Aberrant immunophenotypic expressions in acute lymphoid leukemia: an observational analytical study

Monica Sivakumar*, Atoshi Basu

Department of Pathology, Apollo Gleneagles Hospitals, Kolkata, West Bengal, India

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*Correspondence:
Dr. Monica Sivakumar,
E-mail: monijade159@gmail.com

ABSTRACT

Background: This study aims to find out the expression of aberrant immunophenotypic markers in acute lymphoid leukemia (ALL) and to co-relate its expression with cytogenetic and molecular data.

Methods: Retrospective cum prospective study was carried out in 75 patients of ALL who presented to Apollo Gleneagles hospitals, Kolkata from January 2014 and March 2019. Flow cytometry analysis was done using FC500 (Beckman coulter) All cases were classified according to latest WHO classification.

Results: Out of 75 cases of ALL, 23 cases (30.67%) showed aberrant cross-lineage expression. Amongst the B-ALL cases, the most common aberrant antigen expressed were myeloid antigens 17 cases (77.27%). Aberrant T-antigens were noted in 4 cases (18.10%). Aberrant co-positivity of myeloid as well as T-antigens was seen in 1 case (4.54%). The most common aberrant myeloid antigen expressed was CD33 (77.7%) followed by CD13 (22.2%) and then CD15 (11.1%). Co-positivity of CD13 and CD33 was noted in 2 cases. CD2 and CD33 co-positivity was noted in 1 case. The most frequently expressed aberrant T-antigen was CD2 seen in 3 out of 5 cases (60%).

Conclusions: In B-ALL, the most common aberration was myeloid antigen positivity followed by cross-lineage T-antigen expression. Aberrant CD33 expression was most frequently associated with t(9;22) followed by t(12;21). Aberrant CD15 was most frequently associated with t(4;11). No association with adverse hematological parameters or any significant increase in cytogenetic and molecular abnormality was noted in cases expressing aberrant antigen in comparison to cases not expressing aberrant antigens.

Keywords: ALL, Aberrant immunophenotypic markers, Flow cytometry, Cytogenetic and molecular abnormalities, Adverse haematological parameters

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is characterized by the acquisition of genetic aberrations which drive the proliferation of immature clones. The peak incidence is at the age of 2-5 years. A slight male preponderance has been reported in ALL from several Indian studies. The current male to female ratio in India was found to be 2.5:1. Chromosomal analysis, RT-PCR, FISH and flow cytometry are used to identify leukaemia-specific translocations, sub-microscopic chromosomal abnormalities, and cellular DNA content. Immunophenotyping using flow cytometry is a worldwide mainstay in the diagnosis of acute lymphoblastic leukaemia.

The most frequently expressed antigens are as follows: In T-cell ALL: CD2, cCD3, CD5 and CD7 followed by CD3, CD8 and CD4. In B cell ALL: CD19, cytoplasmic CD79a, cytoplasmic CD22 and PAX-5, while expression of CD20 is variable. Aberrant phenotypes in acute leukaemia are defined as patterns of antigen expression...
on neoplastic cells different from the process of normal hematopoietic maturation.7

In ALL, these aberrancies include: a) Expression of myeloid antigens in ALL, B-lineage antigens in T-ALL or T-lineage antigens in B-ALL, b) co-expression of early (immaturity associated) antigens with mature ones c) antigen over expression or under expression or absence of expression.7

The expression of aberrant myeloid antigen (MyAg) has varied between 5 and 86% in various studies.3 The expression of two or more lineage specific antigens poses a dilemma in assigning leukemia to any one lineage. The term acute leukemia with ambiguous lineage (mixed phenotype AL) is used for such cases.5 This entity is different from expression of aberrant immunophenotypes in acute lymphoid leukemia.6 Specific aberrancies correlate with recurrent cytogenetic abnormalities in B-ALL.9 However, the prognostic significance of myeloid antigen expression in ALL is still in controversy. Some studies have reported worse prognosis for those ALL patients expressing myeloid antigen7-10 whereas other studies have failed to confirm any association with prognosis, good or bad.11-16 The traditional high-risk factors associated with poor outcome in ALL are age <1 year or >12 years, male gender, lower haemoglobin, lower platelet and high total WBC (>50,000/cu.mm), as well as blast counts.16,17 Further there is a large variation in the incidence and prognosis of Acute lymphoid leukaemia patients in Indian population compared to the west.9 Hence, this study was planned to identify various immunophenotypic aberrancies in the Indian population which are identified using flow cytometry.

METHODS

Retrospective cum prospective study was carried out in 75 patients of ALL who presented to Apollo Gleneagles Hospitals, Kolkata from January 2014 and March 2019. In all ALL cases, data of complete blood picture, bone marrow studies, flow cytometry, cytogenetics and molecular studies (if available) was collected and classified according to latest World Health Organisation (WHO) classification.18

Inclusion criteria

All cases of ALL undergoing flow cytometry at the time of presentation were included in the study.

Exclusion criteria

Acute lymphoid leukaemia on treatment or relapsed cases, chronic myeloid leukemia (CML) in blast crisis, the cases that met the current WHO 2016 proposed criteria for ambiguous/mixed phenotype acute leukaemia were excluded from the study.

The flow cytometer makes possible the localization of cells within the electronic window defined on a histogram, which co-relate the orthogonal diffusion of light (side scatter/SS) and the diffusion of narrow angle light (forward scatter/FS). FC500 flow cytometer was used. data acquisition and analysis are done by CXP software (Beckman- Coulter).

Intensity of fluorescence is semi-quantitatively estimated as dim, moderate and high based on the population position on the fluorescence scale. Cells are classified as positive for a given marker when the expression (fluorescence intensity) is greater than that of a negative (isotype) control. The percentage of gated blast cells expressing a particular CD (cluster of differentiation) marker is also used to determine whether expression is negative or positive. Expression of a CD marker by more than 20% of the gated population is considered positive. For terminal deoxytidyl transferase (TdT) the cut off used is 10%.

Fluro-chromes used: FITC (fluoresceine isothiocyanate), PE (phycoerythrin Texas red X), ECD (electron coupled dye), PC5 (phycoerythrin-Cy 5).

Cytogenetic studies using conventional karyotyping and/or FISH are performed. Chromosomal analysis is be done by G-banding karyotype.

For molecular studies, qualitative assay including hybrid transcripts for E2A/PBX1 (also known as TCF3/PBX1), BCR/ABL1, ETV6/RUNX1 (also known as TEL/AML1) and KMT2A/AF4 (also known as MLL gene rearrangement) using RT-PCR is done in selected cases of ALL.

Statistical methods

Sample size was calculated with help of ‘Epi Info (TM) 3.5.3.’ EPI INFO which is a trademark of the centres for disease control and prevention (CDC). Analytical statistics were calculated using GraphPad prism 7 software. Chi square test was used for correlation of aberrant lymphoid marker expression with cytogenetic/molecular abnormalities. 2-tailed binomial test was used to assess probabilities of proportions. Significance level for all p-values were set at α=0.05. Cohen’s d was used to calculate the standardized mean differences. Cut-offs of 0.2, 0.5 and 0.8 were used to designate the effect size as small, medium or large.19 2-sample z test was used to assess whether two populations or groups differ significantly on some single characteristic.

RESULTS

A total of 75 patients were diagnosed as ALL in our institute by flow cytometry in the established time period. Amongst these, 52 were males and 23 were females with a male to female ratio of 2.26 (p value=0.000315). 30
cases presented in the pediatric (0-15 years) age group, followed by 15 cases in the 31-45 years age group and 12 cases each in the age groups of 16-30 years and 46-60 years. Overall, 8% (6/75) of our ALL patients were above 60 years. Out of 75 cases of ALL, 23 cases (30.67%) showed aberrant antigen expression (p value=0.000008).

Amongst the 75 cases, 65 cases were of the B-cell lineage and 10 cases were of T-cell lineage. Out of the 10 T-lineage ALLs, 4 cases (40%) were ETP-ALLs. Out of the 65 ALLs of B-cell lineage, 22 cases (33.85%) showed aberrant antigen expression. One of the ten T-ALL cases showed aberrant antigen expression (10%) (p<0.0001) (Figure 1).

Figure 1: Aberrant expression in the B and T-lineage ALLs.

Amongst the 75 cases, 65 cases were of the B-cell lineage and 10 cases were of T-cell lineage. Out of the 10 T-lineage ALLs, 4 cases (40%) were ETP-ALLs. Out of the 65 ALLs of B-cell lineage, 22 cases (33.85%) showed aberrant antigen expression. One of the ten T-ALL cases showed aberrant antigen expression (10%) (p<0.0001) (Figure 1).

Figure 2: Distribution of B-ALL subtypes amongst cases with aberrant antigen expression.

Amongst the B-ALL cases, the most common aberrant antigen expressed were myeloid antigens 17 cases (77.27%). Aberrant T-antigens were noted in 4 cases (18.10%). Aberrant co-positivity of myeloid as well as T-antigens was seen in 1 case (4.54%) (Figure 3).

Figure 3: Distribution of aberrant markers in B-ALLs.

The most frequently expressed aberrant myeloid antigen was CD33, seen in 14 out of 18 cases (77.7%), followed by CD13 in 4 cases (22.2%). Aberrant CD15 were noted in 2 out of 18 cases (11.1%). The most frequently expressed aberrant T-antigen was CD2 seen in 3 out of 5 cases (60%). CD8 and CD7 expression were seen in 1 case each (20%) (Figures 4 and 5).

Figure 4: Percentage positivity of aberrant myeloid antigens in B-ALLs.

Figure 5: Percentage positivity of aberrant T-lineage antigens in B-ALLs.
In our study, we compared the expression of cytogenetic abnormalities in cases expressing aberrant antigen vs cases not expressing aberrant antigen. Abnormal karyotype and abnormal molecular studies were more in the group of patient expressing aberrant antigen vs cases not expressing aberrant antigen, although this was not statistically significant (Table 1).

Here, t(9;22) as well as t(12;21) was most commonly associated with aberrant CD33 followed by CD13. t(4;11) was commonly noted with aberrant CD15. In complex karyotype, 1 of the 2 cases showed aberrant CD13 and CD33 co-expression (Table 2).

We compared the distributions of certain hematological parameters (hemoglobin, total leucocyte count, platelet count, and peripheral blood as well as bone marrow blast percentage) in cases expressing aberrant antigen versus cases that do not express aberrant antigens. Hemoglobin levels and blast percentages in the peripheral blood smear (PBS) and bone marrow did not vary significantly between the two groups. Total leucocyte counts and platelet counts were more in cases expressing aberrant antigen compared to cases that were not exhibiting presence of aberrant antigen, although this difference was not significant (Table 3).

### Table 1: Cytogenetics and molecular study findings in cases expressing aberrant antigen vs cases not expressing aberrant antigen.

<table>
<thead>
<tr>
<th>Cytogenetics</th>
<th>Cases with aberrant antigen expression</th>
<th>Cases without aberrant antigen expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal karyotype</td>
<td>4</td>
<td>17</td>
</tr>
<tr>
<td>Abnormal karyotype</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>17</td>
</tr>
<tr>
<td>P (binomial probability)</td>
<td>0.1796 (Not significant)</td>
<td>0.2478 (Not significant)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Molecular studies</th>
<th>Normal molecular studies</th>
<th>Abnormal molecular studies</th>
<th>Total</th>
<th>P value (binomial probability)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal molecular studies</td>
<td>3</td>
<td>16</td>
<td></td>
<td>0.09 (Not significant)</td>
</tr>
<tr>
<td>Abnormal molecular studies</td>
<td>10</td>
<td>7</td>
<td></td>
<td>0.0931 (Not significant)</td>
</tr>
<tr>
<td>Total</td>
<td>13</td>
<td>23</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 2: Distribution of cytogenetic abnormalities in cases of B-ALL with aberrant antigen expression.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Nil</th>
<th>t (9;22)</th>
<th>t (4;11)</th>
<th>t (12;21)</th>
<th>t (1;19)</th>
<th>Complex karyotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD33</td>
<td>3</td>
<td>3</td>
<td></td>
<td>1</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>CD13</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD15</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD13 and CD33</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD2</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD2 and CD33</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD7</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>cCD79a</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

### Table 3: Comparison of haemoglobin, total leucocyte count, platelet count, blast percentage between the two cohorts.

<table>
<thead>
<tr>
<th>Variables</th>
<th>ALL with aberrant antigen expression</th>
<th>ALL without aberrant antigen expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>Mean 7.90</td>
<td>8.07</td>
</tr>
<tr>
<td></td>
<td>SD 2.45</td>
<td>2.27</td>
</tr>
<tr>
<td></td>
<td>Z-score (p value) 0.1961 (p: 0.577) (not significant)</td>
<td>0.4519 (p: 0.452) (not significant)</td>
</tr>
<tr>
<td></td>
<td>Cohen’s d 0.05</td>
<td>0.02</td>
</tr>
<tr>
<td>Total leucocyte count (µl)</td>
<td>Mean 61528.26</td>
<td>46530.19</td>
</tr>
<tr>
<td></td>
<td>SD 70545.85</td>
<td>66860.59</td>
</tr>
<tr>
<td></td>
<td>Z-score (p value) -0.6239 (p: 0.2664) (not significant)</td>
<td>0.3788 (p: 0.6476) (not significant)</td>
</tr>
<tr>
<td></td>
<td>Cohen’s d 0.15</td>
<td>0.07</td>
</tr>
<tr>
<td>Platelet count (1,00,000/µl)</td>
<td>Mean 0.54</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td>SD 0.55</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>Z-score (p value) 1.1939 (p: 0.8837) (not significant)</td>
<td>-0.5020 (p: 0.3078) (not significant)</td>
</tr>
</tbody>
</table>

Continued.
DISCUSSION

In our study, the majority of the ALL cases presented in adults (45 cases), 15 cases were seen in the 31-45 years age group and 12 cases each in the age groups of 16-30 years and 46-60 years. 6 of our ALL patients were above 60 years. 30 cases presented in the pediatric (0-15 years) age group. This finding is consistent with the studies conducted by Bhushan and Khalidi et al.\textsuperscript{15,20} This result is in contrast to various studies of Moorman, Foa, Katz and Sharma et al in which ALL was reported to be much more common in children.\textsuperscript{1,7,21,22} The reason for this may be due to the well-established exclusive pediatric hematoclonic units in various other centres in this state which was lacking in our hospital.

Regarding the sex distribution of ALL, we noted a clear male predilection. 52 out of 75 patients were male (69.3%) and the remaining 23 were females (30.66%). The male to female ratio was found to be 2.26:1. This is similar to two studies performed on Indian population by Sharma, (male to female ratio of 2.5:1) and Neelkamal et al (male to female ratio of 2.03:1).\textsuperscript{7,23} A research carried out by Kulkarni et al noted that the male preponderance of ALL was more in Indian population as compared to the west.\textsuperscript{3} This point was also reflected in our study.

Majority of the ALLs were found to be of the B-cell lineage upon immunophenotyping by flow cytometry. Amongst the 75 cases diagnosed by flow cytometry, 65 cases (86.6%) were of the B-cell lineage and 10 cases (13.4%) were of T-cell lineage. This finding is consistent with study done by Jalal SD et al and Neelkamal et al in which immunological subtyping revealed that 85.5% of cases were B-ALL and the remainder T-ALL.\textsuperscript{23,24} Gupta et al gave the distribution of ALL as 81.7% of B-lineage and 17.3% as T-lineage ALL.\textsuperscript{25}

The prevalence rate of aberrant antigens according to various studies ranges from 20 to 55%. Our study shows a prevalence rate of 30.62%, which was well within this range. Our study showed prevalence similar to Kavianpour, Pituch-Noworolska and Momani et al.\textsuperscript{26-28} Bhushan et al opined that there may be several reasons for this wide variation in prevalence.\textsuperscript{15} These include use of different reagents against the CD surface antigens, different cut-off levels, analysis of fresh or frozen cell material, and most importantly the difference between phenotypic characteristics of blast cell of children and adult patients. In our present study, the prevalence rate was noted as 30.62%. This may be due to differences in the ethnicity of the patients and the difference in the flow cytometer instrument and reagents and cut off values for the various antibodies used.

In this study, 33.85% of the B-ALLs and 10% of the T-ALLs revealed aberrant antigen expression. Vitale et al studied 377 cases of adult ALL and noted 38% of B-ALL and 24% of T-ALL expressed aberrant antigens.\textsuperscript{10} Our study showed a lesser percentage of aberrant antigens in T-ALL and this might be due to the fact that limited cases of T-ALL was included in our study. Hence, further studies need to be conducted.

Amongst the B-ALL cases studied by us, the most common aberrant antigen expressed were myeloid antigens 17 cases (77.27%). Aberrant T-antigens were noted in 4 cases (18.10%). Aberrant co-positivity of myeloid as well as T-antigens was seen in 1 case (4.54%). Numerous studies have been done in an attempt to find the exact percentage of aberrant myeloid, as well as aberrant T-antigen in B-ALL.\textsuperscript{6,7,10,13,15} Majority of the studies as discussed above have focused on a single aspect exclusively i.e.: either aberrant myeloid antigen positivity or aberrant T-antigen positivity. Few studies exist in literature which have assessed both these aberrations simultaneously. Our study comprehensively studies the incidence of myeloid as well as T-lineage antigens.

Our study noted that CD33 was the most common aberrant myeloid antigen in B-ALL followed by CD13 (22.2%) and CD15 (11.1%) and CD2 (60%) was the most common aberrant T-antigen elicited in B-lineage ALLs followed by CD7 (20%) and CD8 (20%).

There is a dissimilarity regarding the commonest aberrant myeloid and T-lineage antigen expressed amongst B-ALLs. Few studies report CD13 as the commonest myeloid antigen.\textsuperscript{6,7,10,29,30} While other studies noted CD33

\begin{table}
\centering
\begin{tabular}{|l|c|c|}
\hline
Variables & ALL with aberrant antigen expression & ALL without aberrant antigen expression \\
\hline
Cohen’s d & 0.26 & 0.09 \\
\hline
Peripheral blood blast percentage & & \\
\hline
Mean & 47.65 & 42.35 \\
SD & 35.99 & 37.43 \\
Z-score (p value) & -0.4264 (p: 0.3349) (not significant) & 0.2425 (p: 0.5958) (not significant) \\
Cohen’s d & 0.10 & 0.04 \\
\hline
Bone marrow blast percentage & & \\
\hline
Mean & 81.31 & 84.88 \\
SD & 15.83 & 11.46 \\
Z-score (p value) & 0.6065 (p: 0.7279) (not significant) & -0.4020 (p: 0.3439) (not significant) \\
Cohen’s d & 0.18 & 0.08 \\
\hline
\end{tabular}
\end{table}
as the most frequent aberration.\textsuperscript{15,26,28,31} Few studies report CD2 as the commonest aberrant T-lineage antigen in B-ALL.\textsuperscript{11,32} While other studies noted CD7 as the most frequent aberration.\textsuperscript{27,28} However, there is more or less universal agreement regarding the fact that aberrant myeloid expression is more common than aberrant T-lineage antigen expression in B-ALL. Our study corroborates this fact.

Cytogenetics and molecular studies (wherever available) revealed that aberrant CD33 and CD13 expression was most routinely associated with t(9;22) (q34; q11.2) or BCR-ABL1 hybrid transcript followed by t(12;21) (p13;q22) or ETV6-RUNX1 hybrid transcript. This observation is reiterated by numerous studies by Swerdlov, Seegmiller, Vitale, Khalidi, Gupta and Jaso et al.\textsuperscript{8,10,20,25,33} Aberrant demonstration of CD15 was associated with presence of t(4;11) (q21;q23) and hybrid transcript for KMT2A-AFF 1 (otherwise known as MLL gene rearrangements) respectively corresponding to the studies executed by Seegmiller, Dunphy and Chiaretti et al.\textsuperscript{9,32,34} Aberrant CD2 expression was noted in 3 out of 23 cases of B-ALL (13.0%). 2 out of 3 patients had normal karyotype. These observations are in line with the study done by Dunphy et al which says aberrant CD2 expression is uncommon in childhood and has no known association with any prognostic factors.\textsuperscript{32}

We aimed to find out if there was any associated between aberrant expression and cytogenetic and molecular studies. We found that cases with aberrant antigens showed no significantly increased cytogenetic and molecular abnormalities than those which did not express aberrant antigen (p=0.1796 and p= 0.0931 respectively). This lack of association is re-affirmed in a study done by Vitale et al.\textsuperscript{10}

Low haemoglobin, high TLC, low platelets and high CBC and bone marrow blasts are the haematological parameters have been considered as traditional risk factors for bad prognosis.\textsuperscript{16} Our study agrees with Kurec, Supriyadi, Bhushan and Unal et al who suggested that no significant difference is noted in these hematological parameters between the two cohorts.\textsuperscript{13-16}

\textbf{Limitations}

Cytogenetic and molecular studies were available only in some cases, and hence correlation with hematological parameters were done only for those. Limited number of T-ALLs were included in our study.

\textbf{CONCLUSION}

Acute lymphoblastic leukemia is a clonal hematopoietic myeloid stem cell neoplasm usually originating in the marrow, characterized by a proliferation of immature cells (blasts or blast equivalent). Flow cytometry plays an important role by defining the lineage of blast and by determining how different a blast population is from its normal counterpart (aberrant antigen expression). Cross lineage myeloid expression is most common, followed by T-lineage antigen expression and then combined myeloid and T-antigen expression.

Amongst the myeloid antigen, CD33 is the most frequent aberrant marker, followed by CD13 and CD15. Amongst the T-lineage antigens in B-ALL, CD2 is the most commonly occurring aberrant immunophenotypic expression, followed by CD7 and CD8. Aberrant CD33 and CD13 positivity was associated with t(9;22) and t(12;21). Aberrant CD15 was noted commonly in cases with t(4;11).

Overall, the most often expressed cytogenetic and molecular abnormality was t(9;22) and BCR-ABL1 hybrid transcript respectively. No significant increase in the cytogenetic and molecular abnormalities were noted in the cases expressing aberrant antigens vs cases not expressing aberrant antigens. No statistically significant difference was noted the values of haemoglobin, TLC, platelet count, CBC blast count and bone marrow blast percentage were seen in the two cohorts.

\textbf{ACKNOWLEDGEMENTS}

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\textbf{Ethical approval: The study was approved by the Institutional Ethics Committee}

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