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Original Research Article.

Flow Cytometric Immunophenotyping of Acute Leukaemia in Adult and Its Comparison with Cytomorphology

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ABSTRACT

Background: Immunophenotyping has become an inseparable tool for precise characterization of acute leukemia (AL), which is the corner stone of therapeutic success in these cases. There is paucity of data regarding immunophenotypic profile of adult acute leukemia in Bangladeshi population. This study was carried out to see the immunophenotypic pattern of different types of AL in our population and also to compare between results of flow cytometric immunophenotyping and cytomorphologic assessment.

Methods: A total of seventy (70) morphologically diagnosed adult AL cases (age≥ 18 years) from Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka Medical College & Hospital (DMCH), Sir Salimullah Medical College & Mitford Hospital (SSMC & MH), and National Institute of Cancer Research & Hospital (NICRH) were analyzed by 4-color flow cytometry for immunophenotyping using a predefined panel of antibody. All the samples were reviewed for morphologic features by light microscope before flow cytometric assessment.

Results: Flow cytometry could assign lineage to 64 cases out of 70. Among the 64 cases 31 (48.4%) cases were Acute Myeloblastic Leukaemia (AML), 31 (48.4%) cases were Acute Lymphoblastic Leukaemia (ALL), and 2 (3.12%) were mixed phenotype acute leukemia. CD13 and CD117 were the mostly expressed markers (96.8%) in the AML cases while CD19 was most frequent (100%) in B-ALL, and CD3 was mostly expressed (100%) in T-ALL. Aberrant antigen expression was found in 39.1% of the acute leukemia cases. Complete lineage agreement rate was higher in AML (89.7%) than that of ALL (80.6%). Complete Discordance was found in 9.4% cases between the results of flow cytometry and cytomorphology.

Conclusion: Inclusion of flow cytometry in routine diagnostic workup of acute leukemia ensures proper characterization as well as management of these cases.

Keywords: Immunophenotypic Profile, Flow Cytometry, Adult Acute Leukemia, Cytomorphology.

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INTRODUCTION

Acute leukemia is a heterogeneous group of hematologic malignancies affecting blood and bone marrow cells. The disease follows mostly a poor prognosis but some specific types have better prognoses with specific therapeutic options.[1] Flow cytometry can reliably made distinction between lymphoid and myeloid leukemia, which is crucial for its management. Leukemic blast shows distinct pattern of antigenic phenotype that reflects the pattern of antigen acquisition seen in normal hematopoietic differentiation, yet invariably demonstrate distinct aberrant immunophenotypic features. Detailed understanding of these phenotypic patterns of differentiation, particularly in myeloid leukemia, allows for more precise classification of leukemia than does morphology alone.[2] Multi-parameter flowcytometry is a useful adjunct to morphology and cytochemistry and it is an invaluable tool in the diagnosis of Acute Leukaemia.[3] Flowcytometry of leukemic cells plays an essential role in identification of leukemia cell line, maturation staging and residual disease detection. Several advances in flowcytometry, including availability of an expanded range of antibodies and fluorochromes, improved gating strategies, and multi-parameter analytic techniques, have all dramatically improved our ability to identify different normal cell populations and recognized phenotypic aberrancies, even when present in a small proportion of the cells analyzed.[4] The distribution of leukemia type varies with age of the patient. This study was done to see various types, subtypes, aberrant antigen expression pattern of acute leukemia in adult patients in Bangladeshi population and also to compare the results between flow cytometry and microscopic morphology.

MATERIALS AND METHODS

This cross sectional prospective study was conducted from March, 2015 to February, 2016.

Blood and bone marrow samples were collected from Department of Hematology of Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka Medical College & Hospital (DMCH), Sir Salimullah Medical College & Mitford Hospital (SSMC & MH) and National Institute of Cancer Research and Hospital (NICRH) Dhaka. Morphologic features of all the samples were reviewed by qualified hematologist of Department of Clinical pathology, BSMMU. Rest of the laboratory works were done in the Department of Microbiology & Immunology, BSMMU.

The protocol was approved by the Institutional Review Board (IRB) of BSMMU.

Study Population

Seventy adult patients aged 18years or above, attending the Department of Hematology of BSMMU, DMCH, SSMC & MH and NICRH who were newly diagnosed as acute leukemia by cytomorphology, were included in the study. Informed written consent was taken from all patients. Consecutive sampling procedure was followed for this purpose. Patients who were suffering from chronic myeloid leukemia with blastic crisis, myelodysplastic syndrome, and other myeloproliferative disorders or have received chemotherapy for acute leukemia were excluded from the study.

Sample Collection

Preferred sample was bone marrow aspirate and in case of unavailability peripheral blood was collected. 2 ml of bone marrow aspirate was collected from 29 patients. Collection was performed by experienced personnel of the Hematology Department of the respective institutes from posterior superior iliac spine after ensuring strict asepsis and necessary precautions. 2 ml of peripheral blood from41 patients were collected mostly from antecubital vain with aseptic precautions. All the samples were collected in EDTA tubes.

Morphologic Assessment

All specimens were obtained and prepared for morphologic examination using standard techniques**.** Smears were air dried & stained by Leishman stain followed by light microscopy.

Immunophenotyping

Sample collected in EDTA tube was immediately transported to the lab for immunophenotyping. Measured amount of sample was taken in previously marked tubes to ensure approximate cell concentration of 10^6 per ml. Pre titrated volume of specific antibodies or antibodycocktails were added to specific tubes followed by incubation in dark for 20 minutes. Lysing solution BD FACSLyse™ (1X) was added and incubated further for 10-12 minutes. Then temperature regulated centrifugation was done at 200-300g for 5 minutes at 25^0 C and supernatant discarded. Washing &centrifugation process was repeated once. Cells were finally re-suspended in 0.5ml sheath fluid or PBS with 2% paraformaldehyde. This was done for surface markers. But for staining of intracellular markers 0.5ml of permeabilizing solution Perm2™ (1X) was added to the tubes after centrifugation and incubated for 10 minutes in the dark. Then cells were washed by sheath fluid and centrifuged at 300g for 5 minutes and supernatant discarded. Addition of pre titrated volume of antibodies or antibody cocktail against intracellular antigens was done followed by incubation in dark for 10-15 minutes. Then the steps are same as for extracellular markers as washing, centrifugation, and final preparation in sheath fluid or PBS.

Following marker combinations of fluorochrome tagged monoclonal antibodies (MoAb) were added to different tubes for detection of various cellular markers by flow cytometry

For T – cell: cytoplasmic (cy) CD3, CD5, CD7

For B – cell: CD19, CD10, cyCD79a

For Myeloid cells: CD13, CD33, CD117, CD14, CD15, CD64 and cytoplasmic myeloperoxidase (cyMPO)

Pan leukocyte marker: CD45

Precursor marker: CD34, TdT, HLA-DR

Four color FCM immunophenotyping was performed on BD FACSVerse™ by collecting 10,000 ungated list mode events. The blast gating strategy included using dot plots of CD45 expression versus side scattering (SSC) and also a second gating strategy using forward scattering (FSC). Back gating was also done when required. Analysis of different parameters of the gated cells were done by standard method. Any antigenic marker was considered positive if 20% or more of the blast cells reacted with a particular antibody.

Statistical Analysis

All data after collection by data sheet were checked, edited analyzed by using computer based SPSS (Statistical Package of Social Science, version 20) software. Results were presented in the form of tables and figures. Descriptive analysis of all relevant variables was done by using proportion, central tendency and dispersion. P value was calculated by Chi square test. P value & lt; 0.05 was considered as minimum level of significance.

Table I: Distribution of Acute leukemia cases according to types and sub types (n=64)

Acute leukemia type	No.	%	Acute leukemia subtype	No	%
Acute Myeloblastic Leukemia (AML)	31	48.4			
Acute lymphoblastic Leukemia (ALL)	31	48.4	B-ALL	21	67.7
			T-ALL	10	32.3
Mixed phenotype acute leukemia (MPAL)	2	3.12	B+My	1	50
			B+T	1	50
Total	64	100			

Table II: Immunophenotypic pattern of acute myeloblasticleukemia (n=31)

Markers	No.	%
CD13	30	96.8
CD33	28	90.3
CD117	30	96.8
cyMPO	25	80.6
CD34	23	74.2
HLA-DR	23	74.2
CD14	02	6.5
CD15	02	6.5
CD64	14	45.2
CD235	01	3.2

Table III: Immunophenotypic pattern of patients with ALL (n=31)

Markers	B – ALL 21/31	T – ALL 10/31
	n (%)	n (%)
CD34	17 (80.9)	03 (30)
TdT	10 (47.6)	05 (50)
HLA-DR	19 (90.5)	02 (20)
cyCD79a	20 (95.2)	
CD19	21 (100)	
CD10	17 (80.1)	05 (50)
CD3		10 (100)
CD5		08 (80)
CD7		09 (90)

Table IV: Comparison between cytomorphology and flow cytometric results (n = 70)

Cytomorphology report	No.	Flow cytometric results	No.
AML	31	AML	26
		B – ALL	02
		T – ALL	00
		MPAL	01
		Undetermined*[3]	02
ALL	35	B – ALL	17
		T – ALL	08
		AML	05
		MPAL	01
		Undetermined*[3]	04
MPAL*[1]	01	B- ALL	01
Unclassified*[2]	03	B – ALL	01
		T – ALL	02

*[2] Unclassified: By cytomorphology presence of only blast cells not specified as myeloblast/ lymphoblast

*[3] 6 cases were undetermined by flow cytometry as the blast count was < 20% in the sample.

Table V: Concordance and discordance between results of cytomorphology and flow cytometry (FCM) n=64

Concordance/Discordance	No.	%
Complete concordance	52	81.2
Partial concordance	06	9.4
Discordance	06	9.4
Total	64	100

Concordance: Similar result between cytomorphology and FCM

Discordance: Different result in cytomorphology and FCM

Table VI: Distribution of acute leukemia (AL) patients according to age group (n = 64)

Acute Leukemia cases	Age in years				Total
	(≥18 – 20)	21- 40	41 – 60	61 – 80	No. (%)
	No. (%)	No. (%)	No. (%)	No. (%)	
AML	8 (25.8)	11 (35.5)	08 (25.8)	04 (12.9)	31 (48.4)
B – ALL	14 (66.7)	4 (19.04)	3 (14.3)	0	21 (32.8)
T – ALL	4 (40.0)	6 (60)	0	0	10 (17.2)
MPAL	01 (50)	01 (50)	0	0	02 (3.12)
Median age= 25.5 years					

RESULTS

A total of 70 morphologically diagnosed adult patients with acute leukemia were enrolled in this study. Among them 64 (91.4%) could be characterized by flow cytometry (FCM) while 6 (8.6%) cases remained undetermined as blast cell population was <20%. Out of 64 acute leukemia cases, 31 (48.4%) cases were AML, 31 (48.4%) cases were ALL& 2(3.12%) were of mixed phenotype acute leukaemia (MPAL). Among the ALL cases 21 (67.7%) cases were B – ALL and 10 (32.3%) cases were T – ALL.(Table I)

Among the cases of AML highest expression rate was found 96.8% for both CD13 and CD117 followed by 90.3 % for CD33.cyMPO was positive in 80.6% cases. Markers of immaturity HLA-DR and CD34 were found in 74.2% cases. Among the AML cases 2/31 (6.5%) were both CD34&HLA-DR negative & both were provisionally diagnosed as acute promyelocytic leukemia (APL) by flow cytometry. (Table II)

All of the 21 cases of B – ALL cases were positive for CD19. Expression rate for cyCD79a was 95.2% (20/21) cases, 90.5% (18/21) for HLA-DR. Of the 10 T – ALL cases CD3 is positive in 100% cases, CD7 in 9(90%) cases, CD5 in 8 (80%) cases. TdT, and CD10 were positive in 5 (50%) cases. (Table - III)

Expression of mixed phenotypic markers were found in 3.1% cases (2/64). Between them one was diagnosed as B + My MPAL while the other as B + T MPAL (Table II).

Overall calculation was done on 64 samples as 6 samples have blast count < 20%. Among 31 morphologically diagnosed AML cases 26 were confirmed as AML by FCM while in ALL group 26 cases among 35 were confirmed as ALL by both method. (Table IV) Out of 64 cases 90.6 (58) cases showed total concordance that includes both complete and partial concordance (Table V).

Out of 31 AML patients 35.5%) (11/28) were in 21 – 40 years age group, followed by 25.8% (8/28) in both ≥18 – 20 years and 41 – 60 years age group. Of the 20 B-ALL patients 14 (66.7%) were in ≥18 – 20 years age group, 19.04% (04/21) in 21 – 40 years age group. Out of the 10 T – ALL patients 60% (6/10) were in 21-40 years age group and remaining. Only 02(3.13%) patients were diagnosed as MPAL each of which fell in the ≥18-20 years and 21 – 40 years age group (Table VI).

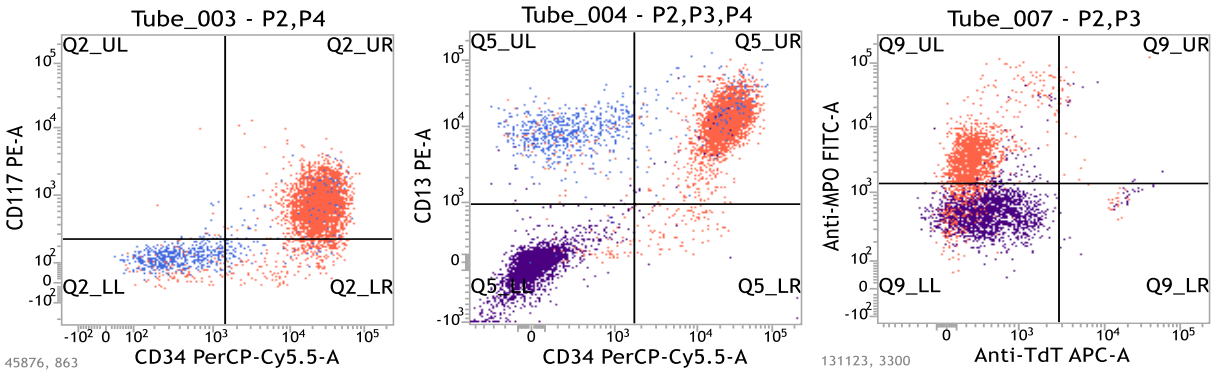

Fig I: Flow cytometric immunophenotypic pattern of Acute Myeloblastic Leukaemia showing positivity for CD34, CD117, CD13, &cyMPO

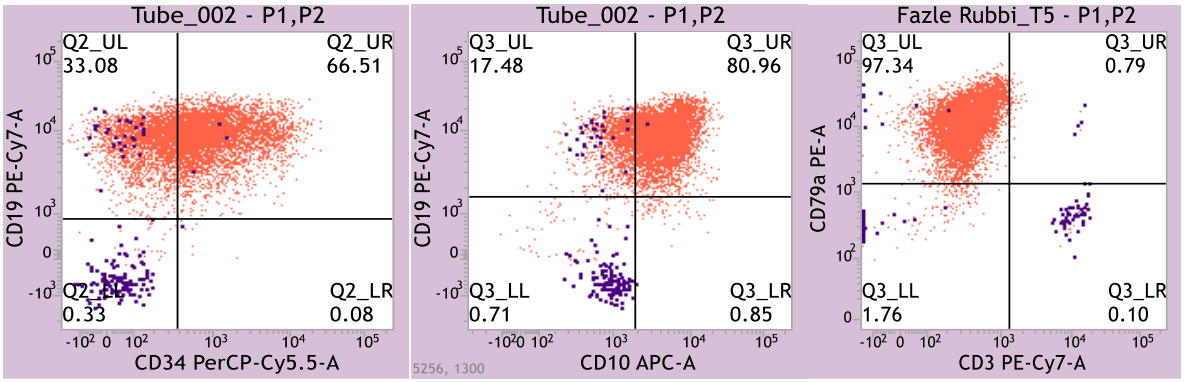

Fig II: Flow cytometric immunophenotypic pattern of acute lymphoblastic leukaemia showing positive CD34, CD19, CD79a & CD10 markers indicating a B-ALL

DISCUSSION

The acute leukemias (AL) are a heterogeneous group of diseases characterized by the rapid expansion of a malignant clone of early hematopoietic progenitors that ultimately replace normal bone marrow tissue resulting in marrow failure. Timely intervention can minimize morbidity in AL they are usually highly responsive to chemotherapy in the initial phase.[5,6] Earlier classification system for acute leukemias was based solely on cytomorphology and cytochemical examination. World Health Organization's in its 2008 Classification of Tumours of Haematopoietic and Lymphoid Tissues incorporated immunophenotyping along with other parameters for best patient outcome. For immunophenotyping of leukemia flow cytometry is the most preferred and convenient method as large number of cells can be assessed accurately within a very short period of time.

This study included 70 adult patients (age ≥18) with acute leukemia diagnosed by cytomorphology. Immunophenotyping was carried out by flow cytometry (FCM) from 29 bone marrow and 41 peripheral blood samples.

Among the 70 samples studied, 64 could be assigned a given lineage indicating that FCM was able to type an acute leukemia in 91.4% cases which is similar to the findings of Feki et al. in 2000 (93.9% cases). In this study, 6 (8.6%) cases were undetermined by FCM as blast count was <20%. Among the 6 samples 5 were peripheral blood and 1 bone marrow. In all these undetermined samples respective bone marrow sample were again sought but not available. It is suggested that ≥30% blast in the peripheral blood in morphology is a prerequisite for a good correlation with FCM findings.[7] The bone marrow sample had 20% blast in the morphologic examination. This variation may be due to errors in sample collection, processing, or due to dilution done during flow cytometric sample processing.

In this study, 64 acute leukemia (AL) cases were diagnosed by flow cytometry (FCM) of which 31 (48.4%) were AML, 31 (48.4%) were ALL and 2 (3.2%) were mixed phenotype acute leukemia (MPAL). But different studies reported the incidence of AML & ALL were 70% & 30%; 53% & 47%; and 60% & 40% respectively.[1,2,8] So this study differs from other studies. In this study 27/64 (42.2%) cases were in ≥18-20 years age group where ALL was 66.7% (18/27). But in the rest 37 cases aged ≥21years AML were found in 23/37 (62.8%) cases and ALL in 13/37 (35.1%) cases. Various studies reported ALL to be the predominant type of acute leukemia in early years of life and this trend continues up to 20 years, incidence of AML rises with age that mainly affects the adult & elderly population.[3,9] So this variation regarding the distribution of leukemia types may be explained in part by younger age of acute leukemia patients.

Expression rate of both CD13 and CD117 were found to be highest (96.8%) among the AML patients which were nearly similar to the results of other studies as CD13 (100% and 95%); andCD117 (100% and 73%) respectively.[10,11] These markers are specifically useful for the identification of morphologically undifferentiated AML cases.[12]

Expression of CD33, an important myelomonocytic marker, was found 90.3% in the AML cases which is consistent with other studies.[1,2]

Cytoplasmic MPO, a very crucial marker for diagnosis of myeloid leukemia, was detected in 80.3 % AML cases. A study done in 2000 showed the positivity of 73%.[13] So the findings of the study is a bit higher than those studies which may be due to very small sample size.

Combination of CD14, CD15 and CD64 is important for differentiation of monocytic origin AML. Combination of any two of these three markers was found in 3/31(9.7%) cases that defines a specific type of AML (AML-M5) which is slightly higher than Harani et al., 2005 (6%).

Hematopoietic progenitor cell markers CD34 and HLA-DR were expressed in 74.2% of AML cases which is nearly similar to this study.[13]

It was found that 6.45% (2/31) AML cases were suggestive of APML by flowcytometric profile which is similar to these studies that showed the incidence was nearly 10%. [2,15]APML has a different therapeutic protocol as well as prognosis.

In the current study 48.4% (31/64) cases were diagnosed as ALL by FCM. Among these ALL cases 67.7% (21/31) cases were B-ALL and the rest 32.3% (10/31) were T-ALL. Findings from these studies showed nearly similar percentage of B-ALL and T-ALL(76% & 24%; 64% & 34% and 75% & 25% respectively).[2,3,16]

Expression of CD19 in B-ALL cases was found 100% in this study which is consistent with other studies.[1,10] CD19 reactivity, for all purpose, is very important for detecting B- lineage leukemia.

Expression rate of cyCD79, very important for lineage assignment in B-ALL, was found 95.2% & that is nearly similar to other studies that reported 100% expression rate.[1,10,16]

CD34 is normally expressed in immature hemopoietic cells or blasts so is an excellent marker for monitoring blast population.[17] CD34 expression was found 80.9%. But variable expression rate were found in different studies as 61.2%, 58.8%, 76.3% respectively.[18,1,10] So the result was within the range.

Expression of HLA-DR in B-ALL cases was 90.5% in this study which is almost similar to the findings of other studies with result around95%.[10,1,18]

TdT, a nonspecific immaturity marker of lymphoid series, was expressed in 47.6% of B-ALL which is similar to this study (51.1%).[10]

T-ALL comprises 32.3% (10/31) of ALL cases. cyCD3, CD5, and CD7 were detected to define these cases.

Expression of cyCD3 in 100% cases of T-ALL was consistent with the findings of these studies.[1,10,2,19] cyCD3 is the most sensitive and specific marker for T-ALL.

Expression of CD7 was 90% (9/10) among the T-ALL cases which is within the range found in these studies (100%, 92.3%, and 85.7% respectively).[10,1, 20]

Expression of CD5 was found in 80% (8/10) of the T-ALL cases which is nearly similar to these study results (84.6%, and 86% respectively).[1,10]

HLA-DR was expressed in 2/10 (20%) of cases which is a bit higher than other studies (14% and 14.2% respectively).[10,20] Small sample size may be responsible for this discrepancies.

CD34 was expressed in 3/10 (30%) of the T- ALL cases which is consistent with the findings of these studies.[19,21]

Expression of TdT was 50% (5/10) in T-ALL cases. Most of the studies showed the expression ranging 80-100% which is higher than current study findings.[1,10,21] Lahjouji et al. reported TdT expression in adult T-ALL was reported 50% in Oman by which is similar to current study.[22] It is well established that TdT expression in T-ALL decreases with maturation and more mature T-ALL don't express this marker. So discrepancy of the current result with

most of the studies may be due to the patients in the current study suffering from more mature T-ALL, which could have been categorized precisely by using extensive panel of monoclonal antibodies. Or it may be due to smaller sample size of the study giving a false impression.

CD10 expression was observed in 50% of the cases in the current study which is nearly similar (43.1%) to this study done in India.[2]

In the study out of 2/64 (3.12%) cases showed the features of MPAL which is consistent with the these studies.[2,10] Flow cytometric assessment is unique in these cases as MPAL diagnosis by morphology is very critical in most of the cases.[23]

Flow cytometric analysis is critical for specific diagnosis of acute leukemia. Concordance between morphology and flow cytometric findings was 90.6 % (58/64) that included both complete and partial similar results. Discordant result was found in 9.4% (6/64) cases where flow cytometry was essential for lineage assignment. Among the 58 concordant cases 81.2% (52/64) had complete concordance and 9.4% (6/64) had partial concordance. Partial concordance was considered when blast type could not be confirmed morphologically or in MPAL cases where additional information regarding the phenotype of the blast lineage was found by FCM. These studies reported similar results regarding concordance between morphology and immunophenotyping (93.9% and 97% respectively).[3,25]

Out of the 64 cases, 31 were diagnosed as AML by cytomorphology. By flow cytometry (FCM), 3 cases were undetermined as there was <20% blast in the collected sample. The complete and partial lineage agreement in case of AML were 92.8% and 3.8% respectively.

Flow cytometric lineage assignment was not possible in 3 out of 35 morphologically diagnosed ALL cases as blast count was <20%. Complete and partial lineage agreement in case of ALL were 81.3% and 3.1% respectively.

1(1.56%) case of morphologically diagnosed MPAL was confirmed as B-ALL by FCM.

Finally complete lineage agreement between cytomorphology and flow cytometry were 92.8% for AML and 81.3% for ALL. A study in India showed concordance between cytomorphology and FCM was 86% and lineage agreement between 2 modalities was 91.6% for AML and 66.7% for ALL.[17] Another study showed concordance rate between morphology and FCM was 95.8%; and lineage agreement for AML and ALL were 89.2% and 80% respectively.[26] Higher concordance rate for AML in these studies is similar to the current study.

In the current study 49/64 (76.6%) acute leukemia cases were 18 – 40 years age group that form the major portion of the study population. Most of the (61.3%) AML patients are in <41 years age. Among the ALL patients 18/31 (58.1%) fall in the 18-20 years age group. These findings are also consistent with these studies that showed AML predominance is seen in <40 years age group and ALL is predominant in <20 age group respectively. [3,9]So the result of the current study regarding age distribution in acute leukemia types is nearly similar to these studies. Median age at the diagnosis of the acute leukemia in adult patients was found 25.5 years in this study. A study in Bangladesh showed that point to be 31 years.[9] An Indian study found the value to be 26 years in India.[27] Various studies done is US and European population revealed median age at diagnosis of acute leukemia in adults is around 55 years.[28,29,30]Difference in result is present with the western studies regarding the age at diagnosis of AL cases which may be possibly explained in part by the different structure of our population as nearly 2/3 of the Bangladeshi population are under 30 and the median age of entire population is 23.4 years.[31] Or it might be due to some environmental factors that needs to be established by further study.

CONCLUSION

AML was the predominant form of leukemia in adults aged more than 20 years. CD13 & CD 117 were the mostly positive marker for AML while for B-ALL and T-ALL they were CD19 & cyCD79a, and CD3 & CD7 respectively. Flowcytometric immunophenotyping could precisely delineate different forms of Acute Leukaemia and is especially important for confirming cytomorphologically diagnosed acute lymphoblastic leukemia.

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