Immunophenotypic Patterns of Childhood Acute Leukemias in Indonesia

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Abstract

Background: Immunophenotyping, as suggested by WHO, may improve diagnosis of childhood leukemia since it offers a better classification of the hematopoietic lineage of malignant cells as compared to morphology. Therefore, we aimed to determine the proportion of the immunophenotypic subtypes of acute leukemia in Indonesian children. Methods. Samples were obtained from patients (0-14 years of age) in 4 hospitals in Indonesia. We analyzed 541 suspected leukemia samples presented over a 4-year period (March 2006 - July 2010) by flow cytometry. Immunophenotyping allowed classification into acute myeloid leukemia (AML) and ALL (B-lineage and T-lineage ALL). Results. Of 541 samples, 136 were tested using a single color method and 405 with a three-color method. Concordance with morphology was very good (κ=0.82) using the three-color method with a panel of 15 monoclonal antibodies (n=387). A relatively high percentage of acute leukemia was classified as AML (23%). Of the ALL samples 83% were B-lineage ALL and 17% T-lineage ALL. Nine out of 239 morphological ALL were labeled AML, and 12/79 morphological AML were in fact ALL. Conclusion. Immunophenotyping in a multi-center study proved feasible and appears particularly important for prognostic assessment of childhood leukemia in low income countries such as Indonesia.

Key words: Childhood leukemia - AML - ALL - immunophenotyping - Indonesia
It is particularly important to resolve an equivocal diagnosis of ALL versus AML, and to determine the lineage of lymphoblasts along the lines of B-lineage-ALL and T-lineage ALL maturation. The latter is important for stratification into Standard Risk (SR) and High Risk (HR) ALL, as well as for determining mature B-ALL, which needs a different treatment schedule. In addition, a specific immunophenotype identified at diagnosis might be useful for evaluating microscopic residual disease by flow cytometry during therapy. Conventional immunophenotyping studies are performed on blood or bone marrow samples by flow cytometry, using an array of monoclonal antibodies to identify cell surface and cytoplasmic antigens. Published guidelines for compositions of monoclonal antibody panels for use with flow cytometry panels in the evaluations of acute leukemias are available (LeBien et al., 1981; Braylan et al., 2001; Kebriaei et al., 2002).

The most common B-lineage ALL is the B lineage phenotype positive for the following: B cell markers CD19, CD22, TdT, cytoplasmic CD79a, CD34 and CD10. B-lineage ALL has been sub-classified according to maturation stage into: early pre B (pro-B), pre-B, transitional (or late) pre-B and (mature) B-ALL (Behm and Campana, 1999; Pui et al., 2002). In different regions, various incidences of B-lineage ALL have been reported (Table 1). Burkitt’s leukemia displays an immunophenotype consisting of mature B cells (Pui et al., 1990b). T-lineage ALL can also be categorized into phenotypic subgroups, correlating to differentiation stages of thymic T cells. T cell markers are cytoplasmic CD3 and CD7 plus CD2 or CD5. This lineage can be further subdivided into early, mid or late thymocyte differentiation (Matutes et al., 1997; Van Dongen, 2003).

The World Health Organization (WHO) classification divides ALL into two main groups only, i.e., B-lineage and T-lineage ALL, without further categorization. To our knowledge, there is no published data on immunological phenotyping of childhood acute leukemias in Indonesia, where stratification for treatment protocols until recently is based solely on clinical criteria and morphology of lymphoblasts. Our study, the first of its kind reported from Indonesia, was designed to investigate the immunophenotyping profiles of childhood acute leukemia and to determine the feasibility of incorporating the results in further defining risk groups for development of treatment protocols.

### Materials and Methods

#### Origin of samples

Samples of blood and bone marrow aspirates from 541 patients, aged 0-14 years, suspected of leukemia between March 2006 until July 2010 were received from Pediatric Cancer Units (PCU) of four Indonesian teaching hospitals: Dr. Sardjito Hospital, Yogyakarta and Kariadi Hospital, Semarang, both are in Central Java, Adam Malik Hospital (Medan, North Sumatra) and Prof. Kandou General Hospital (Manado, North Sulawesi). The laboratory is in Yogyakarta thus samples from Dr. Sardjito hospital, Yogyakarta, were analyzed fresh, and from the other PCU’s samples were sent by airmail and
analyzed within 24 hours.

Single color vs. three-color method

Single color monoclonal antibody (MoAb) panel were used during the period March 2006 till November 2007. One hundred and thirty six samples (118 from Yogyakarta, 18 from Semarang) were analyzed with this single color method and a limited set of 9 monoclonal antibodies (Table 2 old panel). In November 2007, a three-color method was introduced and an expanded set of 15 monoclonal antibodies was used (Table 2 new panel). From December 2007 till the end of this study, immunological phenotyping was performed using the new panel. The PCU’s from Manado and Medan joined in this study from July 2008.

Samples and preparation

Bone marrow and/or blood samples (2-5 ml) were collected in heparinized tubes. Samples from Yogyakarta were analyzed immediately, or stored at 4°C and processed within 24 hours. From outside Yogyakarta transport was in a cool box to maintain an ambient temperature between 10-25°C, and analysis was within 24 hours. A cell bank was set up with cryopreserved cells in liquid nitrogen, and DNA was stored at -80°C.

Immunophenotyping

Monoclonal antibodies (IgG1, CD2, CD7, CD10, CD13, CD19, CD22, CD33, CD34 and CD45) and PBS were added to 35 µl of cell suspension to reach a total volume of 50 µl in polystyrene tubes, washed twice and re-suspended in 300-500 µl of PBS. The suspensions were then incubated in the dark for 15 minutes at room temperature. Following incubation, 16.6 µl of paraformaldehyde (4%) was added to the cell suspensions and incubated for 4 minutes at room temperature, in the dark. One ml of lysing solution was added for 10 minutes at room temperature, also in the dark. Cells were then centrifuged at 1500 rpm for 5 minutes, and supernatant was discharged. For cytoplasmic staining: (cIgG1, cCD79a, cCD3, cMPO and cTdT) cell suspensions were incubated for 15 minutes at room temperature, then washed twice and re-suspended in 2 ml PBS containing 1% FCS. Cells were then centrifuged at 1500 rpm for 5 minutes. The supernatant was discharged, and 250 µl PBS was added, and the tube were wrapped with aluminium foil. Origin of the antibodies used is shown in Table 2. Samples were analyzed in a Becton Dickinson FACS-Calibur flowcytometer. Data were analyzed on Cell Quest program (Becton Dickinson and Company Asia Pacific Division, Singapore). A positive signal was recorded if 20% or more of the cells reacted with the given monoclonal antibody. IgG1 was used as negative control. Samples that gave a positive signal on < 20% cells were recorded as ‘low expression’.

Definition of immunological phenotypes

The phenotype of a case was characterized according to its reactivity with the monoclonal antibodies listed in Table 2. In a case where a sample was positive for more than one lineage, we used a scoring system adapted from The European Group for the Immunological Classification of Leukemias (EGIL). According to this scoring system, a case is considered biphenotypic when the score from two separate lineages is greater than 2. Most leukemia cases can easily be classified into lymphoid or myeloid lineages by light microscopy and flow cytometry studies. Some cases remain difficult to classify, due to co-expression of myeloid and lymphoid antigens. These cases have been designated biphenotypic for mixed AML/ALL or mixed-lineage for acute leukemia having both B- and T-cell characteristics on one cell. Bilineage is defined as mixed lineage acute leukemia expressing different lineage characteristics in two separate cell populations in the same sample (Williams et al., 1997; Yeoh et al., 2002; Kobayashi et al., 2004).

Quality control

All dot-plot data from the beginning of the study until August 2008 (136 old panel and 126 new panel) were re-analyzed at the center of expertise for flowcytometry at the Department of Hematology of the Cancer Center Amsterdam-VU-University Medical Center. Seven of our frozen samples were randomly selected and tested in this laboratory. The results of samples testing are similar, except, during a certain time period, we found 2 incorrect results for cytoplasmic staining, because of different buffers used for cell lysis during that time. However, this finding did not alter our study conclusions.

Results

Samples from 541 patients aged 0-14 years were classified based on immunophenotyping. The flowchart of specific acute leukemia type classifications is depicted in Figure 1 and is based on both the old immunophenotype and new panels.

Figure 1. Classification of Childhood Acute Leukemia Samples in This Study. Susp Leukemia, suspected leukemia
samples were B-lineage ALL, 2 samples were mature B and 64 (17%) were T-lineage ALL. We found mature-B cell phenotype in 2 cases (0.2% of all samples). Among the patients with leukemia, 417 patients had complete data of both morphology and immunophenotyping. In Table 5, the results of morphology and immunophenotyping are cross-tabulated. Kappa score increased from 0.43 (moderate) in the old panel to 0.82 (very good) in the new panel. With the old panel, from morphological ALL cases (n=86), 5 (5.8%) were categorized as AML by immunophenotyping. From 13 AML cases by morphology, 7 (54%) were categorized as ALL by immunophenotyping. With the new panel, from 239 morphology defined ALL cases, 9 (3.8%) samples were categorized as AML by immunophenotyping. From 79 AML patients as determined by morphology, 12 (15%) were categorized as ALL (9 B-lineage and 3 T-lineage ALL) by immunophenotyping.

Peak age for ALL was 2-4 years of age, with a lower second peak around 10 years of age (Figure 2). For AML the first peak was at 3 years of age, while there was no clear second peak around 10-12 years of age. The lower number of patients in the ages of 13 and 14 were probably caused by selective referring some patients to adult wards.

**Discussion**

Besides clinical, morphological, cytochemical and cytogenetic analyses, immunophenotyping has become an essential diagnostic tool for classification, prognosis, patient management and for disease monitoring of acute leukemias (Garand and Robillard, 1996; Rubnitz and Pui, 1997; Van Dongen, 2003; Davis et al., 2007). Reports on the incidence of childhood leukemia by immunophenotyping have been published with various results for the relative frequency of AML and ALL as well as for B- and T-lineage ALL (Table 1).

In our PCU, immunophenotyping on suspected leukemia patients started since March 2006, besides morphological and cytochemical methods. Of the 541 samples immunophenotyped, 497 patients were acute leukemias in which boys were slightly more frequent.

### Table 4. Patient Characteristics Including Immunophenotyping

<table>
<thead>
<tr>
<th>Variable</th>
<th>Old panel</th>
<th>New panel</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>66 (48.5)</td>
<td>244 (60.2)</td>
<td>310 (57.3)</td>
</tr>
<tr>
<td>Female</td>
<td>70 (51.5)</td>
<td>161 (39.8)</td>
<td>231 (42.7)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 1 year</td>
<td>2 (1.5)</td>
<td>12 (3)</td>
<td>14 (2.6)</td>
</tr>
<tr>
<td>1 – 4 years</td>
<td>58 (42.6)</td>
<td>164 (40.5)</td>
<td>222 (41)</td>
</tr>
<tr>
<td>5 – 9 years</td>
<td>37 (27.2)</td>
<td>125 (30.9)</td>
<td>162 (29.9)</td>
</tr>
<tr>
<td>10-14 years</td>
<td>32 (23.5)</td>
<td>99 (24.4)</td>
<td>131 (24.2)</td>
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<tr>
<td>Missing data</td>
<td>7 (5.2)</td>
<td>5 (1.2)</td>
<td>12 (2.2)</td>
</tr>
<tr>
<td>Morphology</td>
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<td></td>
<td></td>
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<tr>
<td>ALL</td>
<td>97 (71.3)</td>
<td>243 (60)</td>
<td>340 (62.8)</td>
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<td>AML</td>
<td>20 (14.7)</td>
<td>83 (20.5)</td>
<td>103 (19)</td>
</tr>
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<td>CML</td>
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<td>2 (0.5)</td>
<td>4 (0.7)</td>
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<tr>
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<td>21 (3.9)</td>
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<tr>
<td>Missing data</td>
<td>14 (10.3)</td>
<td>59 (14.6)</td>
<td>73 (13.5)</td>
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<td>Immunophenotyping</td>
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<tr>
<td>B-lineage ALL</td>
<td>85 (62.5)</td>
<td>230 (56.8)</td>
<td>315 (58.2)</td>
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<tr>
<td>ALL</td>
<td>1 (0.7)</td>
<td>1 (0.2)</td>
<td>2 (0.4)</td>
</tr>
<tr>
<td>T-lineage ALL</td>
<td>5 (3.6)</td>
<td>13 (3.2)</td>
<td>18 (3.2)</td>
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<tr>
<td>AML</td>
<td>16 (11.8)</td>
<td>100 (24.7)</td>
<td>116 (21.4)</td>
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<tr>
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<td>1 (0.2)</td>
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<tr>
<td>Unknown</td>
<td>25 (18.4)</td>
<td>18 (4.4)</td>
<td>43 (7.9)</td>
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</table>

**Table 5. Concordance between Immunophenotyping and Morphology**

<table>
<thead>
<tr>
<th>Morphology</th>
<th>ALL</th>
<th>AML</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunophenotyping</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>(Old Panel)</td>
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<td></td>
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<tr>
<td>ALL</td>
<td>81</td>
<td>7</td>
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<tr>
<td>AML</td>
<td>5</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>Total</td>
<td>86</td>
<td>13</td>
<td>9</td>
</tr>
<tr>
<td>Immunophenotyping</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(New Panel)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALL</td>
<td>230</td>
<td>12</td>
<td>242</td>
</tr>
<tr>
<td>AML</td>
<td>9</td>
<td>67</td>
<td>76</td>
</tr>
<tr>
<td>Total</td>
<td>239</td>
<td>79</td>
<td>318</td>
</tr>
</tbody>
</table>

ALL, acute lymphoblastic leukemia; AML, acute myeloblastic leukemia; CML, chronic myelocytic leukemia
This result was similar to what we have found previously (Supriyadi et al.) and similar with data for other developed countries (Pui et al., 1993). The proportion of AML was relatively high compared to western countries. Presumably it is not because AML incidence is high, but as an effect of a lower incidence of ALL in Indonesia (Supriyadi et al., 2011).

The classification into B- and T-lineage ALL is important for risk stratification and therapy of the patients (Van Dongen, 2003). The proportion of B- and T-lineage ALL shows large differences among countries (Table 1). In our series, the relative proportion of B-lineage ALL was 83%, and T-lineage ALL was 17%, the latter is slightly higher than in most developed countries.

From the beginning of this study until November 2007, a single color method with a set of 9 monoclonal antibodies panel was used to immunophenotype 136 patient samples. The discordance of a diagnosis of AML with this method seemed far too high. Therefore, a new method was set up, by using a three-color method and introducing a set of 15 antibodies. We expanded the old panel by adding antibodies directed against cytoplasmic antigens (Table 2). Cytoplasmic CD3 was introduced, this is considered to be a highly specific marker for T-lineage ALL and cytoplasmic MPO for myeloid lineage cells. Cytoplasmic CD22 and CD79a have been identified as highly specific markers for B-lineages (Garand and Robillard, 1996), although CD79a has been found to be positive in 10% of T-Cell ALL cases too (Sperling et al., 1997). The new panel resulted in less samples (4%) being labeled as ‘non conclusive’, compared to 18% in the old panel. In addition, there were less samples (1%) that showed ‘low marker expression’ as compared to the old panel (14.7%). In conclusion, the three-color method was much better compared to one color method for classification of leukemias.

Khalil et al. (1995) reported concordance between immunophenotyping with morphology and cytochemistry at 97% in King Faisal specialist hospital. They concluded that immunophenotyping by flow cytomtery was a useful and reliable method for classification of acute leukemia (Baersch et al., 1999; Uckan et al., 2000). In this study, we also evaluated the concordance between morphology and immunophenotyping methods and found a good kappa score of 0.82 for the new method. A sizeable number of patients (21/318 = 6.6%) were shifted from ALL to AML protocols or vice versa.

CD117 is more specific for identifying myeloid lineage as compared to CD13 and CD33. An extensive study of about 2000 samples from acute leukemia patients reported CD117 was expressed in over two third of cases of AML, regardless of the FAB subtypes, while less than 5% of cases of ALL were CD117 positive. The specificity of CD117 approximates that of anti-MPO. EGIL has also emphasized the value of CD117 in the recognition of (apparently) undifferentiated acute leukemias. CD117 is also an important myeloid marker for the definition of biphenotypic leukemias. It is recommended to use this marker for routine immunophenotypic analysis of acute leukemias (Kamps et al., 2010). CD117 has also been reported to be positive in approximately 50-60% biphenotypic case (Taskov et al., 1995; Garand and Robillard, 1996; McNally et al., 2002; Moricke et al., 2009; Kamps et al., 2010). In Western countries biphenotypic leukemia is reported to be approximately 3% while we only found 1 case out of the 498 patients. Biphenotypic is considered when the score from two separate lineages is greater than 2. To further improve our monoclonal antibody panel the third generation set of antibody panels additionally uses CD117. One patient in our study was diagnosed as biphenotypic leukemia and this patient expressed CD10, CD19, CD33, CD34, CD79a and MPO. This patient has myeloid score of 3 and lymphoid score was 4 (Table III).

The incidence of ALL is relatively low in Indonesia (Supriyadi et al., 2011), while the incidence of AML is similar to incidences reported from western countries. Therefore, in our study we found that the relatively high percentage of AML patients (23%). Biphenotypic acute leukemia and mature-B ALL were lower compared to Western countries. In conclusion, diagnosis of childhood acute leukemia has been improved in Indonesia by including immunophenotyping in the diagnosis, which was formerly based on the morphology only. Hence, improvement of prognosis is anticipated due to the right risk classification of children and subsequent selection of the most optimal treatment protocols.

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References


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Van Dongen JJ (2003). Immunophenotyping of hematopoietic malignancies (Rotterdam, Departement of Immunology, Erasmus University Medical Center).