Original Article Analysing "tear-drop" prints of acute promyelocytic leukemia (APML): immunophenotypic prognostication of APML by FCM

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Abstract: Introduction: Acute promyelocytic leukemia (APML), although genetically and morphologically distinct from other AML (acute myeloid leukemia) subtypes, is one of the most best responsive acute myeloid leukemia. -Conventional diagnostic methods and morphological hints often fail in the majority of the cases in the peripheral laboratories owing to resource constraints, unavailability of cytogenetic work-up, hypogranular variants, morphological mimicry by AML-monocytic and myelo-monocytic, etc. Flowcytometry (FCM), however, can be utilized as a feasible and reliable immunophenotypic diagnostic and prognostic tool for prompt identification of APML. In order to rapidly and sensitively diagnose APML we intended to suggest a cost effective, sensitive FCM panel and also to prognositicate patients. Material and methods: In this retrospective study, flowcytometry characteristics of 123 cases of acute promyelocytic leukemia were studied including 40 hypogranular variants. The expression of markers was compared with the Mean flurescent Intensity (MFI) and percent expression of markers. A non-statistical comparison was made with cases of acute monocytic leukemia. The cases were grouped according to their immunophenotype characteristics and expression with comparison of MFI by multivariate logistic regression. The aberrant markers positive at diagnostic and remission flow test were compared with the survival outcomes, and their positive predictive values were calculated. Results: The most common feature of side scatter property was the absence of blasts in the window and high side scatter, except hypogranular variants which had low side scatter, Immunophenotypically characterised by positivity for CD117, cMPO, and bright CD33 and CD13 positivity and lack of CD34 and HLA-DR was seen in the majority of APML including hypo-granular variant. We suggest a rapid diagnostic four-tube panel for fast and rapid diagnosis of APML, including hypogranular variants with 100% sensitivity. The study also identified six groups of immunophenotypes with significant prediction values of APML, including hypogranular variants. The study also highlights CD2, CD56, and CD9 as prognostic markers for acute promyelocytic leukemia.

Keywords: Acute promyelocytic leukemia, flowcytometry, prognostication, hypogranular variants

Introduction

APML (10-15% of all AML cases diagnosed in India) is considered a medical emergency owing to the risk of coagulopathy constitutes [1, 2]. Characterized by the presence of a classical fusion transcript PML-RARA (Promyelocytic leukemia retinoic acid receptor-A) or t(15:17), which is often the gold standard for diagnosis by karyotyping, fluorescence in situ hybridization (FISH), and reverse transcriptase-polymerase chain reaction (RT-PCR). Definitive diagnosis is not always affordable/available or feasible financially in resource-poor settings. Although very essential, these services are mostly unaffordable for majority of the patients in low resource settings. It is also labour-intensive process requiring standardization and resources alike.

Flow cytometry (FCM) is a relatively affordable and easily available diagnostic tool for acute leukemia patients in the majority of the centers, central and peripheral. FCM, with its character-

istic "tear drop" side scatter pattern, and other immunophenotypic suggestions for APML (Acute promyelocytic leukemia), has good sensitivity and specificity, but hypogranular variants, as well as other HLA-DR negative AML and acute myelo-monocytic leukemia, pose challenges [3, 4]. Staining for morphology is different in different settings and morphology has never been conclusive in APML, more so in hypogranular variants. Although our setup has RT-PCR for identification of PML-RARA, we wanted to establish the discrepancy and concordance of flowcytometry and RT-PCR and Morphology in accurate and timely identification of APML. We understand that the study might not contribute much to already existing knowledge in flowcytometry but may establish flowcytometry as sensitive tool in rapid iudentification in laboratories where only flowcytometry is available and molecular tests are still outsourced in many low-middle Income countries (LMIC) countries. This adds to delay in diagnosis and increased morbidity and mortality. We hence propose a rapid 4 tube panel for distinguishing AML and APML. Our retrospective survey of all 123 APML cases, morphology suggested APML in only 85 cases, and also morphology suggested APML cannot be ruled out. The confirmatory molecular tests are often not available to answer this diagnostic dilemma in many parts of the world. The comparison of Mean flurescent Intensity (MFI) and Positive predictive value of FCM by simple, reliable, and cost-effective sensitive four-tube panel antibody tube panel for the exclusion of APML has not been studied before, including hypogranular variant and variants with difficult cytogenetic profiles. The cases in our center were diagnosed as APML first on morphology, if there were suspicion of hypogranular variant versus AML M4/M5 flowcytometry was relied upon always. The significant markers that were considered in favour of APML included negativity for CD34, absence of HLA-DR, positivity for CD9. The cases with no consensus were confirmed by RT-PCR for PML-RARA transcript identification. Clinical suspicion of APML was also taken into consideration but in our experience cases where AML induced thrombocytopenia and bleeding were also seen to be misled as APML. Coagulation studies, investigations for disseminated intravascular coagulation (DIC) played an important clinical distinguishing feature of bleeding in these both cases. Hence flowcytometry along with all the findings can pinpoint an impending DIC by correctly diagnosis APML in resource poor settings.

Material and methods

A retrospective study from 2014-2019 of all the cases of APML after obtaining consent from patients/guardians were evaluated for clinico-pathological parameters, flow cytometry, and cytochemistry. Reverse transcriptase PCR (RT-PCR) for PML-RAR alpha was done from private labs by outsourcing in cases with clinical suspicion or where-ever possible. Routine molecular cytogenetic studies for APL specific performed by standard G-banding techniques on unstimulated culture.

Inclusion criteria

Cases of untreated acute leukemia that attended the OPDs of AIIMS and VMMC and SJH hospital in pediatric OPDs which were diagnosed as APML and decided to continue treatment in the same centre. Cases that had given consent to participate in the study.

Exclusion criteria

Cases which had been partially treated outside, cases which did not continue treatment at the same centre, cases which showed reactivity to HIV/HCV and HBsAg were excluded from the study. Also, cases in which consent could not be obtained were excluded from study.

Four-color FCM analysis was performed for all cases using BD FACS Calibur and BC FC500, and the data were analyzed with FACS DIVA software (BD Biosciences, San Jose, CA) in accordance with the international consensus recommendations on flowcytometric analysis of hematolymphoid neoplasia [5]. The antibodies in our routine panel included shown in Table 1 apart from the screening cytoplasmic tube for the exclusion of lymphoid and other myeloid neoplasms. The gating strategy was followed as published before [6]. The percent positivity taken for the categorization of negative, dim positive, and positive is mentioned in Table 1, along with the clone utilized during the experimentation. For the purpose of comparing our results with the literature, we arbitrarily defined in this study staining of 20% or more positivity for the markers like CD34, HLA-DR, CD33,

S No.	CD Marker	Conjugate	Clone	Min Positive Population percent	Dim Positive	Mean MFI Normal/Blast
1	CD34	PC5.5	Immu133	20	10	0.42/0.72
2	CD117	PC7	104D2D1	20	10	0.20/0.35
3	HLA-DR	Pacific blue	Immu-357	20	10	4.33/0.54
4	CD2	ECD	39C1.5	10	10	5.18/1.05
5	CD3	APC 750	UCHT1	10	10	16.77/1.34
6	CD7	APC	8H8.1	10	10	28.63/0.10
7	CD9	APC 750	ALB6	15	15	0.46/62.46
8	CD11b	APC	Bear1	15	15	8.29/3.30
9	CD11c	PC7	BU15	15	15	0.32/0.70
10	CD13	ECD	SJ1D1	20	15	0.20/9.34
11	CD14	APC 750	RM052	20	20	0.31/0.91
12	CD16	FITC	3G8	20	20	0.22/1.05
13	CD18	PE	7E4	20	20	0.68/0.76
14	CD33	APC	D3HL60.251	20	15	0.40/35.54
15	CD56	PE	N901	20	20	0.29/0.22
16	CD64	ECD	22	20	20	0.44/5.42
17	CD65	FITC	88H7	20	20	0.20/0.56
18	cMPO*	FITC	CLB-MPO-1	03	20	0.93/3.67

Table 1. Flow markers with clone and distribution of positivity

*Any positive percent was reported independently regardless of the threshold.

CD13, etc. The minimum positive blast population and the criterion for dim positivity to be considered as positive being mentioned in the table along with other immunomarkers.

We then analyzed the immunophenotypic profiles of these cases in relation to individual antigen expression and karyotype findings. The aberrant markers positive at diagnostic and remission flow test were compared with the survival outcomes, and their positive predictive values were calculated with the ability for the exclusion of other diagnosis from APML by comparing the immunophenotypic results with a different series of AML cases (control for statistical analysis). Clinical management protocol as per NCCN update Version 2.2014 (Annexure 1). Clinical outcomes: As per the standard definitions of CR, OS, RFS, and EFS [2] (Annexure 2), Statistical analysis.

Statistical analysis

Analysis was done on the patients who consented for the study and for treatment. Fisher exact test was used to compare differences between groups with respect to clinicopathological characteristics, clinical outcomes, and response to therapy. The probability of survival was estimated with the use of the Kaplan and

Meier method for overall survival, event-free survival and disease-free survival and compared by the log-rank test among risk groups. All survival estimates are reported ± 1 SE. All P values were two-sided, with values of 0.05 or less indicating a statistical significance. All statistical analyses were performed using SPSS v.16.0 software (SPSS Inc., Chicago, USA). Clinical features are presented as percentages (%) for categorical variables and as mean values ± standard deviation (SD) for normally distributed continuous variables. The chi 2 test was used to analyze differences in the distribution of categorical variables between patient subsets. The Chi 2 test was used to detect differences in the distribution of continuous parametric variables. Multivariateanalyses were performed using a multiple logistic regression analysis. P values <0.05 were considered statistically significant. A cutoff of >10% was used to quantify the presence of a subpopulation of CD34+ and CD56+ cells, and a cutoff of >20% was used for defining positivity for CD34, CD33. CD56 as mentioned in Table 1.

Results

Based on the MFI and the percent positivity of blasts for the immune markers, the positivity of various markers is explained in **Table 2**.

Table 2. Immunophenotypic characteristics

Cases	No.	HLA DR	CD33	CD13	CD34#	cMPO*	CD11b\$	CD11c@	CD117	CD9	CD56	CD2	CD15
All APL cases	123	17 (13.8)	123 (100)	122 (99.1)	30 (24.4%)	123 (100)	26	12	122 (99.1)	120	18	30	40
Hyper granular variant	83	12	83 (100)	82 (98.7)	15 (18.1)	83 (100)	18 (21.7)	11	82 (98.7)	80	10	13	27
Hypo Granular Variant	40	5 (12.5)	40 (100)	40 (100)	15 (37.5)	40 (100)	8 (20.0)	8 (20.0)	40 (100)	40 (100)	8 (20.0)	17	13
AML M4/M5	60	40	49	37	54	58	55	52	42	01	39	02	45

#including heterogenous positive population. *Considered positive even if the threshold for 3% population of interest is present. \$co-positivity for CD117 required to distinguish maturing myeloid precursors or granular monocytes includes dim positivity. @Indicates positivity if more than 5% of population shows moderate to bright positivity

Side scatter properties

We observed that expected variations inside scatter properties among all the cases, with the majority of hyper granular variants extending to the myeloid window with subsets positive in blast window and few heterogeneous populations positive for 11b and 11c. The hypogranular variants, because of less side scatter properties, were localized to the blast widow but had 1 log increase in the MFI for CD45 than other non-APL, AML M4/5 myeloblasts. A number of cases also displayed a very high side scatter with a complete absence of cells in blast window and heterogeneously extending from myeloid widow extending up to 4 logs of side scatter with higher MFI, and a few also displayed classical tear drop appearances inside scatter properties; however, a majority of non-APL cases myelo-monocytic blasts also had a pseudo tear drop appearance because of heterogeneous myeloid and monocytic blasts. To summarize, the most common feature was the absence of blasts in the window and high side scatter. Hypo-granular variants did not show this characteristic.

Immunophenotype properties

A significant majority of cases showed positivity for CD117, cMPO, and bright CD33 and CD13 positivity but so did a majority of Non-APL, with showed lack of HLA-DR antigen in about 86% and negativity for CD34 in about 75.6% cases. The lack of CD34 and HLA-DR was seen in the majority of hypo-granular variants as well, and this feature was less prominent in myelo-monocytic leukemia. Those cases which were HLA-DR+ cases were also positive for CD34 and CD117, indicating myeloid progenitor immunophenotype. The hypogranuar variants showing characteristic diagnostic phenotype based on SSc properties included the absence of HLA-DR, absence of CD11b, the positivity of CD9, positivity for myeloid progenitors like-CD117, CD34 alone, or a combination of these markers identified all cases of hypogranular variant with high sensitivity and positive predictive value. CD2, CD18, CD56, and CD15 were positive in many cases of APL (P>0.05). CD9 served as an accurate diagnostic flow marker in cases where CD11b was dim positive or in hypogranular variants. We observed that grouping the immune characteristics into six types described all studied APL cases, the cases grouped are shown by representative cases in **Figure 1A-G**.

Association of immunophenotypes with clinical and therapy outcomes

We found a non-significant correlation between expression of CD56 and CD2 for poor prognosis between the subgroups (p-value 1.2 and *p*-value 0.92, respectively). With their positivity showing higher relapse rates, higher incidences of complications and deaths. CD34 positivity was also associated with poor prognosis in APL cases, including hypogranular variants. CD9, however, was significantly associated with good prognostic outcome in APML (p-value-0.008) CD14 has been associated with poor prognostic outcome in ALL, but in our study was associated with higher complete remission rates and lower complication rates. The prognostication needs more studies for statistical validation.

Discussion

Immunophenotyping by flow cytometry now has been the standard diagnostics in hematology and also plays a role in the prognosis of acute leukemia. Based on side scatter and phenotypic characteristics of blasts on forward scatter, the scatter properties allow differentiation of major types of cell population and also subclassification of the majority of leukemia. (e.g., minimally differentiated AML versus ALL, acute monoblastic leukemia versus NK-cell lymphoma/leukemia or B-ALL versus T-ALL), The heterogeneity of blasts in AML makes it difficult on forward and side scatter properties to correctly typify the diseases. Still, the population of interest can be specifically indicated in APML based on scatter properties also in acute monocytic leukemia in the majority of cases [3-6]. Borrowitz et al. in their study have defended flowcytometry in the inability to distinguish between AML monocytic and hypogranular APML using better gating strategy and use of scatter properties. We also observed the superiority of flowcytometry by this gating strategy. Classic APL has a well-recognized flow cytometric pattern with increased side scatter, lack of expression of HLA-DR, CD11a, CD11b, CD18, positive CD117, negative or weakly positive CD15 and CD65, negative CD34, often positive CD64, variable (heterogeneous) CD13 and bright CD33 [7]. We also found that classic APML has a very









Figure 1. A: Distribution of Immunophenotypes in cases of APL-Hypogranular vs. Classical vs. Blastic. B: APML Subtype 1- blasts are CD117+, CD33br+, CD34-, HLA-DR-, CD11b-. C: APML Subtype 2- classic tear drop appearance with CD117+, brCD33+, CD34-, HLA-DR+, CD2+, CD11b-, CD56+, CD18+. D: APML Subtype 3- Hypergranular variant with CD117+, CD33+, CD34-, HLA-DR-/+, CD56+, CD11b+. E: APML Subtype 4- CD117+, CD33+br, CD13+, CD34+, HLA-DR+, CD14+, CD34+, HLA-DR+, CD11b+, CD117+, CD33+br, CD13+, CD34+, HLA-DR+, CD11b+, CD115+, CD115+, CD33+, CD34+, HLA-DR-, CD11b+, CD117+, CD33+, dim CD34+, HLA-DR-, CD11b+, dimCD2+. G: Subtype 6: Hypogranular variant with CD117+, CD33+, CD34+, HLA-DR-, CD11b+, CD14-, CD18-.

S No.	Immune group	APL Cases	p-value
1	CD117+, CD33br+, CD34-, HLA-DR-, CD11b-	42	0.04
2	CD117+, CD33+, CD34-, HLA-DR+, CD2+, CD11b-	25	0.001
3	CD117+, CD33+, CD34-, HLA-DR-/+, CD56+, CD11b+	18	0.19
4	CD117+, CD33+, CD34+, HLA-DR+, CD11b-, CD56+/-	16	0.23
5	CD117+, CD33+, CD34+, HLA-DR-, CD11b+, CD2+	14	0.08
6	CD117+, CD33+, CD34+/-, HLA-DR-, CD11b-, CD14+/-, CD18+/-	8	0.91
Total		123	

Table 3. Comparison of immunophenotype class with PPV and MFI of -non-APL cases

strong CD45 scatter pattern and the above markers being very conclusive for APML. However, CD9 served as very distinguishing marker in APML and AML-M4/5 in almost 90% of cases. Kussick et al. described HLA-DR-/CD34phenotype in AML with normal karyotype by conventional cytogenetics and association with the FLT-3 gene internal tandem duplication [8]. Albano et al. reported the association of CD34 expression with the hypogranular APL variant and a higher proportion of CD2+ and HLA-DR+ cases [9]. We also found CD2+ in hypogranular variants in more percentage as compared to classical APML. In the same study, CD34+ APL patients had a significantly higher percentage of peripheral blood leukemic promyelocytes at

S. No	Tube	FIT C	PE	ECD	Pec CP	PC5.5
1	Tube 1	cMPO	CD56	CD34	CD16	CD45
2	Tube 2	CD64	CD2	CD14	CD117	CD45
3	Tube 3	CD9	CD18	HLA-DR	CD33	CD45
4	Tube 4	CD15	CD13	CD19	CD11b	CD45

Table 4. Recommended panel for APML for multiparametric flow cytometry

Abbreviations: MFI, Mean flow intensity; FITC, Fluorescein Isothiocyanate; PE, Phycoerythrin; ECD, Phycoerythrin-Texas Red conjugate (energy coupled dye); Pec CP, Peridinin chlorophyll-A protein; PC, Phycoerythrin-cyanine5 conjugate.

presentation, were more frequently female, and had a higher proportion of bcr3 expression, but there were no differences between the two groups in terms of complete remission, overall survival and disease-free survival [9]. We found that CD34 served as poor prognostic indicator in APML, but the result was not statistically significant as the confounding factors like high TLC, and associated poor risk factors could not be normalized.

FCM diagnosed APML including hypogranular variants with 100% sensitivity prognosticating cases where CD9, CD2, and CD34, CD56 positivity is seen with the specific exclusion of non-APL AML, i.e., AML-M4/M5 with resembling immune profile with strong predictive values. In contrast to other studies, we found a higher incidence of positivity of CD34+, CD56+, CD2+, and CD11b [7-10], posing a diagnostic dilemma. We found a higher positivity and specificity for CD9 in all cases, even for hypogranular variants. In our experience, on multiple logistic regression analysis for survival at day 30/45, a statistically significant positivity of CD9 (P= 0.001) and absence of CD11b (P=0.009) predicts diagnosis along with a non-significant absence of CD34 (P=0.93), HLA-DR (P=1.2) correctly predicts APML including hypogranular variants as compared to divergent results in other published cases [12-18]. CD2 and CD56 were also found to be associated with poor prognosis (p-value 1.2 and p-value 0.92 respectively) and higher relapse rates with lower EFS in our study, as did others [22-24]. Based on the significant values of immunophenotypic groups (Table 3 and Figure 1A-G), we suggest a four-tube cost-effective and sensitive-specific panel for diagnosis of APML, including hypogranular variants that will exclude other AML and predict prognosis in APML for response and relapse (Table 4). A pilot validation analysis on five undiagnosed cases of APML showed panel predicted the diagnosis with 100% specificity [30]. The validation study requires more experimentation.

Conclusion

In this era of molecular subcategorization and personalized medicine, PCR fusion transcript identification is not possible at every diagnostic centre and often time-consuming for this medical emergency with a risk of disseminated intravascular coagulation. Flow cytometric immunophenotypic analysis "tear drop" pattern though characteristic cost-effective four-tube assay can be implemented at any tertiary care set-up. FCM thus serves as an important sensitive, cost-effective, independent diagnostic and prognostic tool for APML in this era of molecular medicine.

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Disclosure of conflict of interest

None.

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