ORIGINAL ARTICLE

Immunophenotyping of T-cell acute lymphoblastic leukaemia: Practical hurdles

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Abstract

Background: The 2016 WHO classification of T cell acute lymphoblastic leukemia suggest that cases of pre and pro T-cell Acute Lymphoblastic Leukaemia (T-ALL) now fall under Early T-cell precursor ALL (ETP-ALL) or Near ETP-ALL. Accurate subtyping is essential as the subsets are characterized by distinct molecular profiles and identifying molecular aberrations allow patients to receive novel treatment agents. *Aim and Objectives:* To study the immunophenotypic characteristics in T-ALL cases and assess the diagnostic difficulties encountered in its subtyping. *Material and Methods:* Thirty-seven cases of T-ALL were analysed on flow cytometry using a 6/8 colour panel of monoclonal antibodies, including B cell, T-cell, myeloid and stem cell markers. The cases were categorized as ETP-ALL, near ETP-ALL, cortical T-ALL and medullary T-ALL. Clinical details were retrieved from patient case files. *Results:* Patient age ranged from 1 to 64 years. Male to female ratio was 2.1: 1 and 35.1% cases were documented to have mediastinal mass. Mean haemoglobin level was 8.6 g/dl, and median total WBC count was $118.3 \times 10^3/\mu l$. Blast percentage ranged from 39-98%, with mean of 84.8%. On flow cytometry analysis, all cases expressed CD7 and cytoplasmic CD3. CALLA+ (CD10+) were 32.4% cases. The aberrant expression of B-cell marker (CD79a) was observed in 5.4% cases. Majority of the cases were classified as medullary T-ALL, constituting 37.8% cases. ETP-ALL were 21.6% cases, 13.5% cases were near ETP-ALL and 24.3% cases were cortical T-ALL. One case could not be definitely assigned to a specific category. *Conclusion:* This study reflects the difficulties encountered in subtyping of T-ALL cases due to antigenic overlap and/or due to lack of an extended panel of secondary markers.

Keywords: Immunophenotype, Acute Lymphoblastic Leukaemia, Flow Cytometry, Blasts

Introduction

T-cell Acute Lymphoblastic Leukaemia (T-ALL) is a neoplasm of lymphoblasts committed to the T-cell lineage, with more than 25% blasts in bone marrow. The unequivocal diagnosis of T-ALL is made on flow cytometric demonstration of cytoplasmic CD3 on the blasts. The blasts show CD7 and dim to moderate expression of CD45. Classically, T-ALL has been subtyped into four stages of intra-thymic differentiation: pro-T-ALL, pre-T-ALL, cortical T-ALL and medullary T-ALL. According to the 2016 World Health Organization (WHO) classification system for tumours of the hematopoietic and lymphoid tissues, most cases of pre and pro T-ALL now fall under ETP-ALL or Near ETP-ALL [1]. T-ALL constitutes nearly 15% of all paediatric ALL cases. With the better understanding of T cell biology, the 5 year survival rate has increased to 65-70%. In contrast to B ALL, the immunophenotype genotype association profile is not as well established in T-ALL [2].

Accurate subtyping is essential as the subsets are characterized by distinct molecular profiles and identifying molecular aberrations allow patients to receive novel treatment agents, such as target

therapies, as the front-line treatment. Hence genomic landscape provides a more logical approach to diagnosis of T-ALL with new therapeutic options [2]. This study was conducted with an aim to study the immunophenotypic characteristics in cases of T-ALL and to assess the diagnostic difficulties encountered in its subtyping.

Material and Methods

This is a retrospective study of 4 years (October 2016 till September 2020) carried out in the Haematology and Clinical Pathology Laboratory in a tertiary care centre in south India after obtaining Institutional Ethics Committee approval. Flow cytometry archives were reviewed for newly diagnosed T-ALL cases, including assay performed on both peripheral blood and/or bone marrow aspirate. Patients of both genders and between 0 to 90 years were included. Cases of acute leukemia of ambiguous lineage, patients who have been treated at a different facility before being referred to our institute and assay performed on body fluids were excluded. Data such as age, gender and presenting complaints were obtained from patient records. The presence of mediastinal mass was documented wherever available. The complete blood picture parameters such as haemoglobin levels, White Blood Cell (WBC) counts, platelet counts, blast percentages were analysed. Cases were analysed on flow cytometry using 6/8 colour panel of monoclonal antibodies. Blasts were gated as dim to moderate CD45 and low SSC. Markers used were T-cell antigens - CD7, cytoplasmic CD3, surface CD3, CD5, CD2, CD1a, CD4, CD8, TCR αβ, TCR γδ, CD25, and CD56, B-cell antigens -CD10, CD19, CD20, and cytoplasmic CD79a and myeloid/monocytic antigens-MPO, CD13, CD33, CD117, CD64, CD36 and CD14. Precursor-cell markers analysed were CD34 and HLA-DR. Positive staining was defined as at least 15% of blast cells showing expression of the marker of interest. Cases were then categorized as ETP-ALL, Near ETP-ALL, Cortical T-ALL and Medullary T-ALL according to the WHO 2016 guidelines.

Statistical analysis

It was performed using Jamovi software version 1.6.23. Categorical variables were represented as frequencies. Continuous variables were assessed for normal distribution by Shapiro-Wilk's test. The normally distributed data were presented as Mean ± SD and skewed data were depicted as median (interquartile range). Means were compared between the different subtypes of T-ALL using one way ANOVA (Fisher's). Value of $p < 0.05$ was considered as statistically significant.

Results

A total of 37 T-ALL cases were analysed during the study period. All continuous variables except haemoglobin % showed normal distribution ($p < 0.001$). Mean age was 19.5 ± 17.6 years. The age range was 1-64 years, with median age being 13 years (Figure 1 shows age distribution of cases) and 62% cases (n=23) were below the age of 18 years. Male to female ratio was 2.1: 1.

The most common symptom was fever, seen in 70.3% cases (n=26). Bleeding tendencies were noted in 35% cases (n=13). On clinical examination, 67.6% cases (n=25) had palpable splenomegaly and 51.4% cases (n=19) had enlarged lymph nodes. Mediastinal mass on radiology were documented in 35.1% cases (n=13).

On flow cytometry analysis, all cases showed CD7 and cytoplasmic CD3 positivity. Table 1 denotes the frequency of expression of different immunophenotypic markers. CALLA+ (CD10+) were found in 32.4% cases (n=12). The expression of one or more myeloid markers were found in 18.9% cases (n=7) and B-cell marker (CD79a) in 5.4% cases (n=2). Cases were classified as Early T-ALL (ETP-ALL), near ETP-ALL, cortical T-ALL and medullary T-ALL. The most common documented subtype was medullary T-ALL, constituting 37.8% cases (n=14) while 21.6% cases (n=8) were ETP-ALL, 13.5% cases (n=5) were near ETP-ALL and 24.3% cases (n=9) were cortical T-ALL. One case did not fit into any of the categories. Two cases that were previously subtyped at the time of diagnosis as Pre or Pro T-ALL were now reclassified as ETP-ALL according to newer guidelines. Similarly, 3 cases of near ETP-ALL previously subtyped as pre or pro T-ALL were reclassified. Among cases where follow up details were available, 5 cases expired, of which 2 were ETP-ALL and one from each of the other subtypes. 9 cases were treated according to the modified BFM-95 regimen. Seven cases of paediatric T-ALL were treated as per the modified ICICLE protocol.

Table 1: Number and percentage of cases showing positivity for various markers

Marker	Cases showing positivity N (%)
T cell markers	
Cd7	37 (100)
Cytoplasmic CD3	37 (100)
Surface CD3	24 (64.9)
CD5	33 (89.2)
CD2	34 (91.9)
CD1a	10 (27.0)
CD4	17 (45.9)
CD8	14 (37.8)
TCR αβ	5 (13.5)
CD19	0
CD20	0
CD10	12 (32.4)
CD79a	2 (5.4)
cMPO	0
CD56	4 (10.8)
CD13	3 (8.1)
CD33	4 (10.8)
CD117	3 (8.1)
CD64	2 (5.4)
CD34	20 (54.1)
HLA-DR	6 (16.2)

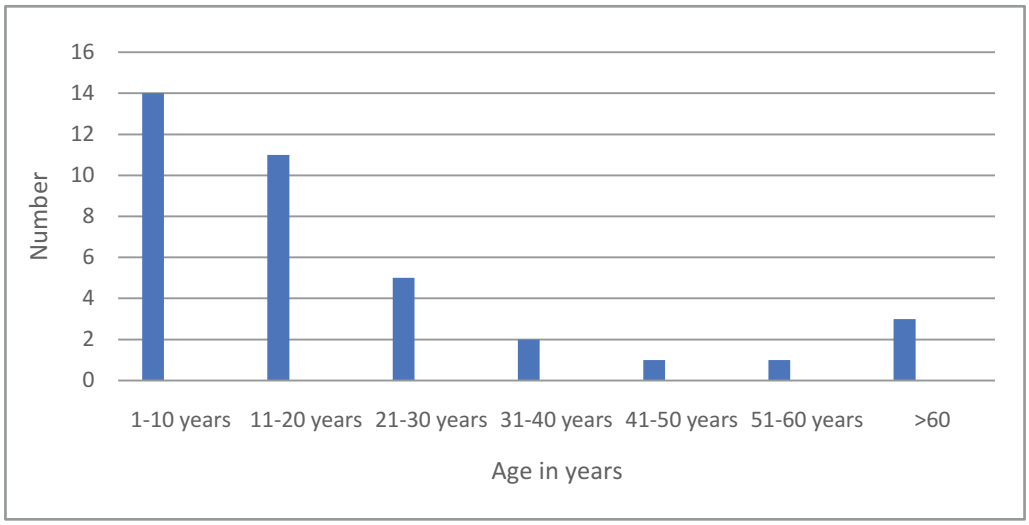

Figure 1: Age-wise distribution of the cases

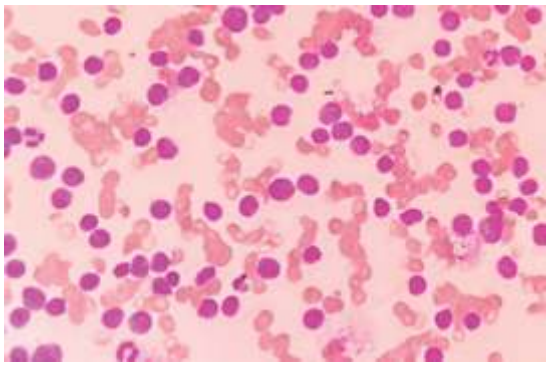

Figure 2: Peripheral smear of T-ALL with 96% blasts (Leishman stain 400X)

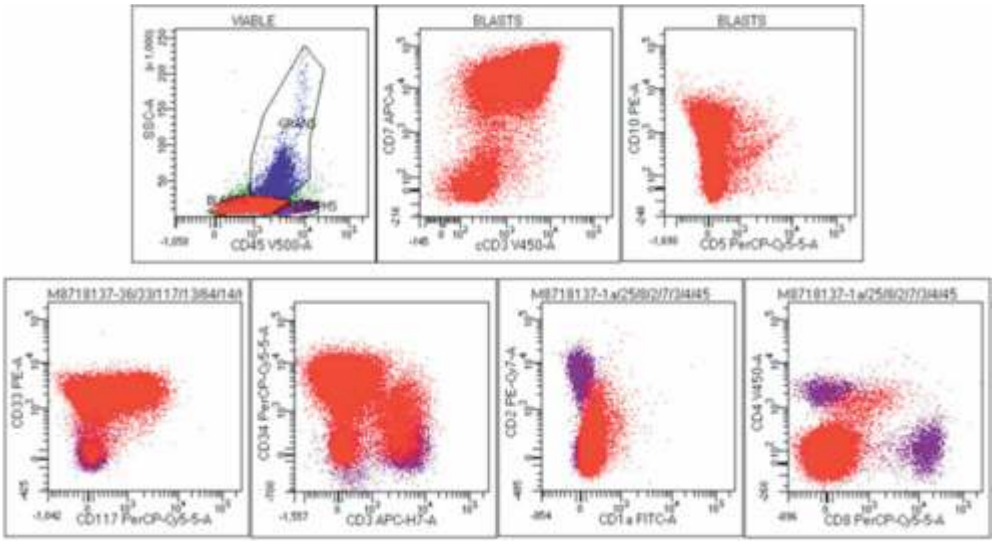

Figure 3: Flow plots of a case of ETP-ALL-blasts gated as dim CD45 and low SSC, and show positivity for CD7, cCD3, CD10, CD33, CD117, CD34 and CD2 and negativity for CD5, sCD3(<20% positive), CD1a, CD4 and CD8

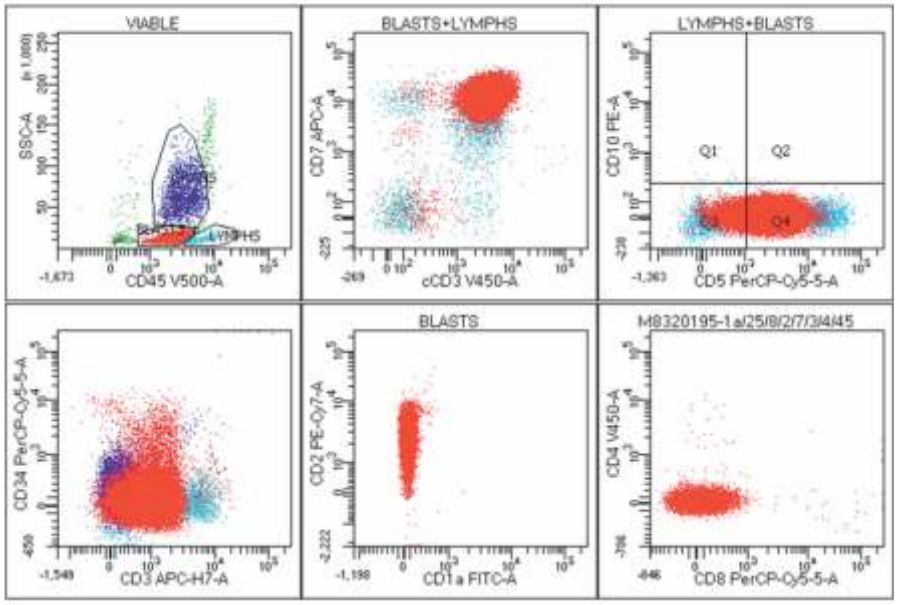

Figure 4: Flow plots of a case of near ETP-ALL- blasts gated as dim CD45 and low SSC, and show positivity for CD7, cCD3, CD5 (bright, >75%), sCD3, CD34 and CD2 and negativity for CD10, CD1a, CD4 and CD8

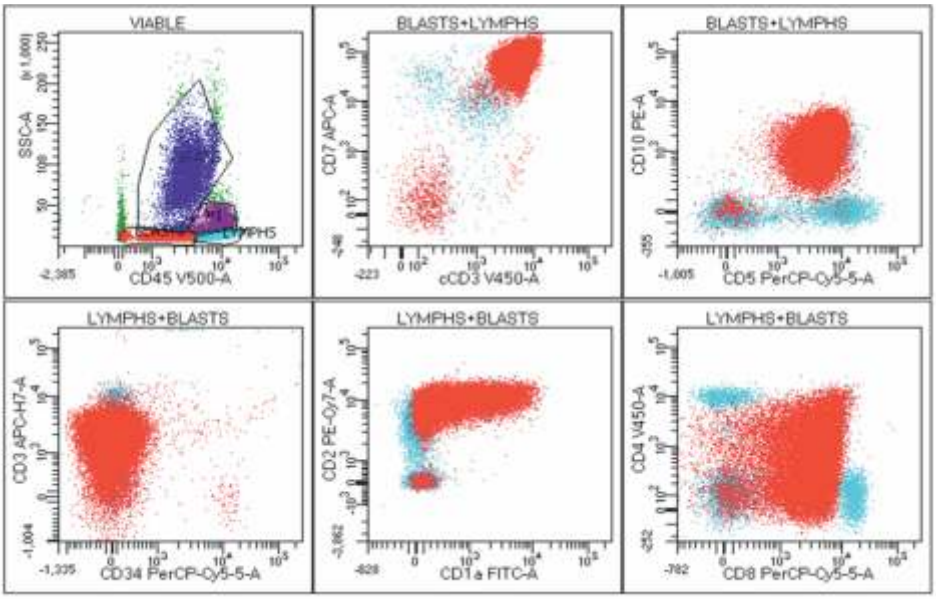

Figure 5: Flow plots of a case of cortical T-ALL- blasts gated as dim CD45 and low SSC, and show positivity for CD7, cCD3, CD5, CD10, sCD3, CD2, CD1a, CD4 and CD8

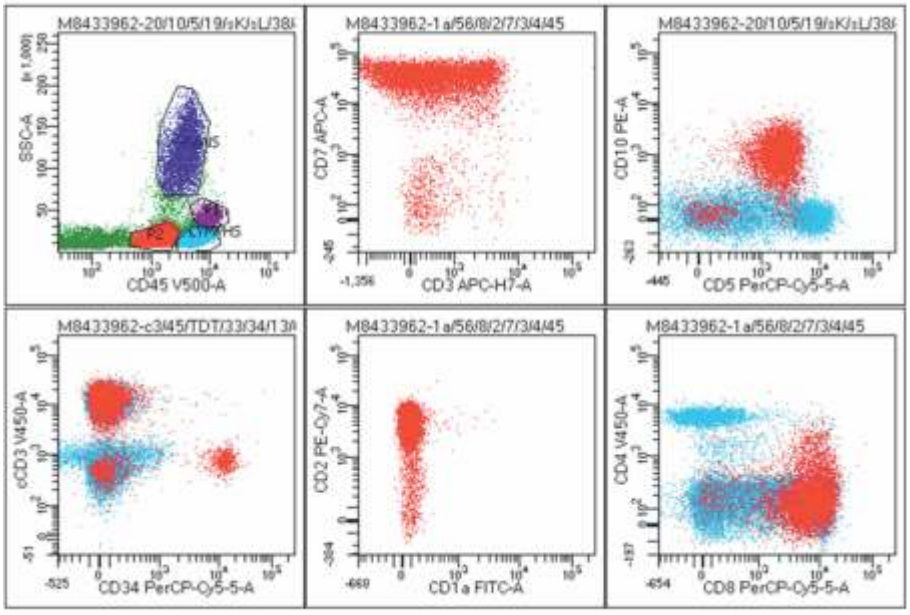

Figure 6: Flow plots of a case of medullary T-ALL- blasts gated as dim CD45 and low SSC, and show positivity for CD7, sCD3, CD5, CD10, cCD3, CD2 and CD8 and negativity for CD34 and CD1a

Discussion

In our study, the clinico-pathological and immuno-phenotypic profile of 37 cases of T-ALL were examined. It was found that 68% of the cases were male, which was similar to what observed in literature. In the study conducted by Brammer *et al*. [3], 75% of T-ALL cases were males while Advani *et al*., in his study on Indian T-ALL cases, observed a percentage of 64.8% [4]. It was observed that more than 50% of cases occurred in children and adolescents, youngest being 1 year of age. Mean age was 19.5 years and median age was 13 years. Al-Mashaikhi *et al*. [5] in his study on Omani T-ALL patients observed a mean age of 19.2 years. Studies have shown that T-ALL is more common in adults than children, although incidence reduces with older age [6].

T-ALL cases typically present with high WBC counts and mediastinal mass. In our study, 35.1% cases had documented mediastinal mass. Genesco *et al*. [7] found mediastinal mass in 47% of patients. The mean and median WBC count in our study was 175.7×10^3/ μL and 118.3×10^3/ μL, respectively and mean blast percentage was 84.8%. Lai *et al*. [8] studied 58 acute leukaemia cases, of which 8 were T-ALL and the mean TLC in these cases was 22.1×10^3/μL and Genesco *et al*. [7] observed a mean of 94.3×10^3/μL. Lahjouji *et al*. [9] found median TLC of 50.5 x 10^3/μL. Median blood blast count and bone marrow blast percentage observed by Al Mugairi *et al*. [10] in adult patients with T-ALL was 15.6×10^9/L and 90% respectively.

On flow cytometry, T-ALL was diagnosed based on CD3 expression in blasts, along with other T-cell antigens. Table 2 shows frequency of markers seen in various studies.

Table 2: Percentage of cases showing positivity for various markers in different studies

Marker	Lahjouji *et al.* [9]	Rezaei *et al.* [11]	Al Mugairi *et al.* [10]	Lewis *et al.* [12]	Our study
sCD3	62.5	38.9	32	38.9	64.9
CD5	82	88.9	93	100	89.2
CD2	-	72.4	70	77.8	91.9
CD1a	31.5	44.4	57	-	27.0
CD4	34	44.4	58	61.1	45.9
CD8	31.5	72.2	71	50	37.8
CD10	14	22.2	32	47.1	32.4
CD79a	20.5	0	0	60	5.4
CD13	42.5	0	23	6.3	8.1
CD33	25	5.6	33	12.5	10.8

In our study, CD10 expression was seen in 32.4% cases, and the mean TLC amongst CD10 positive T-ALL cases was 111.3 × 10^3/µL. Studies have shown that CD10 expression is associated with lower TLC [13]. The significance of CD10 is a matter of debate. Pui *et al*. [14] showed that lack of CD10 expression was independently associated with an adverse prognosis in T-cell ALL. Compared with CD10-T-cell cases, the CD 10+ cases have a high frequency of CD1, CD4, and CD8 antigen expression, but a lower frequency of CD3 expression [15]. Dakka *et al*. [16] found the 5-year survival rate in the group of CD10+ was nearly 35% for T-ALL patients and overall survival was significantly lower for T-ALL CD10−. In the study by Cansolini *et al*. [17] CD10 expression did not have independent prognostic significance.

Classification of T-ALL is based on degree of maturation of blasts. In 1995, EGIL classification subtyped T-ALL into 4 categories [18]:

Pro T-ALL: cCD3+, CD7+

Pre T-ALL: cCD3+, CD7+, CD5/CD2+

Cortical T-ALL: cCD3+, CD1a+, sCD3+/−

Mature T-ALL: cCD3+, sCD3+, CD1a−

In 2009, ETP-ALL was introduced and characterised by presence of lymphoblasts expressing cytoplasmic CD3 and CD7, along with stem cell and myeloid antigens [19]. These cases were described as having a high risk of remission, induction failure or relapse when treated with contemporary protocols of intensive chemotherapy for ALL [19]. However, definite diagnostic criteria were not established. Zubier *et al*. refined immunophenotypic criteria by excluding CD5 expression while adding negativity for CD4 [20].

The WHO 2016 classification for tumours of the hematopoietic and lymphoid tissues has now introduced ETP-ALL as a provisional entity [1]. It defines ETP-ALL as T-ALL expressing CD7, CD3 and lacking CD8 and CD1a. It should be positive for one or more of the myeloid or stem cell markers CD34, CD117, HLA-DR, CD13, CD33, CD11b, and CD65. CD5 is negative or should be present in less than 75% of the blast population. The WHO classification also defined Near ETP-ALL as leukaemia that express 'brighter or more uniform' CD5 in more than 75% of blasts but otherwise meet the criteria for ETP-ALL. With introduction of these entities, it was suggested that most cases of

Table 3: Comparison of age and different CBC parameters between different subtypes

Subtype	ETP-ALL	Near ETP-ALL	Cortical	Medullary	*p*
Percentage of cases N (%)	8 (21.6)	5 (13.5)	9 (24.3)	15 (37.8)	
Age in years (Mean ± SD)	31 ± 21.23	11.2 ± 10.99	14.33 ± 10.54	19.13 ± 18.97	0.15
Total WBC count (× 103/ µL) (Mean ± SD)	127.35 ± 168.61	397.58 ± 167.47	167.79 ± 129.71	132.2 ± 105.06	0.004
Platelet count (× 103/ µL) (Mean ± SD)	107.13 ± 99.88	43.0 ± 28.89	20.67 ± 6.65	98.07 ± 130.69	0.189
Blasts% (Mean ± SD)	81.25 ± 16.12	95.8 ± 2.77	80.33 ± 19.8	85.73 ± 9.71	0.216

pre and pro T-ALL would fall into this category [1]. Using these criteria, we divided our cases as ETP-ALL, Near ETP-ALL, Cortical T-ALL and Medullary T-ALL. Table 3 showed the mean age and mean complete blood count parameters in different subtypes. Medullary T-ALL subtype was the most common (37.8%). This is in contrast to other studies, such as those by Shimizu *et al*. [21] and Lahjouji *et al*. [9] where the immature T-ALL subtypes predominate. In our study, 21.6% cases were classified as ETP-ALL. Reports of ETP-ALL have ranged from 5% to 36% in T-ALL studies [22].

In the present study, median TLC of ETP-ALL cases was observed to be 40.9 × 10^3/μL (range- 0.7-468.5 × 10^3/μL) and was lower as compared to other subtypes. Median TLC of ETP-ALL cases observed by Morita *et al*. [23], Chopra *et al*. [24] and Zuurbier *et al*. [20] were 9.4 ×10^9/L (27 cases, range- 0.8-141.7 ×10^9/L), 7.5 ×10^9/L (9 cases) and 94 ×10^9/L (13 cases, range- 2-435×10^9/L) respectively. Chopra *et al*. [24] and Patrick *et al*. [25] found median TLC to be lower in ETP-ALL as compared to other subtypes. In our study, median age of ETP-ALL was higher compared to other subtypes and similar observation was made in studies by Morita *et al*. [23] and Chopra *et al*. [24] but study done by Saha *et al.* have documented a lower median age and lower M:F ration in ETP ALL as compared to non ETP ALL [26].

In our study, near ETP-ALL cases showed higher median TLC and blood blast percentage. Median TLC of near ETP-ALL cases observed by Morita *et al*. [23] was 7.6 ×10^9/L (24 cases, range- 0.4-151.2 ×10^9/L). They did not find statistically significant difference in TLC between different subtypes. Singh *et al*. [27] found no difference between ETP-ALL, near ETP-ALL and non ETP-ALL in terms of total leukocyte count and age.

Few challenges were experienced during the subtyping of our cases. One case did not fit into any particular criteria. In this case, blasts showed sCD3 and CD5 bright homogenous positivity, CD2 moderate heterogeneous positivity, and was positive for HLA-DR, while CD1a, CD4 and CD8 were negative. Moreover, TCR-αβ was inconclusive. sCD3 bright positivity goes in favour of mature subtype, however since CD1a, CD4 and CD8 were all negative, the case did not fit into cortical or medullary T-ALL subtypes.

The medullary subtype has been described as either CD4 or CD8 positive. However, amongst the cases classified as Medullary T-ALL in the study, it was observed that 4 cases had dual positivity for CD4 and CD8. Cortical subtype was ruled out in these cases as CD1a was negative. Moreover, 2 cases were negative for both CD4 and CD8, but showed strong sCD3 and TCR-αβ positivity and hence classified as medullary subtype.

Shimizu *et al*. [21] and Lahjouji *et al*. [9] classified T-ALL cases as medullary subtype based on positivity for sCD3 and CD7 and negativity for CD1a, irrespective of other markers. Al-Mashaikhi *et al*. [5] classified T-ALL cases into three categories based on the type of antigen expression of CD4 and CD8 (i.e., immature T-cell (CD4-, CD8-), cortical T-cell (CD4+, CD8+), and medullary T-cell (CD4-, CD8+ or CD4+, CD8-). Craig *et al*. [28] mentions that CD4 and CD8 expression patterns are "non-specific", as CD4-/CD8- and CD4+/CD8+ immunophenotypes are also seen in mature T-cell neoplasms.

The overlap between the antigen expressions maybe attributed to overlap between the stages which exists biologically and phenotypically. Patel *et al*. [29] found similar limitation in their study on T-lymphoblastic lymphoma. One case showed negativity for sCD3, CD5, CD34, CD1a, CD4 and CD8 with cCD3, CD7 CD2, CD56 and CD79a positivity. However, myeloid markers that were performed were negative. Though it was categorized as ETP-ALL, possibility of NK-lymphoblastic leukaemia/lymphoma could not be ruled out due to unavailability of CD11b and CD65 markers.

Conclusion

Our study reflects the difficulties encountered in subtyping of T-ALL cases due to antigenic overlap and/or due to lack of an extended panel of secondary markers. It is particularly important to differentiate ETP ALL from non ETP ALL as studies have shown that ETP ALL are more commonly associated with high induction failure and relapse rate. We conclude that a wider panel of markers is required to categorise T-ALL cases, which has potential prognostic and therapeutic implications.

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