RESEARCH COMMUNICATION

Immunophenotypic Patterns of Childhood Acute Leukemias in Indonesia

Eddy Supriyadi[1*], Pudjo H Widjajanto[1], Anjo JP Veerman[1,2], Ignatius Purwanto[1], Yetty M Nency[4], Stefanus Gunawan[5], Selvi Nafianti[6], Dewajani Purnomosari[7], Umi S Intansari[8], Guus Westra[3], Sutaryo[1], Jacqueline Cloos[2,3]

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

Asian Pacific J Cancer Prev, **12**, 3381-3387

Introduction

The diagnosis of acute leukemia is based on morphological and cytochemical investigations of bone marrow samples and/or peripheral blood smears. The French-American-British (FAB) group established criteria of acute leukemia based on morphologic characteristics of the malignant clones. It defines three subtypes of acute lymphoblastic leukemia (ALL): L1, L2 and L3 and 8 subtypes of acute myeloblastic leukemia AML (Lilleyman et al., 1986; Pui et al., 1993; 2008; Bhatia et al., 1999; McNally et al., 2002).

Refinement in classification of acute leukemias is accomplished by immunophenotyping. Differences in expression of surface membrane antigens or cytoplasmic components are used to identify and classify lymphoproliferative disorders by cell of origin and stage of differentiation (Behm and Campana, 1999; Pui et al., 2008; Van Dongen, 2003). This technique improves both accuracy and reproducibility of acute leukemia classification. It is considered particularly useful for identifying acute myeloid leukemia (AML) with lymphoid marker expression and, conversely, for ALL with myeloid marker expressions. Immunological studies of leukemic blasts are essential for identifying biphenotypic and bilineage leukemias. Approximately 6% of ALL cases express two or more myeloid markers. This subtype of acute leukemia is known to respond well to intensive therapy and should therefore be treated according to appropriate risk-based protocols (Pui et al., 1990a; Huh and Ibrahim, 2000). Immunophenotypic analysis is essential for accurately determining the lineage of the malignant clone of leukemic blasts besides the light microscopic diagnosis of childhood leukemia

*[1]Pediatric Hematology Oncology Division, Department of Pediatrics, Dr Sardjito Hospital, Medical Faculty, Universitas Gadjah Mada, Yogyakarta, Indonesia, [2]Pediatric Oncology/Hematology Division, Department of Pediatrics, [3]Department of Hematology, VU University Medical Center, Amsterdam, The Netherlands, [4]Pediatric Hematology Oncology, Kariadi Hospital, Medical Faculty, Diponegoro University, Semarang, [5]Pediatric Hematology Oncology, Prof Kandou Hospital, Medical Faculty, Sam Ratulangi University, [6]Pediatric Hematology Oncology, Adam Malik Hospital, Medical Faculty, University of Sumatera Utara, Medan, [7]Department of Histology and Cell Biology, [8]Department of Clinical Pathology, Medical Faculty, Universitas Gadjah Mada, Yogyakarta, Indonesia *For correspondence: e.supriyadi@yahoo.co.id*

Table 1. Reported Incidence of B- and T-lineage ALL in Various Countries

	B-lineage ALL (%)	T-lineage ALL (%)
Europe		
The Netherlands (Kamps et al., 2010)	88	12
United Kingdom (McNally et al., 2002)	87	13
Germany (Moricke et al., 2009)	87	13
Bulgaria(Taskov et al., 1995)	70	30
North America (Pui et al., 2009)	84	16
Central America (Howard et al., 2005)	90	10
South America		
Chile (Cabrera, 1996)	90	10
Brazil (Rego et al., 1996)	71	29
Australia (Milne et al., 2009)	90	10
Africa		
Morocco (Dakka et al., 2007)	63	37
Egypt (Kamel et al., 1989)	49	51
Asia		
Malaysia-Singapore (Ariffin et al., 2007)	93	7
Thailand (Tiensiwakul et al., 1999)	82	18
Hong-Kong (Shing et al., 1999)	82	18
Saudi Arabia (Roberts et al., 1990)	88	12
Indonesia (this study)	83	17

Table 2. Monoclonal Antibody Panel Used

	Old panel			New panel		
	MoAb	Label	Company	MoAb	Label	Company
B lineage	CD10	FITC	Dako	CD10	PE	Dako
	CD19	FITC	Dako	CD19	PE	Becton Dickinson
	CD22	FITC	Sanguin	CD22	FITC	Sanguin
	IgM	FITC	Becton Dickinson	cCD79a	PE	Beckman Coulter
T lineage	CD2	FITC	Dako	CD2	FITC	Dako
	CD7	FITC	Becton Dickinson	cCD3	FITC	Becton Dickinson
				CD7	PE	Becton Dickinson
Myeloid Lineage	CD33	FITC	Dako	CD13	FITC	Dako
				CD33	PE	Becton Dickinson
				cMPO	PE	Dako
Non Lineage	TdT	FITC	Dako	CD34	FITC	Becton Dickinson
	IgG1	FITC	Becton Dickinson	CD45	PerCP	Becton Dickinson
				cIgG1	FITC	Becton Dickinson
				cIgG1	PE	Becton Dickinson
				cTdT	FITC	Dako

MoAb, monoclonal antibody; FITC, fluorescein isothiocyanate; IgG1, Immunoglobulin G1; PBS, phosphate buffered saline; PE, phycoerythrin; PerCP, peridinin-chlorophyll proteins; TdT, terminal deoxynucleotidyl transferase

(Behm and Campana, 1999; Margolin et al., 2002). It is in particular 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 flowcytometry during therapy. Conventional immunophenotyping studies are performed on blood or bone marrow samples by flowcytometry, using an array of monoclonal antibodies to identify cell surface and cytoplasmic antigens. Published guidelines for compositions of monoclonal antibody panels for use with flowcytometry 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 alumunium foil. Origin of the antibodies used in 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. (Table 3). 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).

Table 3. Scoring System for Definition Acute Biphenotypic Leukemia Adapted from EGIL

Scoring	B-lymphoid	T-lymphoid	Myeloid
2	cCD79a, CD22, cIgM	cCD3	cMyelo Peroxidase
1	CD19, CD10	CD2, CD5	CD33, CD13
0.5	TdT	TdT, CD7	CD11b, CD11c CD14, CD15

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

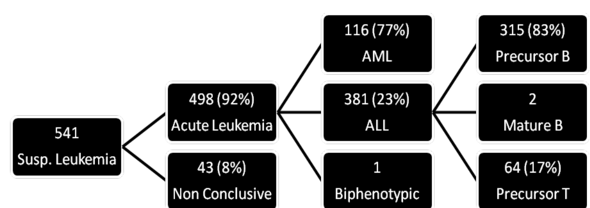

Figure 1. Classification of Childhood Acute Leukemia Samples in This Study. Susp Leukemia, suspected leukemia

Table 4. Patient Characteristics Including Immunophenotyping

Variable	Old panel		New panel		Total	
Sex						
Male	66	(48.5)	244	(60.2)	310	(57.3)
Female	70	(51.5)	161	(39.8)	231	(42.7)
Age						
< 1 year	2	(1.5)	12	(3)	14	(2.6)
1 – 4 years	58	(42.6)	164	(40.5)	222	(41)
5 – 9 years	37	(27.2)	125	(30.9)	162	(29.9)
10-14 years	32	(23.5)	99	(24.4)	131	(24.2)
Missing data	7	(5.2)	5	(1.2)	12	(2.2)
Morphology						
ALL	97	(71.3)	243	(60)	340	(62.8)
AML	20	(14.7)	83	(20.5)	103	(19)
CML	2	(1.5)	2	(0.5)	4	(0.7)
Other	3	(2.2)	18	(4.4)	21	(3.9)
Missing data	14	(10.3)	59	(14.6)	73	(13.5)
Immunophenotyping						
B-lineage ALL	85	-62.5)	230	(56.8)	315	(58.2)
Mature B	1	(0.7)	1	(0.2)	2	(0.4)
T-lineage ALL	9	(6.6)	55	(13.6)	64	(11.8)
AML	16	(11.8)	100	(24.7)	116	(21.4)
Biphenotype	0	(0)	1	(0.2)	1	(0.2)
Unknown	25	(18.4)	18	(4.4)	43	(7.9)

ALL, acute lymphoblastic leukemia; AML, acute myeloblastic leukemia; CML, chronic myelocytic leukemia

Table 5. Concordance between Immunophenotyping and Morphology

		Morphology		
		ALL	AML	Total
Immunophenotyping (Old Panel)	ALL	81	7	88
	AML	5	6	11
	Total	86	13	9
Immunophenotyping (New Panel)	ALL	230	12	242
	AML	9	67	76
	Total	239	79	318

ALL, acute lymphoblastic leukemia; AML, acute myeloblastic leukemia

panel (limited markers and one color panel) and the new panel (three color panel). Forty-three samples were non conclusive. Most were from the old panel 25/136 vs. 18/405 in the new panel (Table 4). Fifteen samples that were received and proved to be non-leukemic were clinically diagnosed as follows: 6 patients with Aplastic anemia, 3 patients with neuroblastoma, 3 samples were normal bone marrow, 1 Non-Hodgkin's Lymphoma patient, 1 Idiopatic Thrombocytopenic Purpura patient, and 1 Burkitt's lymphoma patient. In these samples, a variety of antigen expressions were detected. One patient was documented as a biphenotypic leukemia according EGIL score (Table 3). Four patients with CML showed low expression of CD22 and or MPO.

The old panel gave higher numbers of samples lacking marker expression compared to the new panel, 20 (14.7%) vs. 4 (1%) patients. From the 498 patients with acute leukemia, AML was 116 (23%) and ALL was 381 (77%) (Figure1). Among ALL samples, 315 (83%)

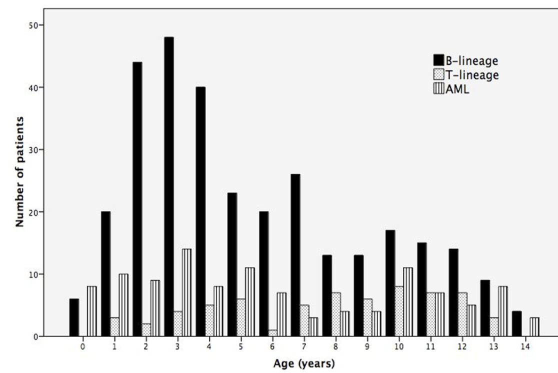

Figure 2. Age Distribution of the Different Subtypes of Childhood Acute Leukemia Diagnosed by Immunophenotyping

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.

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 cytometry 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.

Acknowledgement

This study is supported by Dutch Cancer Society Grant (IN-2006). We thank the Estella Fonds for funding some of chemotherapy in this study. None of the authors has any commercial or other conflicts of interest with regard to this work.

References

Ariffin H, Chen SP, Kwok CS, et al (2007). Ethnic differences in the frequency of subtypes of childhood acute lymphoblastic leukemia: results of the Malaysia-Singapore Leukemia Study Group. *J Pediatr Hematol Oncol*, **29**, 27-31.

Baersch G, Baumann M, Ritter J, et al (1999). Expression of AC133 and CD117 on candidate normal stem cell populations in childhood B-cell precursor acute lymphoblastic leukaemia. *Br J Haematol*, **107**, 572-80.

Behm F, Campana D (1999). Immunophenotyping. In Acute Leukemias, CH Pui, ed. Cambridge University Press), pp. 111-35.

Bhatia S, Ross JA, Greaves MF, et al (1999). Epidemiology and etiology. In Childhood Leukemias, C.H. Pui, ed. Cambridge, Cambridge University Press), pp. 38-49.

Braylan RC, Orfao A, Borowitz MJ, et al (2001). Optimal number of reagents required to evaluate hematolymphoid neoplasias: results of an international consensus meeting. *Cytometry*, **46**, 23-7.

Cabrera MEL, Ugarte S, Matutes E, et al (1996). Clinical and laboratory features as acute lymphoblastic lekemia in Chile. Study of 500 children and 131 adults. *Rev Med Chil,* **124**, 7.

Coebergh JW, van Steensel-Moll HA, Van Wering ER, et al (1985). Epidemiological and immunological characteristics of childhood leukaemia in The Netherlands: population-based data from a nationwide co-operative group of paediatricians. *Leuk Res,* **9**, 683-8.

Dakka N, Bellaoui H, Khattab M, et al (2007). Immunologic profile and outcome of childhood acute lymphoblastic leukemia (ALL) in Morocco. *J Pediatr Hematol Oncol,* **29**, 574-80.

Davis BH, Holden JT, Bene MC, et al (2007). 2006 Bethesda International Consensus recommendations on the flow cytometric immunophenotypic analysis of hematolymphoid neoplasia: medical indications. *Cytometry B Clin Cytom*, **72,** Suppl 1, S5-13.

Dunphy CH (1998). Combining morphology and flow cytometric immunophenotyping to evaluate bone marrow specimens for B-cell malignant neoplasms. *Am J Clin Pathol,* **109**, 625-30.

Garand R, Robillard N (1996). Immunophenotypic characterization of acute leukemias and chronic lymphoproliferative disorders: practical recommendations and classifications. *Hematol Cell Ther,* **38**, 471-86.

Han X, Bueso-Ramos CE (2007). Precursor T-cell acute lymphoblastic leukemia/lymphoblastic lymphoma and acute biphenotypic leukemias. *Am J Clin Pathol,* **127**, 528-44.

Hjalgrim, L.L., Rostgaard, K., Schmiegelow, K., et al (2003). Age- and sex-specific incidence of childhood leukemia by immunophenotype in the Nordic countries. *J Natl Cancer Inst,* **95**, 1539-44.

Howard, S.C., Campana, D., Coustan-Smith, E., et al (2005). Development of a regional flow cytometry center for diagnosis of childhood leukemia in Central America. *Leukemia,* **19**, 323-25.

Huh, Y.O., and Ibrahim, S. (2000). Immunophenotypes in adult acute lymphocytic leukemia. Role of flow cytometry in diagnosis and monitoring of disease. *Hematol Oncol Clin North Am,* **14**, 1251-65.

Kamel AM, Assem MM, Jaffe ES, et al (1989). Immunological phenotypic pattern of acute lymphoblastic leukaemia in Egypt. *Leuk Res,* **13**, 519-25.

Kamps WA, van der Pal-de Bruin KM, Veerman AJ, (2010). Long-term results of Dutch Childhood Oncology Group studies for children with acute lymphoblastic leukemia from 1984 to 2004. *Leukemia,* **24**, 309-19.

Kebriaei P, Anastasi J, Larson RA (2002). Acute lymphoblastic leukaemia: diagnosis and classification. *Best Pract Res Clin Haematol,* **15**, 597-621.

Khalil SH, Jackson JM, Pyle RH, et al (1995). Immunophenotyping of acute leukemia at King Faisal Specialist Hospital and Research Centre. *Ann Saudi Med,* **15**, 137-9.

Kobayashi N, Matsuda K, Sakashita K, et al (2004). Bilineage acute leukemia of T-lymphoid and myeloid lineages. *Haematologica,* **89**, 1139-41.

LeBien TW, McKenna RW, Abramson CS, et al (1981). Use of monoclonal antibodies, morphology, and cytochemistry to probe the cellular heterogeneity of acute leukemia and lymphoma. *Cancer Res,* **41**, 4776-80.

Lilleyman JS, Hann IM, Stevens RF. et al (1986). French American British (FAB) morphological classification of childhood lymphoblastic leukaemia and its clinical importance. *J Clin Pathol*, **39**, 998-1002.

Ludwig WD, Harbott J, Bartram CR, et.al (1993). Incidence and prognostic significance of immunophenotypic subgroups in childhood acute lymphoblastic leukemia: experience of the BFM study 86. *Recent Results Cancer Res,* **131**, 269-82.

Margolin JF, Steuber CP, Poplack DG (2002). Acute lymphoblastic leukemia. In Principles and practice of pediatric oncology, P.A. Pizzo, and D.G. Poplack, eds. (Philadelphia, Lippincott, Williams & Wilkins), pp. 1605-1616.

Matutes E, Morilla R, Farahat N, et al (1997). Definition of acute biphenotypic leukemia. *Haematologica,* **82**, 64-66.

McNally RJ, Alexander FE, Birch JM (2002). Space-time clustering analyses of childhood acute lymphoblastic leukaemia by immunophenotype. *Br J Cancer,* **87**, 513-5.

Milne E, Royle JA, de Klerk NH, et al (2009). Fetal growth and risk of childhood acute lymphoblastic leukemia: results from an Australian case-control study. *Am J Epidemiol,* **170**, 221-8.

Moricke A, Zimmermann M, Reiter A, et al (2009). Long-term results of five consecutive trials in childhood acute lymphoblastic leukemia performed by the ALL-BFM study group from 1981 to 2000. *Leukemia,* **24**, 265-84.

Neame, P.B., Soamboonsrup, P., Browman, G.P., et al (1986). Classifying acute leukemia by immunophenotyping: a combined FAB-immunologic classification of AML. *Blood,* **68**, 1355-62.

Perea G, Altes A, Bellido M, et al (2004). Clinical utility of bone marrow flow cytometry in B-cell non-Hodgkin lymphomas (B-NHL). *Histopathology*, **45**, 268-74.

Pui CH, Behm FG, Crist WM (1993). Clinical and biologic relevance of immunologic marker studies in childhood acute lymphoblastic leukemia. *Blood,* **82**, 343-62.

Pui CH, Behm FG, Singh B, et al (1990a). Myeloid-associated antigen expression lacks prognostic value in childhood acute lymphoblastic leukemia treated with intensive multiagent chemotherapy. *Blood*, **75**, 198-202.

Pui CH, Behm FG, Singh B, et al (1990b). Heterogeneity of presenting features and their relation to treatment outcome in 120 children with T-cell acute lymphoblastic leukemia. *Blood*, **75**, 174-9.

Pui CH, Pei D, Sandlund JT, et al (2009). Long-term results of St Jude Total Therapy Studies 11, 12, 13A, 13B, and 14 for childhood acute lymphoblastic leukemia. *Leukemia,* **24**, 371-382.

Pui CH, Relling MV, Campana D, et al (2002). Childhood acute lymphoblastic leukemia. *Rev Clin Exp Hematol,* **6**, 161-80.

Pui CH, Robison LL, Look AT (2008). Acute lymphoblastic leukaemia. *Lancet*, **371**, 1030-43.

Rego EM, Garcia AB, Viana SR , et al (1996). Characterization of acute lymphoblastic leukemia subtypes in Brazilian patients. *Leuk Res,* **20**, 349-55.

Roberts GT, Aur RJ, Sheth KV (1990). Immunophenotypic and age patterns of childhood acute lymphoblastic leukemia in Saudi Arabia. *Leuk Res*, **14**, 667-72.

Rubnitz JE, Pui CH (1997). Childhood acute lymphoblastic leukemia. *Oncologist,* **2**, 374-80.

Shing MM, Li CK, Chik KW, et al (1999). Outcomes and prognostic factors of Chinese children with acute lymphoblastic leukemia in Hong Kong: preliminary results. *Med Pediatr Oncol*, **32**, 117-23.

Sobol RE, Bloomfield CD, Royston I (1988).

Immunophenotyping in the diagnosis and classification of acute lymphoblastic leukemia. *Clin Lab Med,* **8**, 151-162.

Sperling C, Schwartz S, Buchner T, et al (1997). Expression of the stem cell factor receptor C-KIT (CD117) in acute leukemias. *Haematologica*, **82**, 617-21.

Supriyadi E, Widjajanto PH, Purwanto I, et al (2011) Incidence of childhood leukemia in Yogyakarta, Indonesia, 1998 - 2009. *Pediatr Blood Cancer,* **57**, 588-93.

Taskov H, Dimitrova E, Serbinova M, et al (1995). Immunological subtypes of childhood acute lymphoblastic leukemia in Bulgaria. *Leuk Res,* **19**, 877-81.

Tiensiwakul P, Lertlum T, Nuchprayoon I, et al (1999). Immunophenotyping of acute lymphoblastic leukemia in pediatric patients by three-color flow cytometric analysis. *Asian Pac J Allergy Immunol*, **17**, 17-21.

Uckan D, Hicsonmez G, Yetgin S, et al (2000). CD34/CD117 co-expression in childhood acute leukemia. *Leuk Res,* **24**, 201-6.

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

Williams SR, Wellhausen SR, Barker RL, et al (1997). Acute bilineage leukemia after chronic myelogenous leukemia. *J Ky Med Assoc,* **95**, 393-6.

Yeoh EJ, Ross ME, Shurtleff SA, et al (2002). Classification, subtype discovery, and prediction of outcome in pediatric acute lymphoblastic leukemia by gene expression profiling. *Cancer Cell,* **1**, 133-43.