Original Article

Human leucocytic antigen-DR negative acute myeloid leukemia: A diagnostic dilemma for hematopathologist

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ABSTRACT

Background: Acute myeloid leukemia (AML) blast variably express Human leucocytic antigen (HLA).We retrospectively analyzed immunophenotypic and clinical profile of 12 cases of HLA -DR negative AML and correlated with their morphological, cytogenetics and Molecular findings.There is a paucity of literature mentioning morphological, immunophenotypic and cytogenetics characteristics of HLA DR negative AML. **Aim:** This study was designed to study the morphological, flow cytometric, and cytogenetics characteristics of HLA DR negative AML/non acute Promyelocytic Leukaemia (APML) cases. **Materials and Methods:** Seventeen such cases were diagnosed over a period of 1 year and 8 months. Peripheral blood and bone marrow aspiration smears were stained by Wright giemsa and examined by three hematopathologist independently. Immunophenotyping was done using multicolour flow cytometry on BD FACS CANTO II using FACS DIVA software.Conventional Karyotyping was done using Wright giemsa staining (using IKAROS software) and florescent *in situ* hybridization (FISH) was done using dual color dual fusion probe from Vysis promyelocytic leukemia-retinoic acid receptor alpha (PML-RARA) fusion gene probe. Molecular analysis using reverse transcriptase-polymerase chain reaction (RT-PCR) was done using Thermal Cycler of Applied Biosystem and Gel-Doc by Biorad. **Results:** Of the 12 cases studied ten were classified as French-American-British (FAB) AML-M1. Two case as FAB AML-M2. Morphologically the cells resemble abnormal promyelocytes with bilobation, convoluted and folded nucleus, inconspicuous nucleoli and open chromatin ($n = 11$) and with blastic morphology, open chromatin, and inconspicuous nucleoli ($n = 1$).Karyotyping analysis shows normal karyotype ($n = 10$), del 9q- ($n = 1$), and t (5:9) ($n = 1$) respectively.FISH done using dual color dual fusion probe ($n = 12$) do not show PML-RARA fusion signal.RT-PCR ($n = 12$) revealed a negative result for PML - RARA fusion transcripts. **Conclusion:** HLA-DR negativity does not always imply a diagnosis of APML. A Cytogenetic (FISH/conventional karyotype) or molecular (RT-PCR) evidence of t (15:17) or PML-RARA fusion gene transcript is a must to stamp a case as APML. Morphology and Flow cytometric findings are only complementary to Cytogenetic/Molecular findings.

Key words: Acute myeloid leukemia, hematopathologist, human leucocytic antigen-DR, negative

INTRODUCTION

HLA class II molecules are expressed by the blast in most of the case of acute myeloid leukemia (AML) with the exception of acute promyelocytic leukemia (APML), which is characterized by absence of HLA-DR.[1-3] Absence of HLA-DR antigen expression is rare in non-APML.[4] There is a paucity of literature that describes HLA-DR negative AML cases.In our study, we sought out 12 such cases of AML which lack HLA-DR antigen expression and were negative for t (15:17) by conventional karyotyping ($n = 12$) and also promyelocytic leukemia-retinoic acid receptor alpha (PML-RARA) fusion negative by FISH as well as by reverse transcriptase reaction (RT-PCR) ($n = 12$). In order to fulfill these lacunae 12 such cases have been studied with their morphological, immunophenotypic, cytogenetic and molecular characteristic.

Access this article online	
Quick Response Code:	Website: www.ccij-online.org
	DOI: 10.4103/2278-0513.138055

MATERIALS AND METHODS

Following inclusion and exclusion criteria were followed for the study-

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Inclusion criteria

A total of 12 cases which lack HLA-DR antigen expression with PB and BM were included over a period of one year and eight months.These cases were diagnosed as AML based on morphology substantiated by immunophenotypic findings.FISH and RT-PCR for PML - RARA fusion gene was done in all (n = 12) cases.The characteristic phenotype defined was a cluster of differentiation 13 (CD13), CD-33 positive and HLA-DR negative.

Exclusion criterion

Other cases, which were HLA-DR negative such as classical APML (both macrogranular and microgranular/hypogranular variants), AML-M6, AML-M7 and AML with myelodysplasia related changes were not included into the study.The cases which were HLA DR negative and showed classical abnormal promyelocytes with the presence of faggots turned out to be PML - RARA positive by FISH or RT-PCR and thus were excluded from the study. In AML-M6, AML-M7 and AML with myelodysplasia-related changes although flow cytometry showed them to be HLA_DR negative, but the cells does not resemble the abnormal promyelocyte like morphology.

Immunophenotyping

Immunophenotyping (IPT) was done on ethylenediaminetetraacetic acid anticoagulated PB or BM using standard lyse wash technique. Data were acquired on BD FACS Canto II Flow Cytometer_(BD Biosciences). Data were analyzed using FACS DIVA software. Antibodies tagged to flourochromes such as fluorescein isothiocyanate, Phycoerythrin, Peridinin chlorophyll protein, Peridinin chlorophyll protein conjugated with cyanine dye were used to stain the cells.

Antibodies used were HLA_DR, CD34, CD10, CD19, CD117, CD13, CD33, CD7, cytoplasmic Myeloperioxidase, cytoplasmic CD79a, cytoplasmic CD3. A six color combination of antibodies was used (n = 12).

Cytogenetic analysis

Conventional Karyotyping was done using Wright Giemsa staining (n = 12).FISH was done using dual color dual fusion PML - RARA fusion gene probe (n = 12) by vysis. PML - RARA break apart probe was not used.

Molecular analysis

Nested RT-PCR (qualitative Analysis) was done (n = 12) using Thermal Cycler of Applied Biosystem and Gel Doc by Biorad.

Follow-up and survival

Complete follow-up and survival was noted with the help of electronic medical records and personal communication. A complete follow-up was available in 10 cases in total which were diagnosed to be HLA-DR negative non- APML (n = 12).

RESULTS

There were four males and eight females with age ranging from 11-80 years (median age of 43 years).Fever was the most common presenting symptom (n = 12) followed by petechiae (n = 5).

The percentage of abnormal cells/blast in the differential count ranged from 5 to 87% in peripheral smear and 30-85% in bone marrow aspirate smears. The morphologic categorization and diagnosis of AML were according to the (FAB) classification.[5,6] Commonest morphology [Figure 1] was cells (both in bone marrow aspirate and PB) resembling abnormal promyelocytes with bilobation, convoluted and folded nucleus, inconspicuous nucleoli and open chromatin (n = 12) with the presence of Auer rods (n = 3). The abnormal promyelocyte like cells were positive for myelo-peroxidase cytochemically as shown in Figure 2. 100% of cases (n = 12) had classical immunophenotype with expression of CD13 and CD33 and loss of HLA-DR expression.IPT of one of the HLA-DR negative non APML case (the red population) is shown in Figures 3-5. Conventional Karyotyping done in 12 cases in total revealed a normal karyotype with the absence of t (15;17) (n = 10), presence of deletion 9q (n = 1) and t (5;9) (n = 1).FISH done in all (n = 12) cases showed absence of PML - RARA fusion gene. Molecular analysis was performed in 100% cases in total shows nested RT-PCR for PML - RARA fusion transcript to be negative in HLA-DR non-APML cases (n = 12).The HLA-DR negative non-APML cases which were negative for PML - RARA fusion signal by FISH (n = 12) were also negative for PML - RARA transcript by nested RT-PCR (n = 12).

Follow-up was available for 10 cases.Four cases presented with high total WBC counts and were negative for PML - RARA by FISH remained pancytopoenic for about 1 month after starting standard chemotherapy for AML and did not recovered the counts but on further follow-up (till date) there was a partial recovery of peripheral blood counts. Two case have undergone death.Four cases have fully recovered the peripheral blood counts with bone marrow in complete morphological remission.

DISCUSSION

APML is a distinct AML with morphological, immunophenotypic and molecular features. APML has a distinct immunophenotype:- CD13, CD 33 positive with $CD34^{-/+}$, $CD117^{+/-}$ and HLA-DR negative.[7] Blasts in

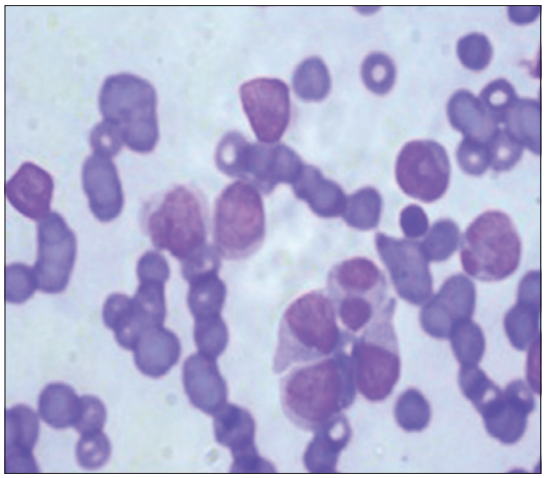

Figure 1: Giemsa staining showing cells resembling abnormal promyelocytes with convoluted and folded nucleus,inconspicuous nucleoli and open chromatin (X 10)

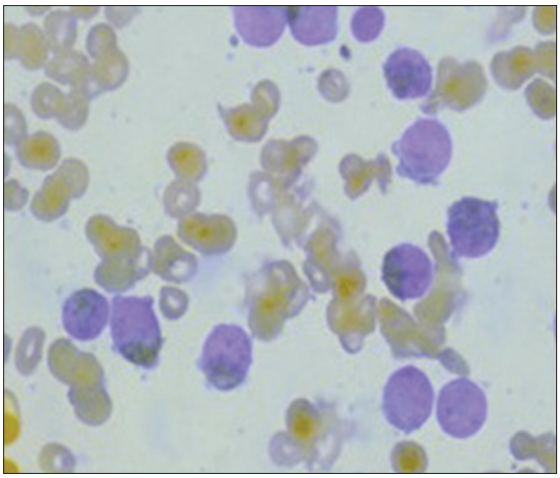

Figure 2: Myeloperioxidase staining showing cells resembling abnormal promyelocytes to be positive for MPO (X 10)

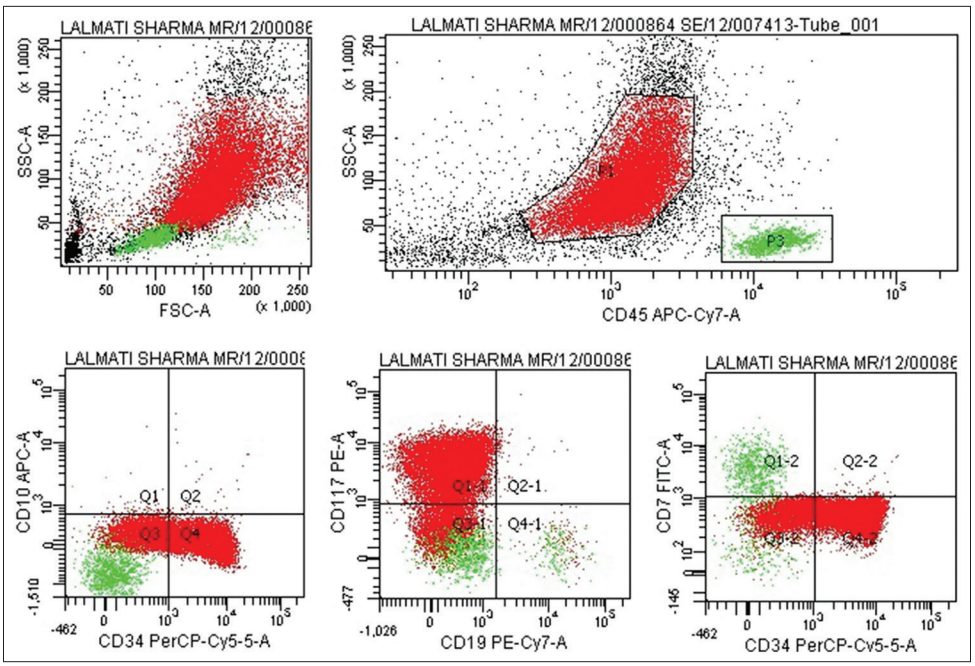

Figure 3: Immunophenotyping analysis showing the gated cells (the red population) with high side scatter to be positive for cluster of differentiation 45 (CD45), CD117 and CD34 while negative for CD10, CD19 and CD7

HLA-DR negative non-APML cases can have morphology resembling the hypogranular variant of APL.Diagnosis of APML is confirmed by the presence of t (15;17) by conventional karyotyping which can be demonstrated by FISH or RT-PCR for PML - RARA fusion gene. Majority of the cases of APML presents with the loss of HLA DR expression but cases presenting with the loss of HLA-DR antigen expression with morphology resembling hypogranular variant of APML should be interpretated cautiously. Such cases should be confirmed for PML - RARA fusion gene by FISH or by RT-PCR. In FISH it is desirable to use break-apart probe for PML - RARA so that variant APMLs in which RARA gene is rearranged with other gene partner[8], can be detected. Identifying variants APMLs is important from the therapeutic point. Two of the APML variants, first in which RARA gene located on chromosome 17 is rearranged with promyelocytic leukemia zinc finger (PLZF) gene on chromosome 11 forming t (11;17)(q23;q21) that is PLZF/RARA fusion transcript and secondly in STAT5b/RARA variant in which the STAT5b gene located at 17q21 is rearranged with RARA gene on chromosome 17[8].These 2 variants are resistant to standard ATRA therapy given to classical APML cases exhibiting PML - RARA fusion transcripts[9]. Further the PML-RARA fusion transcript can be confirmed by molecular techniques as RT-PCR if the cytogenetic analysis is in question. Our finding that lack of HLA-DR antigen expression occurs in non-APML cases corroborates with. Lazarchick and

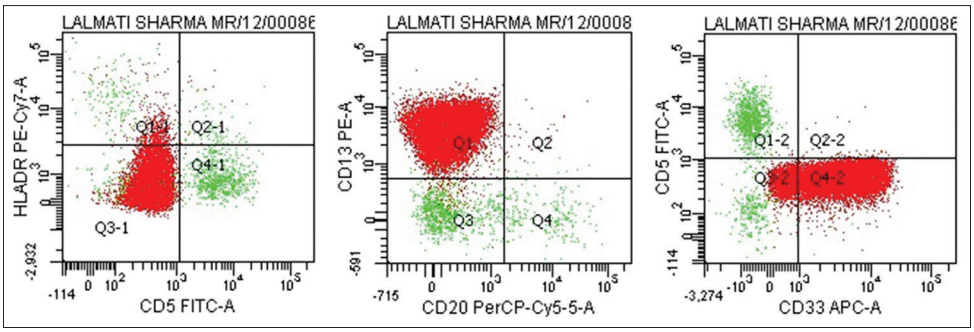

Figure 4: Immunophenotyping analysis showing the gated cells (the red population) to be positive for CD13, CD33 and negative for human leucocytic antigen-DR, CD20 and CD5

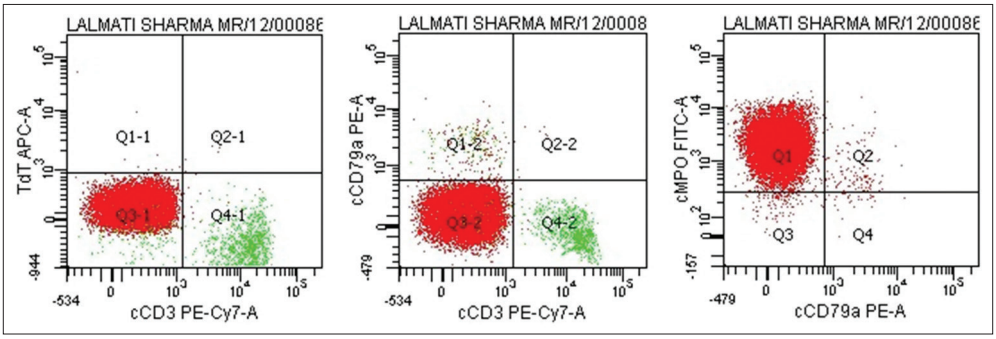

Figure 5: IPT analysis showing the gated cells (the red population) to be positive for cytoplasmic myeloperioxidase and negative for cytoplasmic cluster of differentiation 3 (CD3) and cytoplasmic CD79a

Hopkins who demonstrated absence of HLA-DR antigen expression in AML subtypes other than APL, mainly French-American-British (FAB)- AML - M2.[4] Fenu *et al.* also described three HLA-DR negative AML patients who were suggested to have APL variants based on morphology and immunophenotype, but were reclassified as FAB M2 AML after cytogenetic and molecular analyses were completed[10] Our study has similar results as published by Lazarchick and Hopkins and Fenu *et al.* with only a minor difference as majority of HLA-DR negative cases were reclassified as FAB-subtype of AML-M1 in our study instead of AML-M2. HLA-DR negative non APML cases are associated with FLT-3 internal tandem duplication (ITD) mutation in 84% cases.[9,11] Review of literature from past revealed that FLT-3 ITD mutations are one of the most common mutation found in AMLM1/M2 which corroborates with our finding in this study.[9] FLT-3 ITD are associated with poor prognosis in most of the AML thus HLA-DR negative non-APML which are negative for PML-RARA fusion transcript by FISH and RT-PCR should undergo a FLT-3 ITD mutation analysis for further prognostic characterization. Comparison of clinical and laboratory findings between HLA-DR negative APML and non -APML cases is shown in Table 1.

Table 1: Comparison of clinical, morphological, immunophenotypic and molecular features of APML Vs HLA-DR negative AML

Parameter	APML (*n*=5)	Non-APML/HLA-DR negative AML (*n*=12)
Median age in years	23-64	30-58
Median Hb (gm/dl)	6.8	7.2
Median TLC ($X10^3$/ul)	18-23	20-56
Morphology:	Classical abnormal promyelocytes with Auer rods, buttock cells and Faggots	Cells resemble abnormal promyelocytes with bilobation, convoluted and folded nucleus, inconspicuous nucleoli and open chromatin
Immunophenotype- CD13 and CD33 Positive, CD 34 +/-, CD117-/+ and HLA DR Negative	Present	Present
Conventional Karyotyping detecting t (15;17)	Detected	Not detected
FISH (PML-RARA fusion)	Positive	Negative
RT-PCR (PML-RARA fusion)	Positive	Negative
Median duration of follow up	1 year 8 months	1 year 8 months
Death	1 out of 5	2 out of 12
Median duration from diagnosis to death	1 month	8 months

RT-PCR: Reverse transcriptase-polymerase chain reaction, APML: Acute promyelocytic leukemia, HLA: Human leucocytic antigen, Hb: Hemoglobin, TLC: Total leucocyte count, FISH: Florescent in situ hybridization, PML-RARA: Promyelocytic leukemia-retinoic acid receptor alpha, CD: Cluster of differentiation

ACKNOWLEDGMENTS

We would like to thank for Dr. D.K.Mishra, Dr. Mammen Chandy and Dr. Mayur Parihar.

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Cite this article as: Gupta A, Mishra DK, Chandy M, Parihar M. Human leucocytic antigen-DR negative acute myeloid leukemia: A diagnostic dilemma for hematopathologist. Clin Cancer Investig J 2014;3:383-7.

Source of Support: Nil, **Conflict of Interest:** None declared.