RESEARCH ARTICLE

Characterization of flow cytometric immuno-phenotyping of acute myeloid leukemia with minimal differentiation and acute T-cell lymphoblastic leukemia: A retrospective cross-sectional study [version 1; peer review: 1 approved with reservations, 1 not approved]

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V1 First published: 24 Sep 2020, 9:1170
<https://doi.org/10.12688/f1000research.24929.1>
 Latest published: 24 Sep 2020, 9:1170
<https://doi.org/10.12688/f1000research.24929.1>

Abstract

Background: Acute leukemias (ALs) are a heterogeneous group of malignancies with various clinical, morphological, immunophenotypic, and molecular characteristics. Distinguishing between lymphoid and myeloid leukemia is often performed by flow cytometry. This study aimed to evaluate the immunophenotypic characterization and expression of immuno-markers in both acute myeloid leukemia (AML-M0) and acute T-cell lymphoblastic leukemia (T-ALL).

Methods: A retrospective cross-sectional study was conducted in the Pathology Department/Teaching Laboratories/Medical City/Iraq and included all patients newly diagnosed with AL from 5 January to 10 December 2018. Immunophenotypic analysis was performed on bone marrow samples, freshly collected in EDTA tubes. Flow cytometry (Canto-2 BD) was used, with laser excitation of blue and red wavelengths. A panel of monoclonal antibodies (MoAbs) was used for diagnosis, using a SSC/CD45 gating strategy.

Results: The study showed 41.6% of AML-M0 patients had no aberrant antigen expression, while 33.3%, 16.6%, 8.3%, and 8.3% had aberrant CD7, CD56, CD2, and CD19, respectively. In 16.6% of AML-M0 cases more than one aberrant antigen was expressed. With regard to T-ALL, 7.0% were pro-T type, 58.0% were pre-T, 13.0% were cortical, and 22.0% were mature-T type. In 55.5% of patients with T-ALL there was no aberrant antigen expression.

Conclusion: We concluded that most patients with AML-M0 have no aberrant antigen expression. In patients with T-ALL, the pre-T type is

Open Peer Review

Approval Status

	1	2
version 1
24 Sep 2020	view	view

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most common, according to the European Group for the Immunological Classification of Leukemias (EGIL) classification. Patients with T-ALL also generally lack aberrant antigen expression.

Keywords

Acute leukemia, Immuno-pheno-typing, Acute lymphoblastic leukemia, Multi-parameter flowcytometry

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Author roles: **Al-Saadi EAKD:** Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Resources, Supervision, Visualization, Writing – Original Draft Preparation; **Abdulnabi MA:** Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Project Administration, Resources, Software, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Jaafar FH:** Conceptualization, Data Curation, Investigation, Methodology, Project Administration, Resources, Validation, Visualization, Writing – Original Draft Preparation

Competing interests: No competing interests were disclosed.

Grant information: The author(s) declared that no grants were involved in supporting this work.

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How to cite this article: Al-Saadi EAKD, Abdulnabi MA and Jaafar FH. **Characterization of flow cytometric immuno-phenotyping of acute myeloid leukemia with minimal differentiation and acute T-cell lymphoblastic leukemia: A retrospective cross-sectional study [version 1; peer review: 1 approved with reservations, 1 not approved]** F1000Research 2020, 9:1170 <https://doi.org/10.12688/f1000research.24929.1>

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Introduction

There are many characteristic categories of leukemia, which have well known prognoses and specific treatments. Differentiation between lymphoid and myeloid leukemia is performed using flow cytometry¹. The analysis of acute leukemia immunophenotypic done by flow cytometry, this method recently become a tool for distinguishing between myeloid and lymphoid lineages. Several types of acute leukemias identified morphologically with or without enzyme cytochemical analysis, but immunophenotyping stay a vital for the right identification of myeloid lineages in minimally differentiated acute myeloid leukemia (AML-M0) and the differentiation of B or T-cell lineages in acute lymphoblastic leukemia (ALL)². Flow cytometry (FC) is a newer technology that can be used to detect many CD markers in or on a single malignant cell, and also possesses multi-parametric capabilities for combining the study of physical characteristics, such as cell size and granularity, alongside multiple CD markers. The advantages of FC include simultaneous analysis of multiple antigens on the same cell, assessing small samples, and providing results to make an objective diagnosis within a few hours³. Immunophenotyping permits reproducible lineage detection and assignment of leukemia types. On the other hand, intra-permeable cytoplasmic markers, such as myeloperoxidase, cCD22, cCD3, and cCD79a, are more accurate and specific indicators⁴. Distinguishing AML-M0 from ALL remains a challenge in a minority of cases. The French-American-British (FAB) Cooperative Group recognized that many cases morphologically classified as types of ALL appeared to be of myeloid origin, as determined by monoclonal antibodies and ultra-structural cytochemistry⁵.

This study aimed to evaluate the immunophenotypic characterization and the expression of commonly used immuno-markers in both acute myeloid leukemia with minimal differentiation (AML-M0) and T-cell acute lymphoblastic leukemia (T-ALL), and to define the best use and the role of multi-parameter flow-cytometry in the diagnosis and proper classification of these types of acute leukemia. Also, to evaluate the aberrant antigen expression in both AML-M0 and T-ALL and its correlation with clinical and prognostic criteria.

Methods

Study design and setting

A retrospective cross-sectional study was conducted among 69 participants with newly diagnosed AL (M0 or T-ALL); there were 24 cases of AML-M0, 12 females and 12 males; and 45 cases of T-ALL, 40 males and five females. This study was conducted in the Pathology Department/Teaching Laboratories/Medical City/Iraq, and included all patients newly diagnosed with AL during the period from 5 January to 10 December 2018. Demographic characters of patients like age, and gender were collected from medical records of the participants. General clinical feature of each patients collected either when taking the patients history or during clinical examination included hepatosplenomegaly; lymphadenopathy (LAP); pallor; history of bleeding from gum, gingivae,

and skin; fever; effusion of pleura; and presenting with a mediastinal mass.

Inclusion criteria

1. T-ALL patients aged between (0.5–60) years old.
2. AML-M0 patients aged between (7–76) years old.
3. *de novo* cases, those not receiving steroid or chemo - therapy before sample collection
4. Those with a positive diagnosis of T-ALL or AML-M0 confirmation.

Exclusion criteria

1. Death of patient prior to treatment
2. Those who received chemotherapy in other hospitals either before referral or after finishing induction.
3. Those whom refused treatment.
4. Those with history of steroid intake of than one week.

Diagnostic criteria

1. Those presented have fever, abdominal fullness, and skin rash.
2. Those have pallor, petechiae (bleeding under the skin), sternal tenderness to palpation, lymphadenopathy, and hepatosplenomegaly on physical examination.
3. Those with history of bleeding from gum, and gingivae.
4. Those with elevated WBC count, normal RBC counts, decrease platelets counts in CBC.
5. Those with chest masses on chest radiograph.
6. Those with LAP and organomegaly on CT scans of the chest and abdomen/pelvis.

Sample size calculation

A sample size of 69 participants was calculated by the following formula:

$$n = \frac{z^2 \times \hat{p}(1 - \hat{p})}{\epsilon^2}$$

where

z is the z score for 95% CI=1.96

ϵ is the margin of error=5%

n is sample size

$\hat{p}$ is the population proportion=50%

This means 69 or more measurements/surveys are needed to have a confidence level of 95% that the real value is within $\pm 5\%$ of the measured/surveyed value. We the population proportion based on historical data from our center, whereby we found 50% of those attending were eligible for the study or in other words 50% were diagnosed with T-ALL or AML-M0.

Period of recruitment, follow-up, and data collection

Participants were recruited at their first visit to the center. Each patient attending our center meeting the inclusion criteria were invited to be including in our study after which written informed consent was obtained. Follow-up was performed at every subsequent visit. In EDTA anti-coagulated tubes (ATACO / China, Catalogue No.:443N980100E), the bone marrow aspirate (BMA) samples were collected (2–3 ml). The method of collection as follow: we marked and cleaned the area where the biopsy needle will be inserted. The bone marrow fluid (aspirate) and tissue sample (biopsy) were usually collected from the top ridge of the back of the hipbone (posterior iliac crest). A small incision was made, into which the needle was inserted into the bone and into the bone marrow. A sample of the liquid portion of the bone marrow was then drawn. Following sampling pressure was applied

to the area of insertion to stop the bleeding and then a bandage will be placed on the site. Bone marrow was either aspirated and tested the same day or stored, if necessary, in a refrigerator (2–8°C), for no more than 48 hours. We pre-washed all the sample using at least 25 volumes excess 1X PBS (BD biosciences, Catalogue No.: 554781), mixed well. Then, pelleted cells by centrifugation and re-suspend in 1X PBS with 0.1% sodium azide to the original volume.

Data measurement and quantitative variables

The panel used for diagnosis of both ALs included the following monoclonal antibodies (MoAbs): CD45, CD34, cMPO, CD13, CD33, CD14, CD15, CD64, CD117, cTdT, cCD79a, CD19, CD20, CD10, CD1a, CD2, sCD3, cCD3, CD4, CD5, CD7, CD8, and CD56, with an SSC/CD45 gating strategy, [Table 1](#).

Table 1. Supplier, catalogue number and concentration used of all antibodies.

Antibodies	Supplier	Catalog no.	Concentration
CD45	BD biosciences	332784	6 µL for 100 µL blood
CD34	BD biosciences	333178	6 µL for 100 µL blood
cMPO	BD biosciences	333164	6 µL for 100 µL blood
CD13	BD biosciences	333180	6 µL for 100 µL blood
CD33	BD biosciences	333146	6 µL for 100 µL blood
CD14	BD biosciences	333951	6 µL for 100 µL blood
CD15	BD biosciences	332778	6 µL for 100 µL blood
CD64	BD biosciences	555525	6 µL for 100 µL blood
CD117	BD biosciences	332784	6 µL for 100 µL blood
cTdT	BD biosciences	550756	6 µL for 100 µL blood
cCD79a	BD biosciences	335835	6 µL for 100 µL blood
CD19	BD biosciences	331355	6 µL for 100 µL blood
CD20	BD biosciences	331356	6 µL for 100 µL blood
CD10	BD biosciences	332775	6 µL for 100 µL blood
CD1a	BD biosciences	333167	6 µL for 100 µL blood
CD2	BD biosciences	335821	6 µL for 100 µL blood
sCD3	BD biosciences	332771	6 µL for 100 µL blood
cCD3	BD biosciences	333170	6 µL for 100 µL blood
CD4	BD biosciences	332772	6 µL for 100 µL blood
CD5	BD biosciences	331357	6 µL for 100 µL blood
CD7	BD biosciences	332772	6 µL for 100 µL blood
CD8	BD biosciences	333171	6 µL for 100 µL blood
CD56	BD biosciences	335826	6 µL for 100 µL blood

Immunophenotypic analysis was performed. Cells were stained using fluorescence-conjugated MoAbs and analyzed using a four-color Canto-2 BD flow cytometry device⁶. The device software was based on the **FACS DIVA-8** operating system for multi-parametric data acquisition, display, analysis, and instrument control. The Canto-2 BD is a high performance, accurate, and cost-effective flow cytometry device that employs two lasers (red and blue), six optical parameters, and offers four colors with forward scatter (FSC) and side scatter (SSC) in combination with four fluorescence channels (FL1–FL4)⁷.

Addition of surface antibodies

Each surface antibody (6 µl) was added to 100 µl of well-mixed EDTA (BD biosciences, Catalogue No.: 347689) anti-coagulated blood in labeled tubes, then incubated at room temperature in the dark for 15 minutes. Then, 2.5 ml 1X BD FACS lysis solution (BD biosciences, Catalogue No.: 349202) was added, and the samples were again incubated at room temperature in the dark for 15 minutes. The cells were washed three times by centrifugation at 1500 × g for 5 minutes before decanting the supernatant. FACS permeabilizing solution (BD biosciences, Catalogue No.: 347692) (0.5 ml) was added. Here, we vortex gently and incubated for 15 to 20 minutes in the dark at room temperature (20°C–25°C), and then the solution was re-suspended by vigorous shaking to mix. We then added 2 mL of CellWASH solution (BD biosciences, Catalogue No.: 349524) and centrifuged at 1200g for 5 minutes. Finally, we added 0.5 mL of CellFIX solution (BD biosciences, Catalogue No.: 340181) and mixed thoroughly. Then the samples were analyzed using the flow cytometer.

Addition of surface and cytoplasmic antibodies

After adding surface markers, 0.5 ml of permeabilizing reagent was added to each tube. The tubes were vortexed then incubated for 15 minutes in the dark. Then, 6 µl of each cytoplasmic marker was added and the samples were incubated at room temperature in the dark for 30 minutes. Then, 0.5 ml of FACS solution was added, and the samples were re-suspended by vigorous mixing before being analyzed using the flow cytometer.

Permeabilizing reagent⁸

Permeabilizing Solution 2 (BD FACSTM) is a premixed concentrate formulated specifically for use in flow cytometry. It is intended to permeabilize cell membranes prior to intracellular immunofluorescence staining with monoclonal antibodies.

Lysing reagent⁹

Cell Lyse (BD biosciences, Catalogue No.: 559759), which is an erythrocyte lysing reagent kit for wash and no-wash procedures was used for cell lysis. Residual debris was removed by centrifugation and cell washing. Fixative reagent was used to fix and stabilize leukocytes. The fixed samples were stored for up to 24 hours at 2°C to 8°C before analysis.

Reagents

1. BD FACSTM lysing solution (10X) (Catalog No. 349202)
2. BD CellWASH™ (Catalog No.349524)

3. BD CellFIX™ (Catalog No. 340181)
4. BD FACSTM permeabilizing solution 2 (Catalog No. 347692)
5. Wash buffer of PBS with 0.1% sodium azide (Catalog No. 554781)
6. Distilled water (BDH Ltd., Catalogue No.:DW005HH2).

Equipment

1. Adjustable pipettes and their tips (Gelson/ France, Catalogue No.: 111-898-3).
2. Beakers (BD Biosciences, Catalogue No.: 33350410).
3. Flasks (BD Biosciences, Catalogue No.: 645392).
4. Centrifuge (Elite – Medichem/ (India), Catalogue No.: 153-33-IND).
5. Disposable slides (BD Biosciences, Catalogue No.: 335630).
6. EDTA tubes (ATACO / China, Catalogue No.:443N980100E).
7. Racks (BD Biosciences, Catalogue No.: 333489).
8. Refrigerator (HITACHI, Catalogue No.: HRPK0284A_E118-600L4D-STD-EX).
9. Stop watch (CTIZ / Japan, Catalogue No.:44448FHD).
10. Syringes (ATACO / China, Catalogue No.: CCD35560001).
11. Vortex mixer (Memmert/ Germany, Catalogue No.: CR076662088).
12. Wash bottle (BD Biosciences, Catalogue No.: 33635107).
13. Falcon® disposable 12 x 75-mm polystyrene test tubes (BD Biosciences, Catalogue No.: 343514).

Gating strategy

The dominant population of leukemic blasts was identified using side scatter versus CD45 dot plots to isolate the blast population. A 20% cut-off value was used to indicate the positivity of surface markers, while the cut-off value for cytoplasmic markers was 10%.

Software

Data acquisition and analysis was performed using FCS DIVA-8, made by the BD Software Company.

Ethical considerations

Written informed consent was obtained from all participants, or the parents of those aged less than 18 years. The Medical Ethical Committee at the Oncology Center, Al-Kindy College of Medicine, Baghdad University, Iraq, approved this study (code: 52).

Statistical analysis

We used Microsoft Excel (v. 2010) to calculate frequencies and percentages of variables, and to calculate means and standard deviations (SDs).

Results

A total of 69 patients were newly diagnosed with AL; 24 patients with AML-M0, 12 females and 12 males; and 45 patients with T-ALL, 40 males and 5 females, as shown in Figure 1, the age and sex of patients is shown in Table 2 and Table 3¹⁰.

Regarding clinical findings, we listed in Table 4¹⁰ as follow: hepatomegaly in 24.6%; splenomegaly in 36.2%; LAP in

Figure 1. The frequency of both AML-M0 and T-ALL patients. AML-M0: acute myeloid leukemia, T-ALL: acute T-cell lymphoblastic leukemia.

Table 2. Patient diagnosis and gender.

Gender	All patients N = 69		AML-M0 N=24		T ALL N=45	
	N	%	N	%	N	%
Male	52	75.4	12	50	40	88.9
Female	17	24.6	12	50	5	11.1

Table 3. The age groups differences between AML-M0 and T-ALL patients.

Age	N	Min-Max	Mean±SD	P value
AML-M0	24	7-76	43.73±18.62	< 0.001
T-ALL	45	0.5-60	18.26±14.74	

44.9%; Pallor in 68.1%; bleeding in 71%; fever in 60.9%; pleural effusion in 1.4%; mediastinal mass in 5.8%.

This study identified 10 (41.6%) patients with AML-M0 who showed no aberrant antigen expression, while 33.3%, 16.6%, 8.3%, and 8.3% had aberrant CD7, CD56, CD2, and CD19 expression, respectively. In four (16.6%) cases of M0, there was more than one aberrant antigen expressed: two cases (8.3%) had both CD7 and CD56, one case (4.2%) had CD7 plus CD19, and one case expressed CD7 plus CD2. With regard to patients with T-ALL, we found the following: 7.0% of T-ALL patients had pro-T type, 58.0% had pre-T type, 13.0% had cortical type, and 22.0% had mature-T type, according to the European Group for the Immunological Classification of Leukemias (EGIL) criteria¹¹. Our study also showed that 55.5% of all patients with T-ALL lacked aberrant antigen expression, while 13.3%, 13.3%, 11.1%, 6.7%, and 2.2% had aberrant CD13, CD117, CD33, CD19, and CD56 expression, respectively. Six cases (13.3%) showed expression of more than one aberrant antigen, mainly CD13 plus CD10, CD19, or CD56; CD13 plus CD117 in two cases; and one case with aberrant CD33 plus CD19, Table 5, Table 6 and Table 7¹⁰.

Discussion

Flow cytometry of leukemic cells plays an essential role in the identification of leukemia cell lines, maturation stage, and detection of residual disease. Immunophenotyping can improve both the accuracy and reproducibility of leukemia classification and is considered particularly useful for identifying AML with lymphoid marker expression and ALL with myeloid marker expression¹². Some cases of AML associated with the expression of CD7 have a poor prognosis¹³, while other studies have reported CD2 and CD19 are better prognostic indicators in cases of AML¹⁴. In this study, 69 participants with *de novo* AL were enrolled, 45 of whom had T-ALL and 24 of whom had AML-M0. Most patients (88%) with T-ALL were male, which was in accordance with a study by Abid Salih and colleagues¹⁵ and also similar to another study that showed a predominance of males among patients with T-ALL¹⁶. The male:female ratio was 1:1 in patients with AML-M0 in our study, which was consistent with earlier work¹⁷.

In the present study, the most common features patients with T-ALL presented with were pallor and fever^{14,17-19}, followed by hepato-splenomegaly and lymphadenopathy, which is in accordance with the findings of other studies^{14,17,18}. The least common presenting features were CNS involvement, pleural effusion, and mediastinal mass^{19,20}.

Regarding the clinical features of AML-M0, the current study found that pallor and fever were present in 16 (66.7%) of patients, consistent with the findings of Ghosh *et al.*¹⁷. Anemia is a common feature of all acute leukemias and is due to bone marrow infiltration; again, our results are comparable with other studies¹⁶. Pahloosy *et al.* reported pallor and fever were prominent symptoms²⁰. The current study showed that bleeding occurred in 19 (79.2%) of patients with

Table 4. The distribution of clinical features in patients with AML-M0 and T-ALL.

Clinical features		All patients N = 69		AML\M0 N= 24		T ALL N=45	
		N	%	N	%	N	%
Hepatomegaly	Absent	52	75.4	24	100.0	28	62.2
	Present	17	24.6	0	0.0	17	37.8
Splenomegaly	Absent *	44	63.8	21	87.5	23	51.1
	Present	25	36.2	3	12.5	22	48.9
LAP	Absent *	38	55.1	20	83.3	44	97.8
	Present	31	44.9	4	16.7	1	2.2
Pallor	Absent*	22	31.9	8	33.3	14	31.1
	Present	47	68.1	16	66.7	31	68.9
Bleeding	Absent*	20	29.0	5	20.8	15	33.3
	Present	49	71.0	19	79.22	30	66.7
Fever	Absent*	27	39.1	8	33.3	19	42.2
	Present	42	60.9	16	66.7	26	57.8
Pleural effusion	Absent*	68	98.6	24	100.0	44	97.8
	Present	1	1.4	0	0.0	1	2.2
Mediastinal mass	Absent*	27	94.2	24	100.0	41	91.1
	Present	4	5.8	0	0.0	4	8.9

AML-M0. Such bleeding may be due to a number of causes, such as thrombocytopenia due to bone marrow suppression by infiltrating malignant leukemic cells, and by platelet dysfunction that accompanies leukemic involvement in the bone marrow¹⁶. Gaydos *et al.* were the first to report this in 1962, when they found that there was a linear relationship between bleeding and platelet count²¹. Qazi *et al.* correlated this feature with thrombocytopenia alone or disseminated intravascular coagulopathy (DIC)²². Extramedullary infiltration by leukemic cells may result in lymphadenopathy, splenomegaly, and/or hepatomegaly. In our patients, we observed hepatomegaly in 24.6% of them. We concluded that pleural effusion was absent in AML-M0 patients but present in one (2.2%) patient with T-ALL. Regarding mediastinal mass, this was absent in cases of AML-M0, and this agree with that noted by Ghosh *et al.*¹⁷.

The incidence of lymphoid antigen expression in AML-M0 cases was 58.4%, which was higher than that seen by El-Sissy *et al.*¹² and Khalidi *et al.*²³, who found aberrant lymphoid antigen expression in AML to be 47% and 48.1%, respectively. The incidence of CD56 expression was 16.6% in this

study, which was similar to rates recorded in previous studies^{24–28}. Many studies have concluded that overall survival was significantly decreased in individuals who expressed CD56^{24–26}. Regarding CD7, it was expressed in (33.3%) of patients with AML-M0, which was in agreement with other studies that found that CD7 to be aberrantly expressed in 10%–40% of blasts in AML patients²⁶. Kita *et al.*¹³ showed that CD7 expression on cells is indicative of phenotype, is functional when immature, and is regarded as a prognostic risk factor.

Here, the most commonly expressed lymphoid marker was CD7 (33.3%), which is the same as recent reports by Bahia *et al.*²⁹ and Zheng *et al.*³⁰, although it is higher than the results reported by Venditti *et al.*³¹. CD4 was expressed in 25% of patients with AML-M0, which is comparable to one other study³² but higher than values reported by some other studies, e.g., 17.5%³³ and 16%²³.

In the current study, CD19 was expressed in 8.3% of patients, which is in accordance with the range of 8.6%–10% found in other studies like Khalidi *et al.*²³, Bahia *et al.*²⁹ and Venditti *et al.*³¹ but contrasts with other reports of 14%³⁴ and even lower

Table 5. Patterns of markers expression in both AML-M0 and T-ALL.

		Negative No (%)	Dim Positive No (%)	Moderate positive No (%)	Bright positive No (%)	Heterogeneous in positive No (%)	Total Positive No (%)
CD45	AML\M0 (24)	1 (4.2)	1 (4.2)	22 (91.7)	0	0	23 (95.8)
	T-ALL (45)	0	1 (2.2)	36 (80.0)	8 (17.8)	0	45 (100)
HLADR	AML\M0 (24)	4 (16.7)	4 (16.7)	9 (37.5)	4 (16.7)	3 (12.5)	20 (83.3)
	T-ALL (45)	41 (91.1)	1 (2.2)	2 (4.4)	1 (2.2)	0	4 (8.9)
CD33	AML\M0 (24)	4 (16.7)	5 (20.8)	11 (45.8)	0	4 (17)	20 (83.3)
	T-ALL (45)	40(88.9)	2 (4.4)	1 (2.2)	0	2 (4.4)	5 (11.1)
CD13	AML\M0 (24)	4 (16.7)	6 (25.0)	11 (45.8)	2 (8.3)	1 (4.2)	20 (83.3)
	T-ALL (45)	39 (86.7)	4 (8.9)	0	0	2 (4.4)	6 (13.3)
CD117	AML\M0 (24)	3 (12.5)	4 (16.7)	9 (37.5)	2 (8.3)	6 (25.0)	21 (87.5)
	T-ALL (45)	39 (86.7)	2 (4.4)	2 (4.4)	0	2 (4.4)	6 (13.3)
CD10	AML\M0 (24)	0	0	0	0	0	0
	T-ALL (45)	39 (86.7)	3 (6.7)	3 (6.7)	0	0	6 (13.3)
CD19	AML\M0 (24)	22 (91.7)	1 (4.2)	1 (4.2)	0	0	2 (8.3)
	T-ALL (45)	42 (93.3)	3 (6.7)	0	0	0	3 (6.7)
CD56	AML\M0 (24)	20 (83.3)	0	3 (12.5)	0	1 (4.2)	4 (16.6)
	T-ALL (45)	44 (97.8)	0	0	0	1 (2.2)	1 (2.2)
Cytoplasmic TdT	AML\M0 (24)	22 (91.7)	0 (91.7)	2 (0.0)	0 (8.3)	0	2 (8.3)
	T-ALL (45)	29 (64.4)	13 (28.9)	3 (6.7)	0	0	16 (35.6)
CD1a	AML\M0 (24)	0	0	0	0	0	0
	T-ALL (45)	39 (86.7)	0	5 (11.1)	0	1 (2.2)	6 (13.3)
CD2	AML\M0 (24)	22 (91.7)	1(4.2)	1 (4.2)	0	0	2 (8.3)
	T-ALL (45)	6 (13.3)	4 (8.9)	29 (64.4)	4 (8.9)	2 (4.4)	39 (86.7)
Surface CD3	AML\M0 (24)	0	0	0	0	0	0
	T-ALL (45)	19 (42.2)	6 (13.3)	13 (28.9)	5 (11.1)	2 (4.4)	26 (57.8)
CD4	AML\M0 (24)	18 (75.0)	2 (8.3)	3 (12.5)	0	1 (4.2)	6 (25.0)
	T-ALL (45)	19 (42.2)	3 (6.7)	14 (31.1)	2 (4.4)	7 (15.6)	57.8
CD5	AML\M0 (24)	0	0	0	0	0	0
	T-ALL (45)	16 (35.6)	10 (22.2)	16 (35.6)	1 (2.2)	2 (4.4)	29 (64.4)
CD8	AML\M0 (24)	0	0	0	0	0	0
	T-ALL (45)	22 (48.9)	1 (2.2)	14 (31.1)	2 (4.4)	6 (13.3)	23 (51.11)
CD34	AML\M0 (24)	11 (45.8)	1 (4.2)	7 (29.2)	3 (12.5)	2 (8.3)	13 (54.16)
	T-ALL (45)	29 (64.4)	4 (8.9)	4 (8.9)	1 (2.2)	7 (15.6)	16 (35.56)
CD7	AML\M0 (24)	16 (66.7)	3 (12.5)	5 (20.8)	0	0	8 (33.3)
	T-ALL (45)	2 (4.4)	2 (4.4)	28 (62.2)	13 (28.9)	0	43 (95.6)
Cytoplasmic CD3	AML\M0 (24)	0	0	0	0	0	0
	T-ALL (45)	19 (42.2)	6 (13.3)	9 (20.0)	10 (22.2)	1 (2.2)	26 (57.8)

Table 6. The frequency of aberrant antigen expression in T-ALL patients.

	N	%
T-cell Acute lymphoblastic leukemia without aberrant expression	25	55.5
T-cell Acute lymphoblastic leukemia with aberrant CD13	6	13.3
T-cell Acute lymphoblastic leukemia with aberrant CD117	6	13.3
T-cell Acute lymphoblastic leukemia with aberrant CD10	6	13.3
T-cell Acute lymphoblastic leukemia with aberrant CD33	5	11.1
T-cell Acute lymphoblastic leukemia with aberrant CD19	3	6.7
T-cell Acute lymphoblastic leukemia with aberrant CD56	1	2.2

*Some cases had more than one aberrant antigen expression.

Table 7. The frequency of aberrant antigen expression in AML-M0 patients.

	Frequency*	Percent 100%
AML/M0 without expression	10	41.6
AML/M0 with aberrant CD7 expression.	8	33.3
AML/M0 with aberrant CD4 expression	6	25
AML/M0 with aberrant CD56	4	16.6
AML/M0 with aberrant CD2	2	8.3
CD19	2	8.3

*Some cases had more than one aberrant antigen expression.

than 2.5%³⁵. The current study showed that CD2 was expressed in 8.3% of cases of AML, similar to the proportion reported by Thalhammer-Scherr *et al.*³⁶, who found the expression of CD2 to be 3%, while Bahia *et al.*²⁹ and Venditti *et al.*³¹ found higher values, of 11.4% and 14%, respectively.

The pattern of myeloid expression is correlated with some genetic features of blast cells. CD15, CD33, and CD65 are expressed in ALL cases with a rearranged MLL gene, and CD13 and CD33 are expressed in cases with the ETV6-RUNX1 gene³⁷. The presence of these antigens lacks prognostic significance in contemporary treatment programs³⁸.

The CD13 and CD33 were expressed in 6 (13.3%) and 5 (11.1%) of cases, respectively, which is consistent with another study³⁹. Acute T-cell lymphoblastic leukemia with aberrant CD117 presented in 6 (13.3%) patients and is also comparable with the proportion seen in other studies^{40–43}. Three (6.7%) patients with T-cell acute lymphoblastic leukemia expressed aberrant CD19, a lower value than other studies which found

the expression of CD19 in cases of T-ALL to be 22%⁴⁴. T-cell acute lymphoblastic leukemia expressing aberrant CD56 was only seen in one (2.2%) patient, and this is consistent with other studies⁴⁵.

Conclusion

We concluded that aberrant expression of CD markers was present in some cases of acute leukemia. The incidence of aberrant antigen expression was comparable with other studies and represents an indicator of poor prognosis. CD13, CD33, and CD117 were the most frequent aberrant myeloid antigens expressed in T-ALL cases, while CD19 and CD56 were expressed in fewer cases. A large number of AML cases had aberrant lymphoid phenotypes, with T-cell markers more common than B-cell markers. In addition, CD7 was a common aberrantly expressed lymphoid marker in AML. In the future, correlation studies of cytogenetic and lymphoid phenotypes should be performed to determine if there is any association between specific cytogenetic anomalies and the aberrant expression of certain lymphoid markers in patients with

AML, or between specific cytogenetic anomalies and myeloid markers in patients with ALL.

Data availability

Underlying data

Zenodo: Leukemia data. <http://doi.org/10.5281/zenodo.4021334>¹⁰

This project contains the following underlying data:

- Pdf files for each participant including a immunophenotyping report with scatter plots

- Data.xlsx (Dataset containing immunophenotyping results for all participants)

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](#) (CC-BY 4.0).

Acknowledgments

Great thanks for **Adam Bodley, MAEd** from Peerwith for his copyediting services of this work.

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Buldini Barbara

Laboratory of Hematology-Oncology, University of Padova, Padova, Italy

The authors submitted an original paper on immunophenotypic characterization of acute myeloid leukemia with minimal differentiation and T-ALL.

The paper must be a 'Not Approved' due to the following points:

- The Aim of the study is not clear.
- Criteria for Antigen expression is not defined.
- Introduction: The cornerstone of leukemia diagnosis, according WHO includes also morphology and cytogenetics. This should be mentioned. The definition of the AML subgroup included in the study is confounding: the authors used indifferently the terms "M0-AML" (FAB classification) and "AML with minimal differentiation" (WHO classification). Whereas the two terms are not interchangeable and refer to different AMLs subtypes. Moreover, authors mentioned to study also "the correlation with clinical and prognostic criteria": in the paper, survival analysis are missing.
- The study cohort is small and very heterogeneous, including both pediatric and adulthood ALs.
- Inclusion and exclusion criteria are not clear: e.g., Exclusion criteria 4 contrasts with inclusion criterion 3; Inclusion criteria 4 should mention the diagnostic criteria (according WHO Classification?); Exclusion criteria 1 and 3 are not clear: this is an IF study on AL at diagnosis, why these patients should be excluded?
- Diagnostic criteria are not relevant to the study purpose since the authors did not associate them to the study topic (ALs immunophenotype).
- The statistical explanation of sample dimension calculation is not clear. It seems unlikely that T-ALL and M0-AML represent half of the patients with a newly diagnosed AL in the study period.

- Samples preparation and acquisition are not accurately described:
 - to obtain accurate staining, the total amount of MoAbs should be calculated on samples WBC counts rather than blood volume.
 - No data on the total number of stained and acquired cells per sample are available.
- The results are not accurately presented and include data that went beyond the aim of the study:
 - The authors did not report the total number of newly diagnosed ALs (BCP-, T-, myeloid) in the study period. 69 patients with newly diagnosed AL were included while Figure 1 includes only AML M0 and T-ALL.
 - The authors included clinical data without correlating them with immunophenotypic results.
- References are obsolete (29/45 date more than 10 years) and incomplete. WHO classification is not included, and this is the basis for leukemia diagnosis.
- The manuscript needs copy editing for English language.

Is the work clearly and accurately presented and does it cite the current literature?

No

Is the study design appropriate and is the work technically sound?

No

Are sufficient details of methods and analysis provided to allow replication by others?

No

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Pediatric leukemias

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Reviewer Report 20 January 2021

<https://doi.org/10.5256/f1000research.27503.r77230>

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John K. Choi

Department of Pathology, University of Alabama at Birmingham, Birmingham, AL, USA

The authors present a well written article of a single institution review of their AML-M0 and T-ALL cases in regards to immunophenotype and clinical findings. They conclude that most AML-M0 have no aberrant antigen expression and most T-ALL are in the pre-T development phase, findings compatible with previous publications.

There are concerns that question the validity of their conclusions.

1. The authors appear to use EGIL classification scheme for assigning lineage to their leukemia. Unfortunately, the most current classifications use the WHO criteria with the latest being the 2016 publication. Many cases appear misclassified and need to be reclassified using the most current scheme. For example, only 42.2% of the T-ALL cases are cCD3 positive although the current WHO criteria require cCD3 positivity for a diagnosis of T-ALL. It is very likely that the CD3 negative cases would be reclassified as AML-M0 with potentially different conclusions.
2. The authors should specify the diagnostic criteria for AML-M0 and T-ALL in their method section.
3. Given that the manuscript details the clinical findings and blast immunophenotype at diagnosis, it is unclear why the authors should exclude patients with poor outcome or treatment issue.
4. In the introduction, last sentence, the authors wanted to "...its correlation with ... prognostic criteria." The results for this is not presented (see concern 3 above).
5. The authors cite the large AML Iraqi study (ref 33) but the authors should also cite the large ALL Iraqi study by Jalal *et al.* ISLH 2017;39:625.
6. Although the results are not novel, they do document the findings in Iraq and the authors are encouraged to include this info in the title for ease of searching.
7. The authors conclude that "most AML-M0 have no aberrant antigen expression" However, this represent only 41.6% of AML-M0 cases. That would mean that 58.4% or most of AML-M0 cases have aberrant expression.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Flow cytometry based MRD analysis of acute leukemia.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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