IOSR Journal of Dental and Medical Sciences (IOSR-JDMS)
e-ISSN: 2279-0853, p-ISSN: 2279-0861.Volume 18, Issue 1 Ser. 18 (January. 2019), PP 55-58
www.iosrjournals.org

Prognostic Significance of Flowcytometric Immunophenotype In Acute Leukemias: Insights Gained At A Tertiary Care Hospital

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Date of Submission: 16-01-2019 Date of acceptance: 02-02-2019

I. Introduction

Acute Leukemia (AL) is one of the few hematopoietic neoplasms in which medicine offers complete cure with as high as 70% children going into complete remission post treatment. Flow cytometric immunophenotyping (FCI) of the blasts is pivotal in the diagnosis and prognosis of AL[1]. Newer moAbs, improved gating and analytical techniques have improved the utility of FCI in diagnosis and classification of leukaemias[2]. Many types are known to carry predictable prognosis and specific therapy is warranted. In addition, immunophenotyping can also show blast cell heterogeneity and detect antigen associations rarely seen normally. AL can clearly be assigned a lymphoid or non-lymphoid lineage by immunophenotypic analysis. However, the situation is more complex in cases of morphologically difficult or undifferentiated leukemias, when 'unusual' patterns of phenotypic expression are found, when the results of immunophenotype do not correlate with other data, or in cases of disease relapse[1]. Inspite of its relevance, in India, very few studies have been done to study the phenotype of AL in Indian population and study the demographics and therapeutic outcomes in its regards. This study aimed to study the immunophenotypic profile using FCI in AL and find its relevance in disease behavior, treatment and outcomes.

II. Materials And Methods

A total of fifty cases of AL diagnosed based on morphological diagnosis on the peripheral blood smears over a period of three years in hospital were included. Diagnosis of Acute Myeloid Leukemia (AML) or Acute Lymphoblastic Leukemia (ALL) was based on French American British (FAB) classification. FCI was performed and diagnosis was based on WHO classification. The classification of Biphenotypic AL was done on EGIL classification.

Bone marrow aspirates and peripheral blood were used. Surface antigens processing was done prior to cytoplasmic and nuclear antigens with Lyse and wash technique. Three coloured FCM immunophenotyping was performed on FACS Calibur (Becton-Dickson, San Jose, CA) by collecting 10,000 ungated list mode events, selecting an appropriate blast gate on the combination of forward and side scatter, and analyzing cells with the most appropriate blast gate. Antigen expression was considered to be positive when the percentage of positive blast cells is $\geq 20\%$.

The typical combinations in the primary panel was three colours with forward scatter channel/side scatter channel (FSC/SSC) and included CD5, CD7, CD19, CD10, CD34, CD33, CD13, CD14, CD117, cMPO, CD79a, CD22, CD3. CD45 conjugates were available with FITC and gating was based on this strategy. Additional markers (secondary panel) were done in the following circumstances: (i) The primary panel did not yield sufficient marker expression for unequivocal lineage assignment, (ii) When two or more lineage associated markers of two or more lineage were positive in the primary minimal panel, (iii) for subtyping of AML, and (iv) in some cases as per choice of the reporting hematopathologist. Antiglycophorin were done in cases with strong morphological suspicion of AML M7 and AML M6, respectively or else when rest of the markers did not reach a conclusive diagnosis.

Statistical Analysis

The results were analyzed using SPSS software version 14. Correlation was calculated by applying the Chi square test and Fischer exact test and p values were obtained. A p value of less than 0.05 was considered significant.

III. Results

Patient and clinical parameters

Amongst the 50 cases, 27 (54%) were male and 23 (46%) were female with male to female ratio of 1.17:1. The age ranged from 04 days to 70 years (mean 31.30 years). Fourteen (28%) were of the pediatric population (≤15 years). B-ALL was the predominant leukaemia in the children. Three out of four cases of BAL diagnosed were in the pediatric age group. AML was the predominant leukaemia in the adult population. All cases of T-ALL were found in the adults as shown in table 01.

96% had insidious disease onset with one patient presenting with acute onset intraventricular hemorrhage. All patients had anemia with hemoglobin range 3.9 g/dL to 12.6 g/dL, weakness, easy fatigability and pallor. The WBC count varied from 3,990/mm^3 to 4,55,000/mm^3. All the patients, except one, had thrombocytopenia with platelet counts ranging from 5,000 to 1,10,000/mm^3. 40% of the patients had bleeding manifestations with petechiae and purpura. Lymphadenopathy was seen in 26% of patients.

Expression of Immunophenotypic markers

B-cell ALL (n=20): 90% patients were CD10 (CALLA) positive (18/20) and 10% pro B-ALL (2/20) cases. CD22 was expressed in both cases of pro B-ALL, while cCD79a was expressed in one out of two cases of pro B-ALL. Both were positive in only one out of the two cases. 70% (14/20) expressed CD34 with strength of expression ranging from 22.22% to 99.90%. TdT was expressed in 75% (15/20) cases. HLA-DR was expressed in all the cases.

T-cell ALL (n=06): CD3 and CD7 were universally positive in all six cases with 100% positivity. CD5 and TdT were found in four and five cases respectively. 50% (03/06) cases expressed CD34. HLA-DR expression was not seen in any.

AML (n=20) : CD33 and CD13 were most commonly expressed (20/20) markers. CD117 was positive in 75% cases and mostly negative in AML-M4 and AML-M5. CD14 was commonly associated with monocytic leukaemias, being highest in M4 subtype. CD33 was the most sensitive marker for AML M0. MPO positivity was in 75% (15/20) cases as it was negative in AML M0 and weakly expressed in AML-M5. CD34 was seen in 60% (12/20) cases of AML, in all cases of AML-M0 alongwith HLA-DR. HLA-DR positivity was highest in case of AML-M2 and was least in the M4 subtype.

Acute leukaemias of ambiguous lineage (n=04) : 04 (08%) cases of Biphenotypic Acute Leukemia (BAL) were diagnosed based on the EGIL criteria (Fig 01). Two cases showed a B-cell and T-cell mixed phenotype and two cases showed a T-cell and myeloid mixed phenotype. Three cases were in the pediatric age group and one was adult. BAL was associated with higher TLC counts and stronger expression of CD34.

Expression of aberrant markers

Out of 20 cases of B-ALL, only 08 cases were conventional B-ALL and 12 cases expressed an aberrant marker with CD13 in 06 cases, CD 33 in other 06 cases. CD13 and CD33 were co-expressed in 04 cases. One case co-expressed CD33 and CD14. 04 cases expressed CD7. CD117 and CD14 were expressed in one case each.

Out of the 06 cases of T-ALL, CD10 was expressed in 50% (3/6) cases. CD19 expression was found in 16.66% (1/6) cases and CD22 expression in 16.66% (1/6) cases. CD117 and CD13 ware expressed in one case each.

In our study, lymphoid antigens were seen in 60% (12/20) cases of AML. CD7 was the most commonly expressed lymphoid antigen in 60% (12/20) cases followed by TdT expression in 30% (6/20) and CD22 in 20% (4/20) cases. One case of AML-M2 showed aberrant positivity of CD19 and one case of AML-M0 showed aberrant expression of CD79a.

Correlation of Response and Relapse to Immunophenotypic Markers

We correlated the response and relapse rate in patients in respect to the immunophenotypic markers expression profile. In B-ALL subgroup, sixteen cases (80%) showed response to chemotherapy. The other three cases showed relapse after treatment, while one case died of disease after therapy started. Conventional ALL responded better to treatment with lesser relapse than patients with aberrant antigen expression which was statistically significant with a p value of 0.02 as in table 02. 75% patients with CD34+CD19+CD10 expression responded to treatment, alongwith 02 patients of CD34+CD19+CD10- phenotype also. However, no significant correlation was found between expression of CD10 with either response or relapse to treatment. Only 66.66% in pediatric and 75% of adults who expressed CD34, responded to treatment whereas, 100% of pediatric and adult

patients who did not express CD34, responded to treatment. This was however not statistically significant both in the adult and pediatric age group.

In the AML subgroup, CD34 was found significant (p=0.03) in predicting response to intensive chemotherapy as shown in table no.03. Patients with leukemic cells exhibiting CD34 responded to chemotherapy in 41.66% cases whereas 100% patients with negative expression of CD34 responded to chemotherapy. It was associated with worse prognosis in the form of lower response rates following institution of therapy. CD7 was found significant (p=0.03) as patients with CD7 negative AML responded 100% to treatment as compared to CD7+ AML, where only 41.66% patients responded to treatment. The other markers CD117 and TdT were not associated significantly with response to treatment or complete remission in patients of AML.

Cases of BAL responded very poorly to therapy with fatal outcomes and a rapid downhill disease course.

IV. Discussion

The current standard of care is to perform immunophenotyping by multicolor flow cytometry to further sub- classify lineage (e.g. myeloid leukaemia, B and T-cell leukaemia), pick up CD34 positivity in blast cells and to supplement the findings with worldwide studies [2,3]. Aside from identification of blasts, FCI is especially useful in identification of AML M0, differentiation of APL from AML M1/M2, and identification of TdT-negative ALL and unusual variants, like transitional B-cell ALL and undifferentiated and BAL[4]. Amongst 26 cases of ALL, 20 (80%) showed a B-cell phenotype, out of which, 18 were CALLA positive B-ALL. This is relatively higher than reported in previous Indian study[5]. T-ALL was found in 12% (06/50) of cases of AL and 23.08% (06/26) cases of ALL. This is in concordance with the Indian study done which quotes an incidence of 12.4%. However, all the cases were seen in the adult population with mean age of 30.16 years which is higher than the studies done before. Gujral et al have quoted that T-ALL is more common in adolescent males [5]. Naresh et al have quoted a higher incidence of T-cell lymphomas and leukaemias in India compared to the West[6]. However, in our study it was found in equal incidence in both males and females with an overall higher age at presentation.

Expression of immunophenotypic markers in ALL

In ALL, precursor B-ALL was found in 18 (69.23%) and T-ALL was found in 06 (23.07%) cases. World over, approximately 70% of adult ALL cases are precursor B-cell ALL, 25% are precursor T-cell ALL, and 5% are mature B-cell (Burkitt's cell) ALL7. However, no case of mature B-cell ALL was seen in our study. The most commonly expressed markers included CD10, CD19, CD20, and CD22 as proposed by EGIL group8 which was also seen in our study.

CD10 expression has been associated with good prognosis in cases of B-ALL9. No such association was found in our study. CD10+ CD19+ CD34+ immunophenotype is associated with favorable characteristics and better event-free survival for children and a subset of infants with better outcomes 10. However, we could not find any such correlation. This can be attributed to small sample size in our study.

CD34 was seen in 75% of adults and 66% in the pediatric cases. Such high expression in adult ALL have been reported by the CALBG group11. CD34 expression has been identified as a favorable prognostic factor for both disease free and overall survival in childhood ALL12. In our study, no significant correlation was found between CD34 expressions either in adult or pediatric population.

58.82% adult ALL exhibited myeloid antigen expression which is slightly higher than reported incidences ranging from 15 to 54% in adults and 4 to 35% in children13,14. The commonly expressed myeloid antigen expressed in ALL was CD13 in 26.72% (7/26) cases, as has been seen by Den Boer et al and Pui et al13,14. Intra lineage aberrance was found in 20% of B-precursor ALL, which expressed T-cell markers, which is slightly lower than studies quoted before15. However, aberrant antigen expression was found in 62.5% cases of ALL which is in concordance with studies cited earlier16. Aberrant expression of myeloid antigens was found to be an adverse prognostic factor in childhood B-ALL in our study with a p value of 0.02. It is usually associated with short disease free survival, a short duration of first remission and a high relapse rate at all treatment phases 17-19 which was also found in our study. However, studies from St. Jude and Italy have reported no effect of aberrant antigen expression on overall prognosis in ALL17,18 .

In T-ALL, 50% cases showed intralineage antigen variation whereas inter-lineage antigen variation was found in 33.33% cases only. This has been documented earlier on also with higher incidence of intra lineage variation than inter lineage aberration in T-ALL20.

Expression of immunophenotypic markers in AML

In AML, CD13, CD33 and MPO were the most consistent markers as has been seen in previous studies[5,21] followed by CD117 in 80.6% of M3 cases. Moreover, compared with CD33 and CD13, CD117 had much higher specificity since it was rarely observed in ALL. This is in concordance with studies done in India and abroad [22,23] . The differentiation of AML-M1 was difficult from AML-M2 as the markers shared by the two are almost the same. Though a set of markers and their expression pattern has been proposed to differentiate the two i.e. CD11b and CD15 [22], we could not do so as markers like CD11b and CD15 were not done in our study.

A lot of research has been done on the expression of lymphoid markers in AML patients, most of which indicated that the positive rates of CD7, CD2, and CD19 were between 16% and 20% [24,25]. Kaleem et al saw CD7 frequently being expressed in various AML subtypes, whereas CD19 expression as per them was related to M2 [26]. This was consistent with our results too. CD7 was the lymphoid marker that was frequently expressed in various AML subtypes seen in our study, was also seen by Legrand et al[27]. CD7 negative AML had a better prognosis in our study population with a p value of 0.03. There have been variable reports on expression of CD7 and its relation to disease behavior in AML[4,25,27]. Expression of CD3 was not seen in any case of AML, which is consistent with findings of Lewis et al [29].

Expression of CD34 was associated with poor prognosis in our study population ($p = 0.03$). This has also been reported by Zheng et al [30], Chang et al[17] and Raspadori et al[31]. However, studies like Kyoda et al found no significant differences in the outcome and rate of complete remission between CD34+ and CD34- AML patients[32]. CD117 has been associated with reduced complete remission rate in earlier reports by Ashman et al[33]. However, no such correlation was found in our study.

There are no consistent findings with respect to the relationship between TdT positivity and prognosis. Similarly, in our study, we could not find any correlation with a p value of 0.68, though co-expression of CD7 and TdT has been associated with poor prognosis by Suggs et al[4].

Acute leukaemias of ambiguous lineage

In our study, we found 08% (04) cases of BAL. These were diagnosed as AML or ALL based on FAB. However, on FCI these were diagnosed as BAL and Bilineage AL. This percentage is high as compared to literature where it is reported between 2% to 5%[33]. The reason could be that our study was a small sample size.

In our study, 50% cases expressed a mixed B and T-cell phenotype and 50% expressed markers of T-cell and myeloid phenotype. Gujral et al have reported the most common immunophenotypes, representing 60-65% of all BAL, are a co-expression of myeloid and B-lymphoid markers, while 25-30% of BAL cases show co-expression of myeloid and T-lymphoid markers[34]. No case of triphenotypic AL was seen in our study. These patients had an overall poor prognosis with high fatality rates and poor response to therapy as has been reported by previous studies[33].

V. Summary and Conclusions

FCI is imperative nowadays in AL but needs critical analysis and insight to predict disease behaviour, progress and outcome. Definitive treatment decisions are to be based on exact AL subtype. From our study, some important conclusions were obtained. In ALL, antigen expression is prudent for response to therapy as conventional ALL responds better to treatment than ALL with aberrant phenotype expression. Expression of hematopoietic markers is statistically significant in AML and leads to poorer therapy outcomes. However, they are of no significance in ALL, both in the adult and pediatric age group. CD7+ AML phenotype has poorer outcome. This strengthens our opinion that CD7 causes poorer prognosis as against studies which have attributed no significance to this marker. Thus, immunophenotypic profile in AML, in addition to diagnosis and sub-classification, can be of prognostic significance too.

Our study highlights the necessity of FCI with regards to the importance of immunophenotype in prognosis. Also, the results necessitate the usage of a prognostic scoring system comprising of morphology, clinical symptoms, cytogenetic abnormalities, and FCI parameters to be devised for AL patients. This would be a helpful guide for estimation of prognosis in AL patients. We propose that FCI still is more relevant in the Indian scenario as molecular studies are not routinely chromosomal rearrangements. Case specific mutational analysis can be done where immunophenotypic ambiguity exists.

Limitations of the study

There are a few limitations of our study. Firstly, the dependent population of our centre and the number of cases is more of the reproductive age group and hails from a more healthy background. This could have partially skewed our study data especially the demographic patterns and statistical analysis. In few, markedly hyper cellular or 'packed' BM may have yielded too few cells for adequate analysis. Moreover, the antibodies used were of primary and secondary panel as per defined guidelines. If we had a more extensive panel of antibodies, more findings could have been generated from our study.