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Comparison of Peripheral Blood versus Bone Marrow Blasts Immunophenotype in Pediatric Acute Leukemias

Saeeda Almarzooqi, Jill Crumbacher, Edward Firgau II, Samir Kahwash

Nationwide Children's Hospital, 700 Children's Drive, Columbus, OH 43205

*Corresponding author: Dr. Samir Kahwash Email: samir.kahwash@nationwidechildrens.org
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Abstract

Due to continued improvement in molecular and immunodiagnostic methods, more leukemia subtypes are being defined and diagnosed by their genetic and immunophenotypic profiles rather than by morphologic features alone. These advances, while relegating morphologic review and bone marrow (BM) blast counts to a lesser relevance, have elevated expectations of a full diagnostic work up using any specimen containing blasts, regardless of BM blast status. In some clinical situations, the pathologist is often asked to render a complete diagnostic and prognostic work up of leukemia on a peripheral blood (PB) sample, due to poor specimen quality or blast yield in a BM sample, with the intuitive assumption that PB and BM blasts, in the same patient and at a given point of time, are identical.

In an attempt to evaluate the immunophenotypic aspects of this assumption, we searched our records for cases of acute leukemia that had immunophenotyping of both PB and BM at the time of diagnosis, and found five cases: two acute myeloid leukemia (AML) and three acute lymphoblastic leukemia (ALL) cases. Utilizing similar pre-analytical conditions and similar FC gating strategy, positivity of the blasts in PB vs BM for some commonly used markers was compared. Significant differences were seen in several myeloid, lymphoid, and platelet markers in all patients. This discordance may carry significant clinical implications.

Keywords: Pediatric leukemia, Immunophenotype, Flow cytometry .

This study was presented in part at the Society for Pediatric Pathology annual meeting March 19-20, 2010, Washington, DC, USA

Table 1: List of antibodies used for Flow cytometry
* Fluorochrome used for CD 5 is PC5/PeCY5 in patients 1 and 3 and is FITC in patients 4 and 5.
# Fluorochrome used for CD 34 is PC5/PeCY5 in patients 4 and 5 and is FITC in patients 1 and 3.
Fluorochrome PC5/PeCY5 was used to label CD20 in case 5 as a gating marker for Kappa and Lambda.

Antibody	Fluorochrome	Source
	FITC	
CD19	FITC	Beckman Coulter/Immunotech
CD20##	FITC	Beckman Coulter/Immunotech
CD22	FITC	Beckman Coulter/Immunotech
CD34#	FITC	Beckman Coulter/Immunotech
CD41	FITC	Beckman Coulter/Immunotech
CD8	FITC	Beckman Coulter/Immunotech
CD5*	FITC	Beckman Coulter/Immunotech
CD2	FITC	Beckman Coulter/Immunotech
CD11b	FITC	Beckman Coulter/Immunotech
CD42B	FITC	Beckman Coulter/Immunotech
CD61	FITC	Beckman Coulter/Immunotech
TdT	FITC	Beckman Coulter/Immunotech
MPO	FITC	Beckman Coulter/Immunotech
SIgM	FITC	Dako
Kappa		Dako
	PE/RD1	
CD10	PE/RD1	Beckman Coulter/Immunotech
CD33	PE/RD1	Beckman Coulter/Immunotech
CD235A	PE/RD1	Beckman Coulter/Immunotech
CD4	PE/RD1	Beckman Coulter/Immunotech
CD11c	PE/RD1	Beckman Coulter/Immunotech
CD7	PE/RD1	Beckman Coulter/Immunotech
HLA:DR	PE/RD1	Beckman Coulter/Immunotech
CD14	PE/RD1	Beckman Coulter/Immunotech
CD79a	PE/RD1	Beckman Coulter/Immunotech
CD15	PE/RD1	Beckman Coulter/Immunotech
Lambda		Dako
	PC5/PeCY5	
CD20##	PC5/PeCY5	Beckman Coulter/Immunotech
CD5*	PC5/PeCY5	Beckman Coulter/Immunotech
CD3	PC5/PeCY5	Beckman Coulter/Immunotech
CD56	PC5/PeCY5	Beckman Coulter/Immunotech
CD13	PC5/PeCY5	Beckman Coulter/Immunotech
CD19	PC5/PeCY5	Beckman Coulter/Immunotech
CD34#		Beckman Coulter/Immunotech

Introduction

Technological advances of recent years have resulted in a growing reliance on immunophenotyping and genetics in the diagnosis and classification of hematopoietic neoplasms. Some types of acute leukemia (AL) are now defined more by their immunophenotypic and molecular genetic features rather than morphologic features, and can be diagnosed primarily based on the blast immunophenotype regardless of the source (BM, PB or body fluid). The pathologist is often asked to render a complete diagnostic and prognostic work-up on a peripheral blood sample rather than a more invasive bone marrow aspirate or biopsy, particularly when dealing with the very ill or very young patient. Underlying this trend is not only the desire to spare the patient an

invasive procedure, but also an intuitive assumption that PB and BM blasts, in the same patient at a given point of time, are identical. Our review aims to evaluate the immunophenotypic aspects of this assumption.

Table 2: Patient characteristics

Abbreviations: yrs.: years; F: female; M: male; AML: acute myeloid leukemia; ALL: acute lymphoid leukemia.

	Age (yrs.)/ Gender	Clinical presentation	Leukemia subtype	Percentage of blasts	Follow-up (years)
1	9/ F	leukocytosis	Acute myelomonocytic leukemia	BM 87% PB 82%	- Relapse 6 months later - BMT 9 months post diagnosis - died 11 months post diagnosis
2	14/ M	leukocytosis	B-lymphoblastic leukemia	BM 94% PB 82%	- Chemotherapy/BMT - died 10 months post diagnosis
3	9/M	leukocytosis anemia thrombocytopenia	Acute monocytic leukemia	BM 90% PB 90 %	- relapse 8 months post diagnosis
4	2/M	leukocytosis anemia thrombocytopenia	B-lymphoblastic leukemia	BM 90% PB 15%	On chemotherapy
5	1.5/ M	leukocytosis, thrombocytopenia	B-lymphoblastic leukemia	BM 90% PB 89%	On chemotherapy

Materials and Methods

This study has been approved by Nationwide Children's Hospital institutional review board (IRB09-00482). Records were searched for cases of AL that had immunophenotyping of both PB and BM at the time of diagnosis. In each case, specimen preparation and flow cytometric analyses were completed in an identical manner: PB samples were collected in EDTA tubes, and BM samples were collected in sodium heparin coated tubes. Whole peripheral blood samples with adjusted white count were used without washing and pipetted straight into the tubes containing the monoclonal antibodies (MAB). BM was washed twice with phosphate buffered saline that contains 1% fetal calf serum. The BM was then incubated for 10 minutes in AB sera. The BM (with adjusted white count) was then pipetted into the MAB tubes. CD45-ECD was utilized on all patients for gating, and was present in each tube listed for the monoclonal antibodies run on our patients (Table 1). All monoclonal antibodies were purchased from Beckman Coulter/Immunotech except for IgM, Kappa and Lambda, which were acquired from Dako.

A Beckman-Coulter FC500 flow cytometer with a Beckman Coulter CXP Software was used for analysis of our samples, except for case 2 where Beckman Coulter EPICS XL-MCL using System II software was utilized. The calibration of instruments at our laboratory is checked with fluorescent beads provided by Beckman Coulter (instrument manufacturer). In addition, a weekly "auto standardization" panel is performed that allows the instrument to account for "bleeding" of the fluorescent dyes into wavelengths other than the desired one for detection.

Both instruments utilized four-color analysis with gating on CD45 and side scatter characteristics to identify blasts (Figure 1). The panel of markers tested in our laboratory included: CD2, CD3, CD5, CD7, CD4, CD8, CD56, CD19, CD20, sIgM, CD13, CD33, CD11b, CD11c, CD10, CD34, HLA:DR, Glycophorin A, CD45, CD41, CD42b, CD61, CD14, TdT and CD22.

Utilizing similar gates for the same patient, the percentage of blasts positive for each of our AL panel markers in PB and BM were compared. The panel of markers tested in our laboratory included: CD2, CD3, CD5, CD7, CD4, CD8, CD56, CD19, CD20, sIgM, CD13, CD33, CD11b, CD11c, CD10, CD34, HLA:DR, Glycophorin A, CD45, CD41, CD42b, CD61, CD14, TdT and CD22.

Differences in expression for each marker between PB and BM were calculated by subtracting the percentage of positive blasts in peripheral blood from those in the bone marrow. A difference of ≥10% was arbitrarily considered significant.

Review of Nationwide Children's Hospital records from

Table 3: A list of percentage positivity of blasts for each marker in all five cases.
Abbreviations: BM bone marrow; PB peripheral blood; ALL acute lymphoblastic leukemia; AML acute myeloid leukemia; ND not done.

CASE/ MARKERS	CASE 1 (AML, M4)		CASE 2 (ALL, L1)		CASE 3 (AML, M5a)		CASE 4 (ALL, L1)		CASE 5 (ALL, L1)	
	BM	**PB**	**BM**	**PB**	**BM**	**PB**	**BM**	**PB**	**BM**	**PB**
CD2	14.4	3.2	1.2	0	0.8	0	0.2	0.5	0	0.1
CD3	0	0.9	1.8	14.9	0.1	0	0.1	0.8	0	0
CD5	0	0.1	0	2.1	0	0	0.4	2.5	0.3	0.5
CD7	17.8	1.4	1.2	0.2	3	0	0	0.4	0.6	0.8
CD4	0.2	1.3	0	0	6.8	0.6	0	0	0.4	0.1
CD8	0	0.9	0	0	0	1.3	0.2	0.2	0	0
CD56	0.1	0.2	ND	ND	0	0	0.2	0.1	0	1.4
CD19	4.2	0.2	99.2	98.5	37.3	19.4	75.3	74	95.2	85
CD20	0	0.9	30	34.6	0	0.2	0.7	0.2	85.9	79
sIgM	0.2	3.1	0.4	3.7	0.3	0	0.6	4.2	0	0.5
CD13	15.7	7	1.9	0.7	0.7	2.9	0.9	0.2	0	0.5
CD33	94.5	84.4	1.2	13.9	64.9	34.8	0	3.7	0	1.5
CD11b	55.5	17.4	0.4	1.1	19.5	3.6	0	0.2	0	0.5
CD11c	32.6	1.4	ND	ND	22.8	1	0.2	3.7	0	0.1
CD10	0.2	0.2	99.1	98.5	0.2	0	93.8	80.6	98.9	94.2
CD34	86.3	97.2	32.4	38.7	78.8	95.6	86.6	76.9	20.2	27.5
HLA:DR	87.6	78.3	35.8	94.3	55.9	42.3	64.1	69.1	63.4	0.6
Glycophorin A	2.1	5.4	ND	ND	1.5	1.8	8.6	1.1	1.1	4
CD45	83.9	92.1	ND	ND	88.5	99.1	96.7	99.3	96.9	99.3
CD41	0.8	10.8	ND	ND	1.4	10.5	0.3	10.8	0	0.1
CD15	0.3	1.4	ND	ND	0.2	0				
CD42b	1.4	57	ND	ND	3.8	6.5				
CD61	16.2	97	ND	ND	78.5	6.6				
CD14	ND	ND	0	0	0.3	2.2				
TdT							74.8	50.2	61.5	54
CD22							80.1	13.4	ND	66.8

November 1999 through January 2010 yielded 576 cases of AL. Among these were 520 BM and 56 PB samples. Only five patients had concurrent immunophenotyping performed on both PB and BM at time of diagnosis (two acute myeloid leukemia (AML) and three acute lymphoblastic leukemia (ALL) cases). Collection of BM took place following the acquisition of the PB sample. In all cases, due to clinical variables beyond the laboratory's control, the initial work-

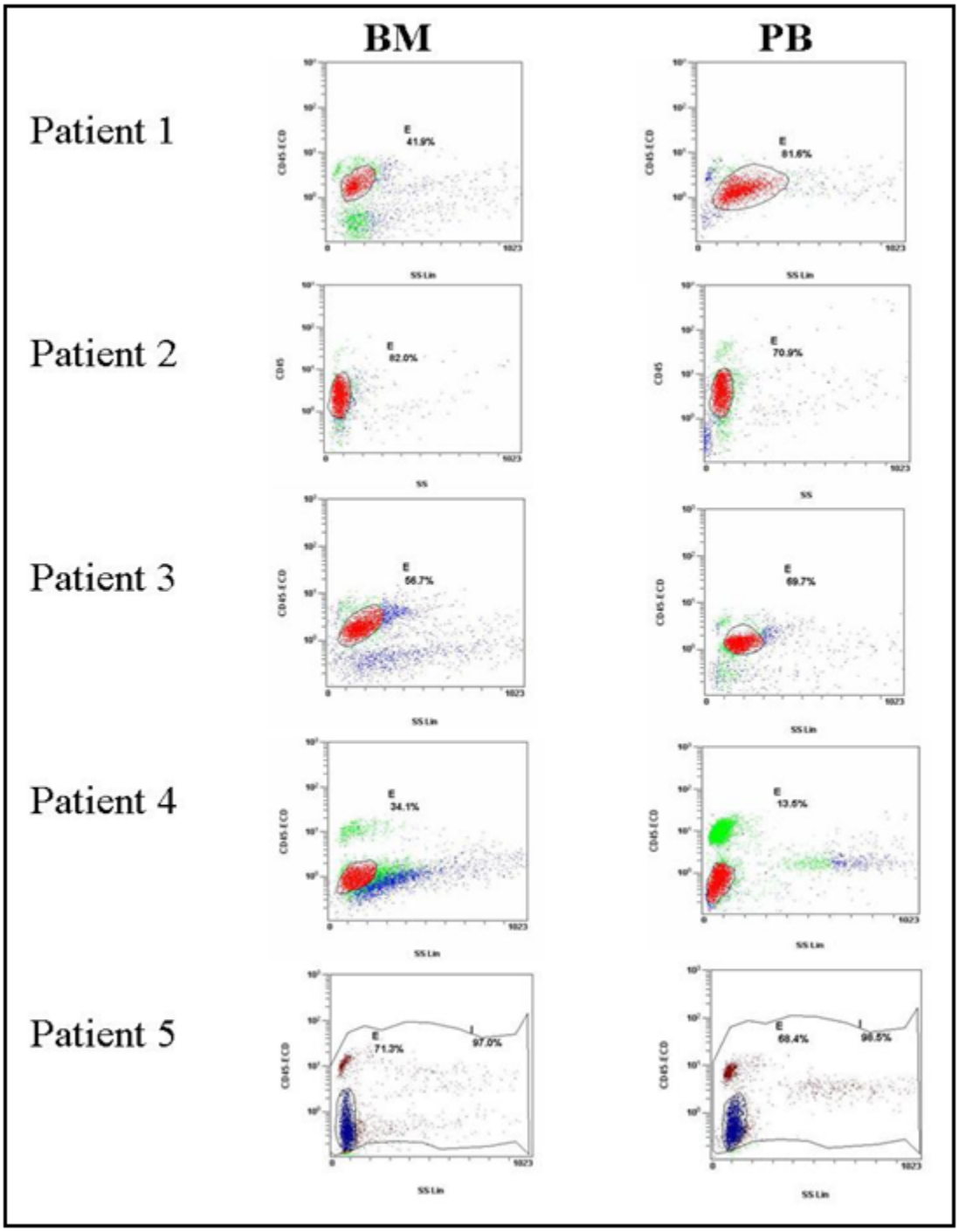

Figure 1: Scattergram of patients 1- 5 using gating on CD45 for both peripheral blood and bone marrow. (Abbreviation: BM bone marrow; PB peripheral blood; y axis side scatter; x axis CD45.)

up including flow cytometry was done on PB. The time interval between collection of PB sample and collection of BM sample were as follows: 41 hours in patient 1; 24 hours in patient 2; 26 hours in patient 3; 69 hours in patient 4; and 28 hours in patient 5. No leukemia treatment was given during these intervals

Results

Patient characteristics are summarized in Table 2. Flow cytometry data are summarized in Table 3. Significant differences were seen in several myeloid, lymphoid and platelet markers across all patients (Figures 2-5), as follows:

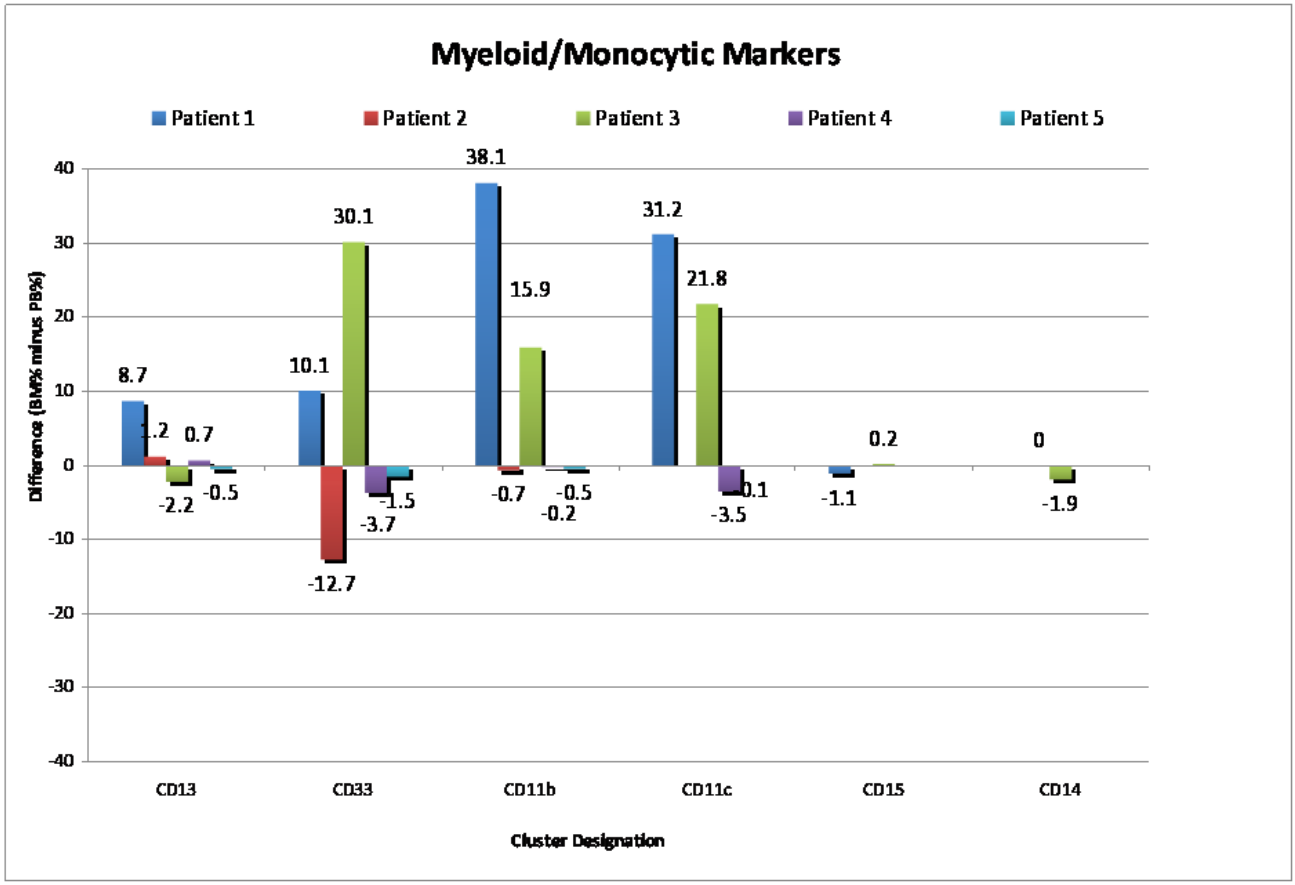

Figure 2: Expression of myeloid/monocytic markers using delta value in cases 1-5

Patient 1 (AML): CD2, CD7, CD11b, CD11c, CD33, CD34, CD41, CD42b and CD61
Patient 2 (ALL): CD3, CD33, and HLA:DR
Patient 3 (AML): CD11b, CD11c, CD19, CD33, CD34, CD45, CD61 and HLA:DR
Patient 4 (ALL): CD10, CD22, CD41 and TdT
Patient 5 (ALL): CD19 and HLA:DR.
The largest difference was seen in patient 1 (Δ = 80.8% for CD61). These differences created initial challenges in interpretation, which were ultimately resolved by incorporating other findings.

In cases 1 and 2, findings of flow cytometry on PB and BM were consistent with the diagnosis of acute myelomonocytic leukemia and B lymphoblastic leukemia respectively. Discrepancies in the percentages of blast positivity on flow cytometry did not pose a difficulty in the diagnosis. Similarly, in case 5, the findings in the PB flow cytometry were consistent with those on the BM. Flow cytometry showed a 90% blast population that was positive for CD10, CD19, CD20, CD22, TdT, CD34 and cytoplasmic Mu with negative sIg. Thus, a diagnosis of B lymphoblastic leukemia was rendered.

In case 3, blasts constituted 90% of peripheral blood cells with a morphology that is consistent with monoblasts. They were reactive for CD33, HLA:DR and CD34. The blasts were negative for CD14, CD15, CD4, CD11b and CD11c. raising the diagnosis of AML with minimal differentiation. However, a subset of blasts (20% of blasts) was reactive with some B cell markers including CD19 and CD79a and myeloperoxidase was seen in < 2% of PB blasts. Thus, the flow cytometry findings on PB raised the possibility of minimally differentiated or mixed lineage leukemia. Subsequent work-up including cytochemical staining on the bone marrow confirmed the diagnosis of acute monocytic leukemia.

In case 4, PB flow cytometry showed a blast population constituting only 15% of cells. Blasts were positive for CD19, CD10 CD34 and HLA:DR. In addition, 50% of the blasts were positive for TdT but only 13% were positive

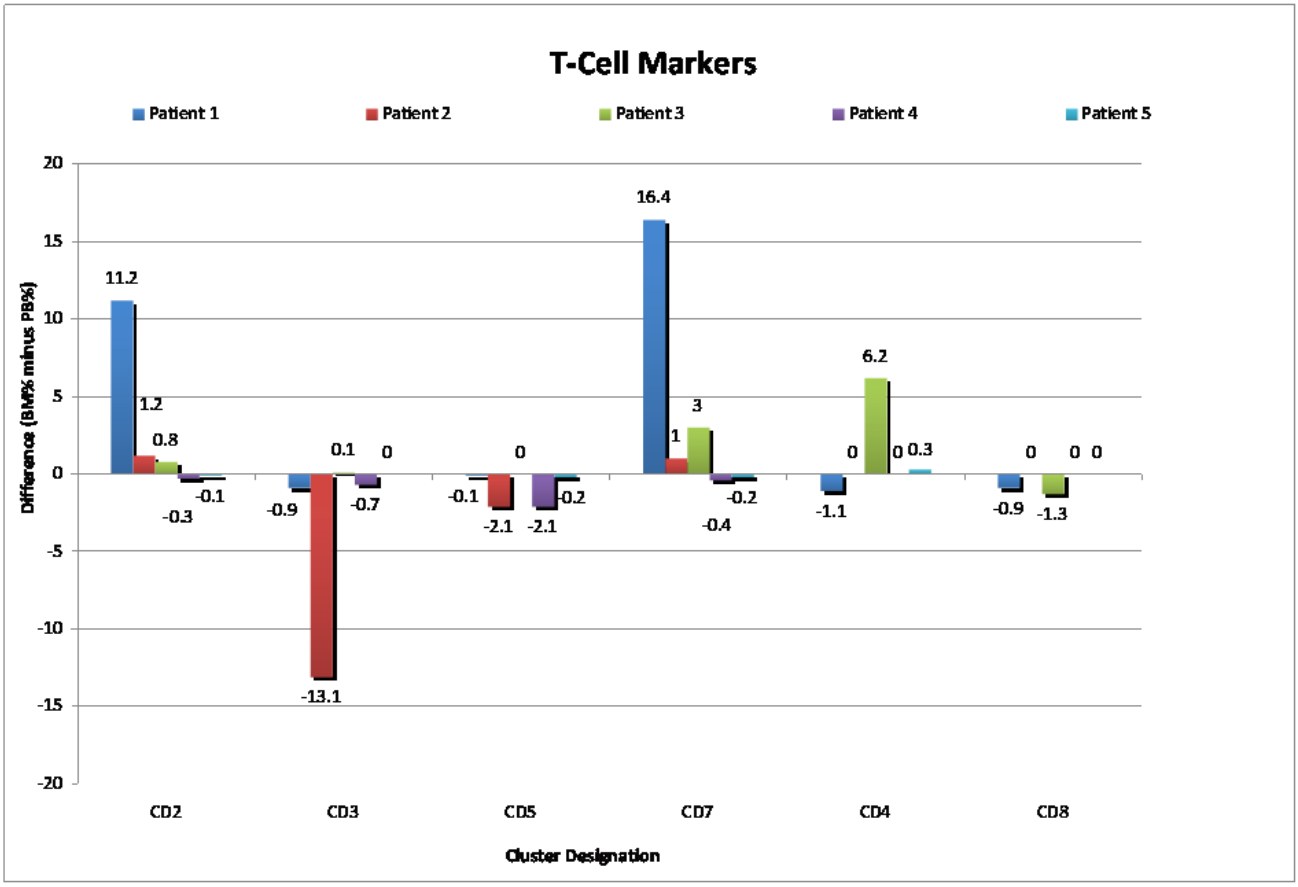

Figure 3: Expression of T-cell markers using delta value in cases 1-5

for CD22. Subsequent BM biopsy and flow cytometry disclosed a 90% blast population with positivity for CD10, CC19, CD34, HLA:DR and TdT rendering a definite diagnosis of precursor B-cell lymphoblastic leukemia.

Discussion

The 2008 WHO Classification of Hematopoietic and Lymphoid Tissues describes two broad categories of AML (1). The first includes cases with recurrent cytogenetic abnormalities, which constitute an estimated 55% of all cases. In this category, the cytogenetic findings trump all other clinical, laboratory, and immunophenotypic findings, and relegate blast source and percentage to lesser importance. The second includes AML with no specific genetic findings, where the diagnosis is based on a constellation of morphologic and immunophenotypic findings aided by ancillary studies such as cytochemical, cytogenetic and molecular genetic data. In the latter category, morphologic examination and determination of blast percentage in a bone marrow aspirate sample is crucial in making the diagnosis and subtyping the leukemia. This situation is exemplified by cases where the differential diagnosis is between AML and a myelodysplastic disorder (MDS). In this category, the diagnosis of AML requires the presence of ≥ 20% blasts in the BM, which cannot be rendered without a BM morphological evaluation.

In the context of ALL, with the exception of Burkitt's leukemia (representing <5% of cases), there is no comparable group of cases where morphologic features may help predict a specific genetic abnormality.

In the initial diagnostic work up, it is natural to start with simpler tests (e.g. morphologic evaluation as compared to immunophenotyping) and less invasive procedures (e.g. peripheral blood sample rather than bone marrow examination), followed methodically and in an algorithmic fashion by more invasive procedures and more specific tests.

Morphologic criteria for the diagnosis of AL have stood the test of time, but current disease classifications are based on integration of morphologic, clinical, genetic, and immunophenotypic data. On the other hand, utilization of immunophenotypic and molecular techniques outside the context of complete morphologic and clinical data may be problematic. Under less than ideal or urgent clinical

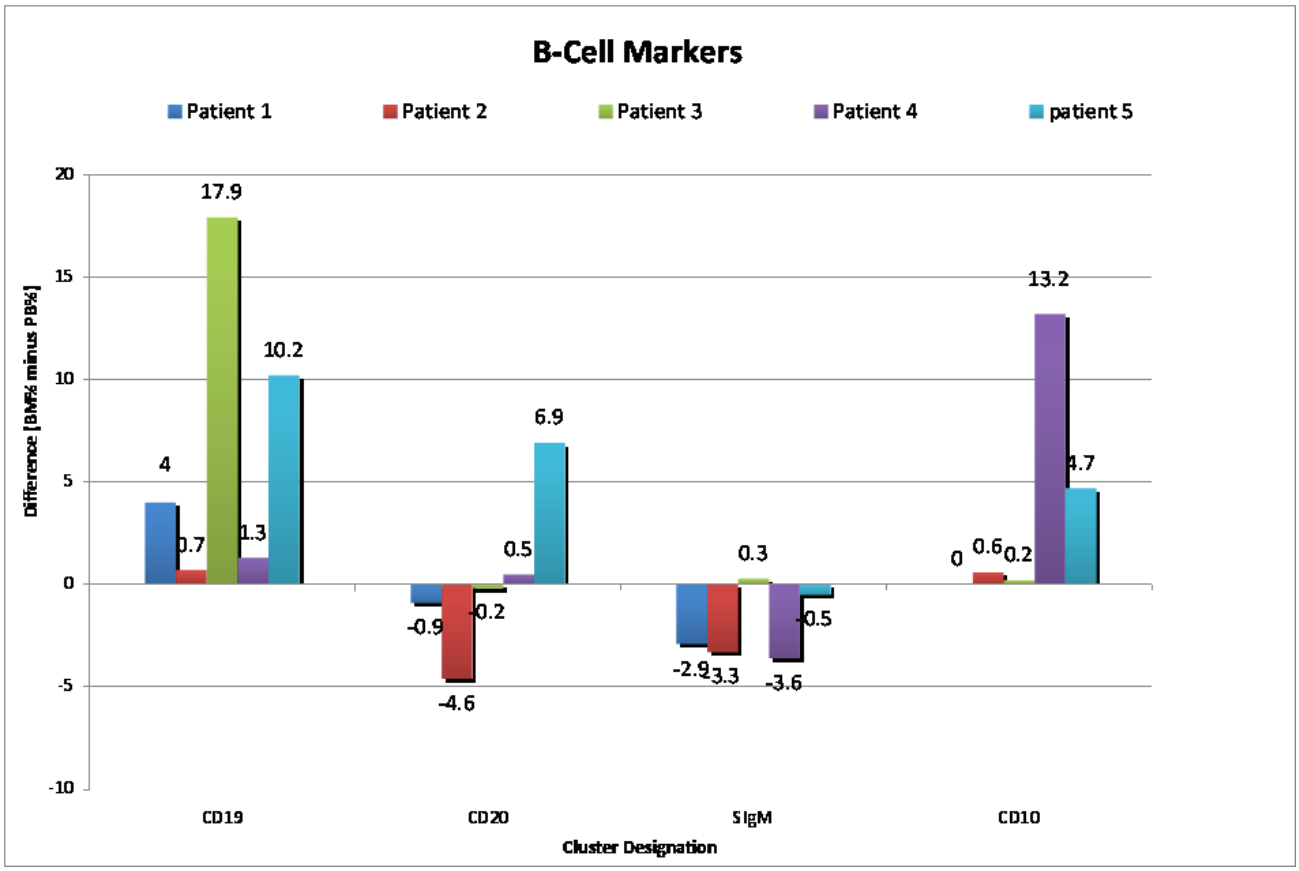

Figure 4: Expression of B-cell markers using delta value in cases 1-5

conditions, mounting pressure to rapidly establish a diagnosis of AL can preclude obtaining optimal marrow samples for molecular, genetic, and research. Establishing a diagnosis on PB or body fluid samples is an accepted alternative, but blasts from the latter sources tend to be physiologically older, exhibiting degenerative changes that can be seen by simple morphologic comparison. It is even possible that they may represent a subclone of the original marrow blasts, i.e. a type with overlapping, but non-identical characteristics to those in the BM, including variably different immunophenotype.

While the overlapping features of PB and BM blasts may not affect the ability to render a specific diagnosis in most cases, the difference may be significant when expression of a single, or few, aberrant phenotypic markers can dictate management options.

Characterization of blast immunophenotype has implications in terms of diagnosis, prognosis, and with the increased utilization of targeted immunotherapy, may impact the choice of treatment within the same diagnostic category. For example, in patients with AML, gemtuzumab ozogamicin is being tried as a target therapy in subtypes that are presumed to have CD33 overt or cryptic marking (2). Additional examples include trials evaluating rituximab and alemtuzumab as targeted monoclonal antibodies against CD20 and CD22 positive blasts respectively in cases of ALL (3).

Few studies in the literature have addressed differences in BM vs. PB blasts. These studies have focused largely on the diagnostic features. Weinkauff, et al., found a strong correlation between BM and PB blast in a study of 28 patients (4). They utilized three-color flow cytometry where the panel of markers included: CD13, CD33, CD34, CD10, CD19, TdT, CD2 and CD41. In addition, extra markers were evaluated in some cases to facilitate diagnosis, and these included: CD64, CD4, CD8, CD7, CD38, CD138, Glycophorin A and c-kit. They detected a difference for some of the markers that did not impact the diagnosis. In two of their patients with ALL, the marker CD33 showed a difference between BM and PB. Another patient with AML

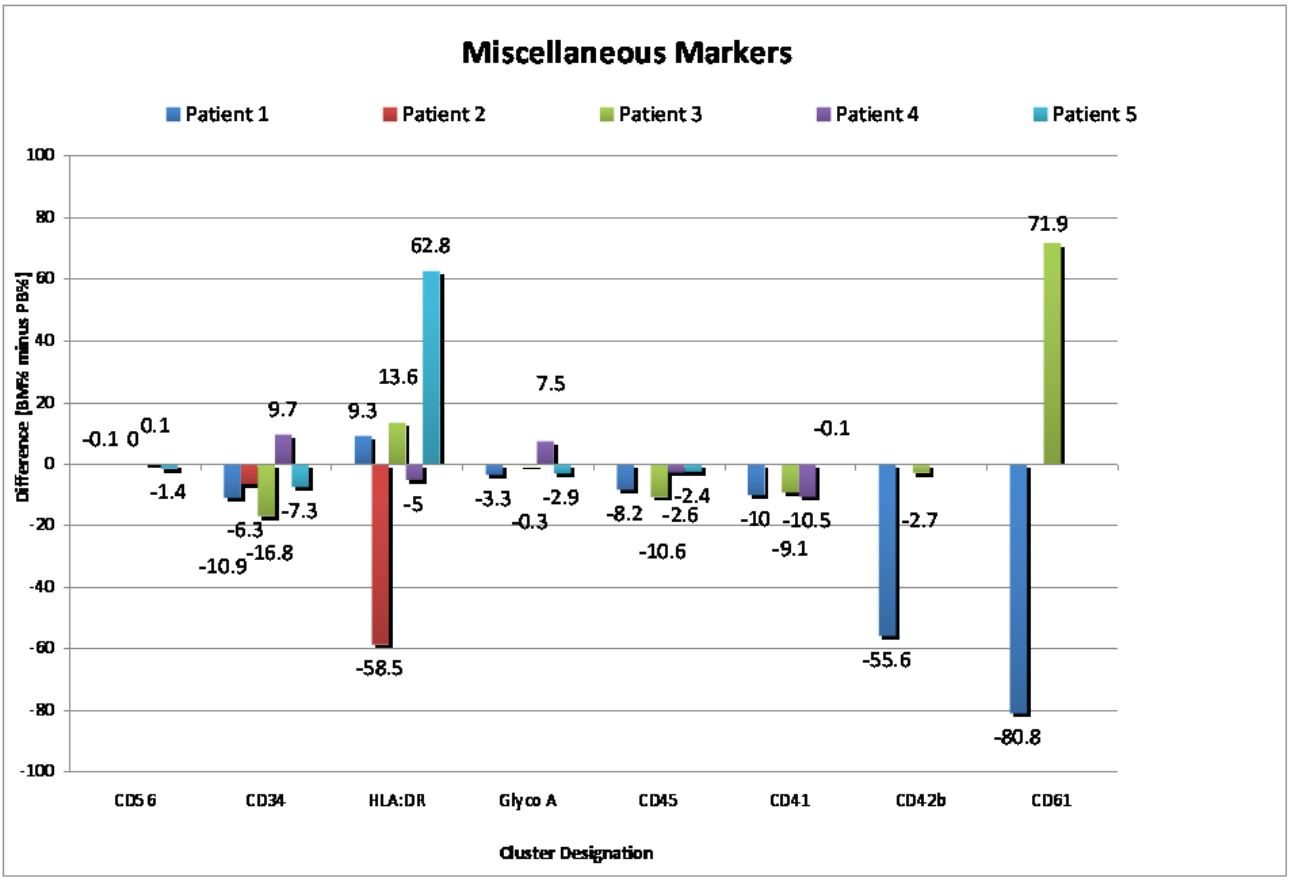

Figure 5: Expression of miscellaneous markers using delta value in cases 1-5

demonstrated a discrepancy for CD64.

Our findings appear to contrast with a study by Rezaei, et al. (5) that performed an immunophenotypic comparison of BM vs. PB blasts utilizing two-color analysis with flow cytometer. They used 13 markers including CD3, CD7, CD5, CD22, CD20, CD10, CD19, CD13, CD33, CD14, CD45, HLA:DR AND TdT. All their cases had a PB blast count of ≥30%. They studied 18 patients with AML and 13 patients with ALL and found good correlation between PB and BM, which was better in AML compared to ALL cases (5).

The importance of detecting a difference between PB vs. BM blast characteristics is exemplified in the study by Sheridan, et al., which looked at the expression of ZAP-70 in patients with chronic lymphocytic leukemia by flow cytometry (6). This marker has been demonstrated to correlate with the unmutated IgVH in these leukemias, hence a poorer prognosis. The authors demonstrated that in their 311 cases of chronic lymphocytic leukemia, a greater percentage of marker positivity was detected in BM compared to PB samples (6).

In our study, four of the five cases had a blast percentage ≥ 30% in the PB (suggested by Rezaei as prerequisite for good correlation). Case 4 had a PB blast percentage of 15%. Even though the majority of our cases fulfilled the criteria of ≥ 30% blasts in the peripheral blood, significant differences in several markers were detected by immunophenotyping (Table 3). Platelet markers are perhaps notorious in this regard due to the known phenomenon of platelet adhesion to leukemic blasts in PB, causing false positivity (7). This can explain the higher percentage detected for CD61 in the PB of case 1 compared to that in the BM. However, the same explanation may not be used in case 3 where CD61 was higher in the BM sample.

Another possible explanation for these discrepancies is that PB blasts may represent a sub-clone of BM blasts. This carries implications in terms of molecular differences that may exist between the two blast populations. Blast

heterogeneity may further help explain some intriguing clinical presentations and blast affinities. For example, the lack or paucity of circulating PB blasts in some cases, despite very high blast counts in the bone marrow or other tissues, may perhaps indicate that PB blasts are not simply a "spillover" of BM blasts (8).

It is noteworthy that this discordance in blast marker expression is not limited to peripheral blood versus bone marrow, but can be seen in the context of tissue leukemic involvement (i.e. myeloid sarcoma). For example, in a study by Chang, et al., the authors found that myeloperoxidase, usually absent in AML-M5 in the bone marrow, was detected in myeloid sarcoma in two of the five cases of AML-M5 in their study (9).

In conclusion, immunophenotyping of blasts by flow cytometry from simultaneous PB and BM sources can show significant immunophenotypic differences. We hypothesize that PB blasts are merely a subset of BM blasts, with overlapping but variably different immunophenotypes. The differences can be significant enough to change the interpretation of some key markers (positive vs. negative) resulting in diagnostic difficulties, and influencing management. In addition, since future treatments and targeted therapies may depend on expression of specific markers, protocol designs will need to specify a consistent specimen source in order to allow uniformity and accurate comparison. A wider prospective study may help in shedding more light on these issues.

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