to DNCH Clinical Pathology Laboratory from January 2005 until December 2007. This study confirmed that immunophenotyping was especially useful in determining the diagnosis of lineage, to distinguish between ALL and AML, especially in cases with negative SBB as in M0 and M5a, to differentiate between B-lineage ALL and T-ALL, and to diagnose cases of biphenotypic/mixed lineage leukemia.

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