Utility of Flow Cytometric DNA Ploidy Analysis in the Diagnosis of Pure Erythroid Leukemia: An Illustrative Case Report

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Authors' contributions

This work was carried out in collaboration among all authors. Authors NG and AH compiled the data, analysed the cytogenetics and flow cytometry data and wrote the manuscript. Author TD analysed the patient demographic data, cytogenetics and flow cytometry data. Author AM analysed the patient demographic data and laboratory investigation. Author DC provided clinical information. All authors read and approved the final manuscript.

Article Information

Case Study

ABSTRACT

Introduction: The classification and definition of leukemic proliferations pertaining to erythroid lineage have undergone modifications since their first description in the early 19th century. Pure erythroid leukemia (PEL), despite the stringent current WHO definition, is a difficult diagnostic entity owing to problems in distinction from non-neoplastic and neoplastic mimics and lack of lineage specific flow cytometry (FCM)/ immunohistochemistry (IHC) markers. However, almost all the cases of PEL have complex cytogenetics with chromosomal aneuploidy.
Case Study: Here we present a case of 54 year male, known case of Polycythemia Vera since 12 years who developed pain in both the lower limbs for the past 10 days. Peripheral blood smear revealed leuko-erythroblastic picture. With the clinical query of myelofibrosis/ leukemic transformation, bone marrow examination was done. Both aspirate and biopsy revealed marked

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erythroid hyperplasia with many cells having immature proerythroblastic morphology. Flow cytometric immunophenotyping revealed 35% erythroid cells expressing CD58 and CD71 along with 5% myeloid blasts expressing CD34, CD38, CD117, CD71, CD13, CD33, HLADR along with dim myeloperoxidase (MPO) and aberrant CD7. FxCycle™ violet (FCV) based FCM DNA ploidy, performed as a part of routine, revealed a major high-hyperdiploid clone with DNA Index (DI) 1.35 and minor diploid clone with DI 1.01 for both erythroid cells and myeloid blasts. Cytogenetic analysis also confirmed both these clones with a modal karyotype 58~63,XY,-5,-7,-12,-14,-17,-20,+21[cp16]/46,XY[4]. Karyotype analysis is technically challenging and not available at many centres.

Discussion and Conclusions: FCV based FCM DNA ploidy is a rapid, simple and inexpensive assay which has strong correlation with cytogenetic ploidy and can provide specific results in immunophenotypically selected cells, in hemodilute samples, even in cases with cytogenetic culture failure and can supplement the diagnosis of PEL.

1. INTRODUCTION

Pure erythroid leukemia (PEL) is characterized by hyper-proliferation of immature erythroblasts with maturation arrest in the bone marrow, an aggressive clinical course with a short overall survival [1-4]. However many times, the diagnosis and recognition of this condition becomes difficult, attributable to both non-neoplastic and neoplastic mimics associated with marrow erythroid hyperplasia and dyserythropoiesis, stringent WHO diagnostic criteria and lack of definitive lineage specific flow-cytometry (FCM)/ immunohistochemistry (IHC) markers to delineate the immature proliferating erythroblasts of PEL [5-7]. Almost all the PEL cases however, are associated with a complex karyotype with multiple structural and numerical chromosomal abnormalities [8,9]. Flow cytometric DNA ploidy has been documented to have an excellent correlation with cytogenetic analysis in haematolymphoid neoplasm [10-12]. Here we present a case which emphasizes the utility of FxCycle™ violet based flow cytometric DNA ploidy analysis in ascertaining the diagnosis of PEL.

2. CASE STUDY

A 54 year old male, presented to the outpatient department of our hospital with complaints of severe back pain and pain in bilateral lower limbs for the past 10 days. Previous medical records revealed that he was a known case of Polycythemia Vera (PV), JAK2-V617F positive, diagnosed 12 years back and managed with phlebotomy (last done 4 months back) and hydoxyurea (500 mg/day) along with acetylsalicylic acid. Magnetic Resonance Imaging done outside unveiled the possibility of infiltrative marrow disease. On examination, he had an enlarged spleen (8 cm below left costal margin) along with mild bone tenderness while the rest of the systemic examination was un-remarkable. There was no pallor, icterus or palpable lymph nodes. Total blood counts and peripheral smear revealed a white blood cell count (WBC) of 6.32 x 10^3/µL, (reference range 4.0 to 10.0 x 10^3/µL), haemoglobin 10.1 g/dL (reference range 13.0 to 17.0 g/dL) and platelets 68 x 10^3/µL (reference range 150 to 400 x 10^3/µL) with leuko-erythroblastic picture having 6% blasts, 2% myelocytes, 3% metamyelocytes, dysgranulopoiesis and 7 nRBCs/100 WBCs (Fig. 1A). Serum lactate dehydrogenase (LDH, reference range 135-225 U/L) was 915 U/L. With the clinical query of myelofibrosis/ leukemic transformation, bone marrow examination was ordered and performed. The bone marrow aspiration revealed hypercellular particles with erythroid hyperplasia comprising of 85% erythroid cells exhibiting marked dyserythropoiesis and 52% of these cells having immature proerythroblastic morphology (Fig. 1B) along with PAS positivity (Fig. 1B inset). Bone marrow biopsy was markedly hypercellular according to the patient's age and displayed near total replacement by sheets of erythroid precursors (Fig. 1C) highlighted by CD71 (Fig. 1D), E-Cadherin (Fig. 1E) with dim expression of CD117 (Fig. 1F) and negative for CD34 (Fig. 1G) and myeloperoxidase (MPO) (Fig. 1H).

Flow cytometric immunophenotyping done on bone marrow aspirate revealed 35% erythroid cells expressing CD58 and CD71 along with 5% myeloid blasts expressing dim CD45 with CD34, CD38, CD117, CD71, CD13, CD33, HLADR along with dim MPO and aberrant CD7 (Fig. 2).
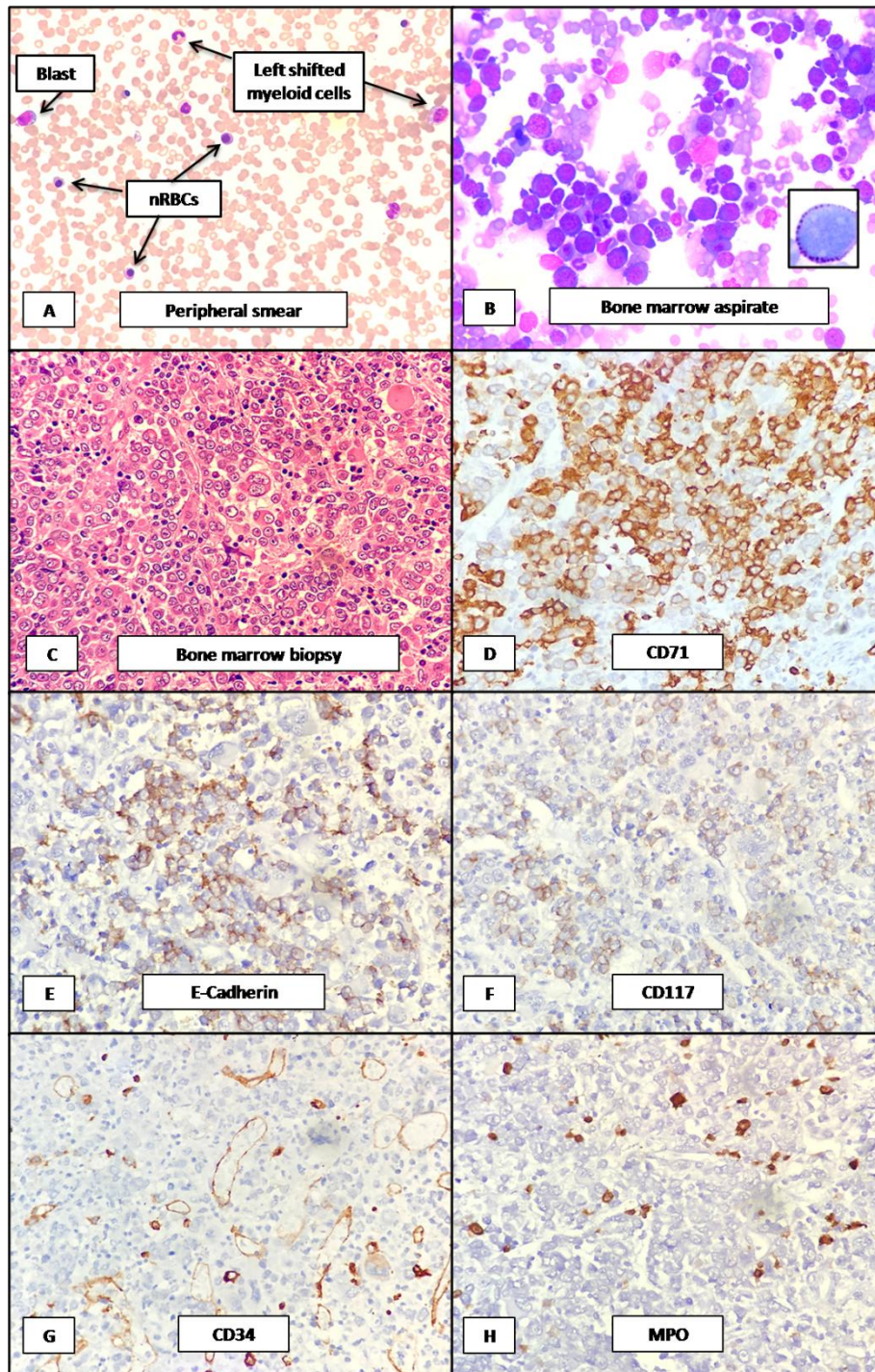
Fig. 1. A Peripheral Smear showing leuko-erythroblastic picture; B Bone marrow aspirate showing erythroid hyperplasia (85% erythroid cells) with dyserythropoiesis and 52% immature proerythroblasts (inset showing PAS positivity in erythroblasts); C Bone marrow biopsy showing replacement by sheets of erythroid precursors; D CD71 immunostain highlighting erythroid precursors; E E-Cadherin immunostain highlighting erythroid precursors; F CD117 immunostain showing dim expression on some of the erythroid precursors; G CD34 immunostain highlighting ~5% blasts, negative on erythroid precursors; H MPO immunostain negative on erythroid precursors
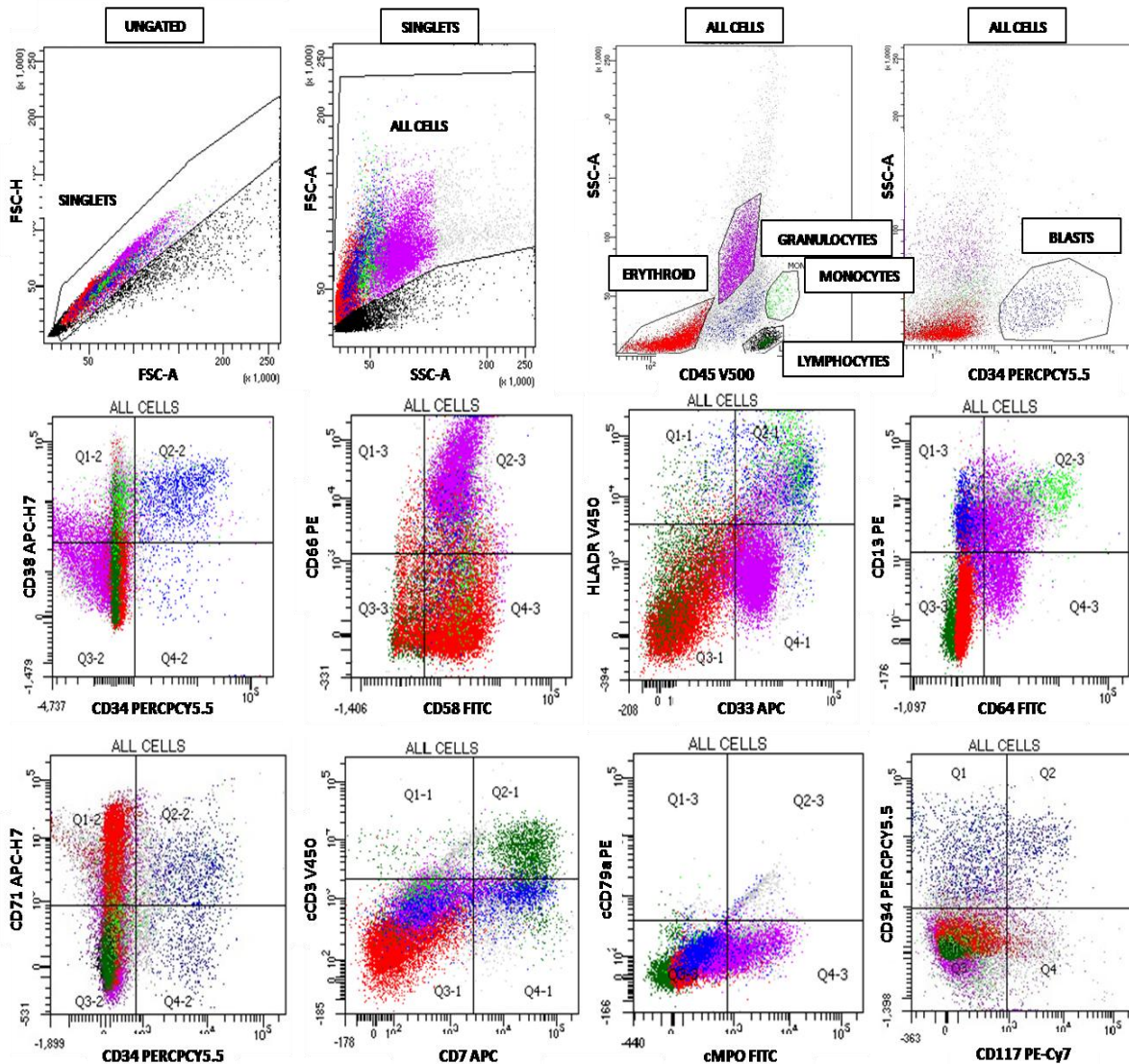
Fig. 2. Flow cytometric immunophenotypic scatterograms showing erythroid cells (Red) expressing CD58 and CD71 and Myeloid blasts (Blue) expressing CD34, CD38, CD117, CD71, CD13, CD33, HLADR along with dim MPO and aberrant CD7. Granulocytes (Pink) and Lymphocytes (Dark green) are seen in their respective areas

FxCycle^{TM} violet (FCV) based flow-cytometric DNA ploidy analysis was simultaneously performed and revealed two clones of both erythroid population and myeloid blasts, a major high-hyperdiploid clone with DNA Index (DI, geometric mean of G_0/G_1 peak of abnormal cells divided by geometric mean of G_0/G_1 peak of lymphocytes) 1.35 and a minor diploid clone (DI 1.01) (Fig. 3).

The karyotype as well confirmed high-hyperdiploidy in 16 metaphases and diploidy in four metaphases, with modal karyotype 58~63,XY,-5,-7,-12,-14,-17, 20,+21 [cp16]/ 46,XY [4] (Fig. 4). Molecular testing (by PCR) was negative for AML ETO gene rearrangement t(8;21), BCR-ABL gene rearrangement t(9;22), PML-RARA gene rearrangement, Inv16(p13q22) / t(16;16) gene rearrangement, NPM1 gene mutation and FLT3-ITD/TKD gene mutation. So a final diagnosis of Pure Erythroid Leukemia transformed from an underlying Polycythemia Vera was considered. The patient however did not opt for further treatment at our hospital and left against medical advice.
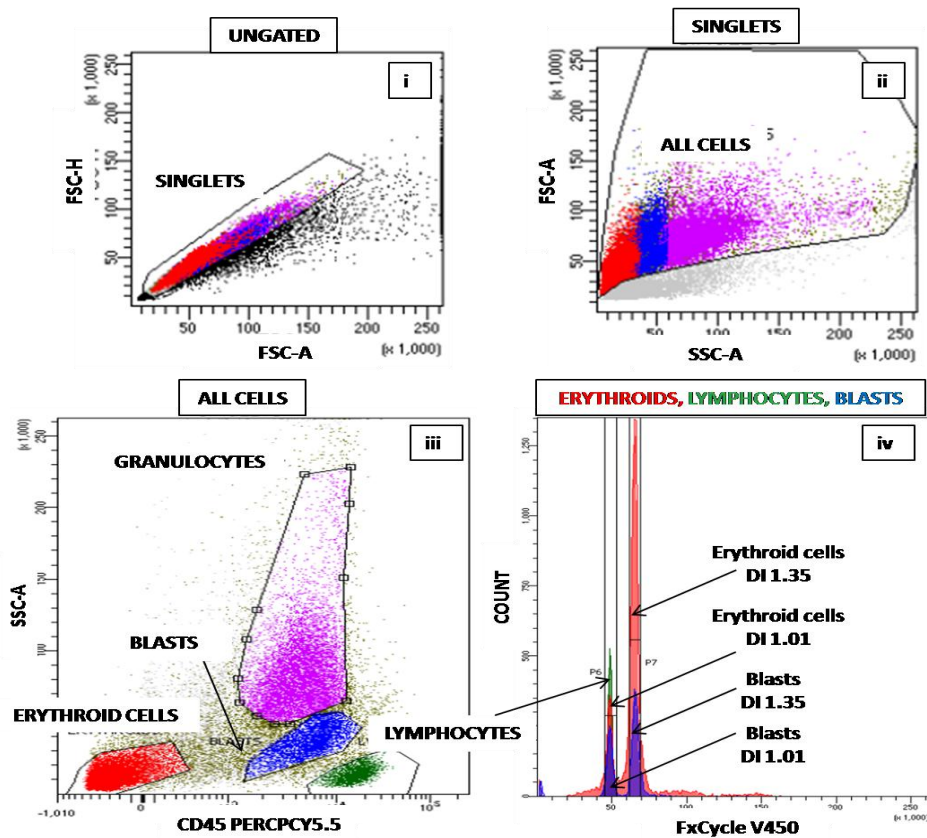
Fig. 3. Gating strategy: Dot plot "i" shows gating singlets using forward scatter (FSC) area and FSC height plot; Plot "ii" shows gating viable events using FSC area and side scatter (SSC) area; Plot "iii" shows CD45/ SSC to gate granulocytes (pink, moderate CD45 with high SSC), erythroid cells (red, CD45 negative area), blasts (blue, dim to moderate CD45 region with low to moderate SSC) and lymphocytes (green, bright CD45 expression with low SSC). Histogram overlay plot" iv" showing DNA cell cycle analysis using FxCycle Violet (V450). Geometric mean (GM) of G_0/G_1 peak (abnormal cells) is divided by GM of G0/G1 peak (lymphocytes) to calculate DNA index (DI). GM lymphocytes 47,223; GM Blasts 47,820 and 65,014; GM erythroid cells 47,860 and 65,020. Coefficient of variation (CV) of G0G1 peak of lymphocytes, blasts, erythroid cells ranged between 3.2 -3.5%

3. DISCUSSION

Consequent to greater understanding of the disease patho-biology, integration of clinical features with molecular and immunophenotypic profiles, leukemias arising from erythroid lineage have undergone changes in the disease definition since its first description by Coppelli in 1912 and documentation by Di Guglielmo in 1928 [1-4]. It was first included as a separate entity in French-American-British (FAB) Cooperative group in 1976. The World health organization (WHO) classification published in the year 2001 recognized two subtypes 1) Erythroid/ Myeloid Subtype and 2) Pure erythroid leukemia, both of which continued to exist in WHO 2008 classification, until the WHO 2017 revision, from which the erythroid/ myeloid subtype was removed [2]. Cases previously classified as erythroleukemia (erythroid/myeloid type) on the basis of counting myeloblasts as a percentage of non-erythroid cells when erythroid precursor cells constituted ≥ 50% of the marrow cells are now classified on the basis of the total bone marrow or peripheral blood blast cell count. Such cases are currently classified as myelodysplastic syndrome with excess blasts if blasts constitute < 20% of all marrow or blood cells and as acute myeloid leukaemia (AML) with myelodysplasia-related changes if blasts constitute ≥ 20% of the cells, regardless of the erythroid cell count [9].
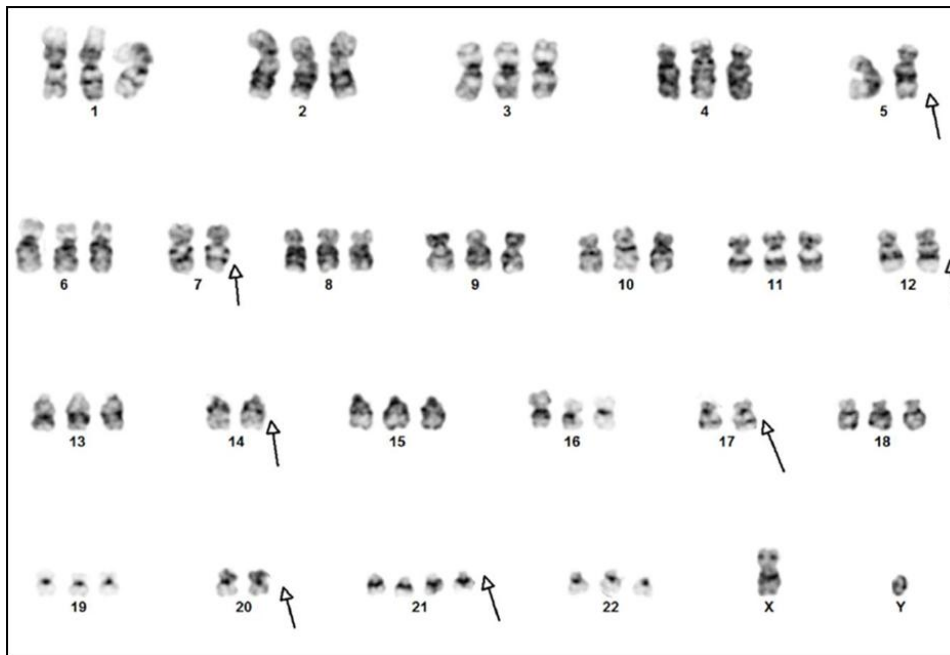
Fig. 4. Chromosomal analysis with Modal karyotype 58~63,XY,-5,-7,-12,-14,-17,-20,+21[cp16]/46,XY[4] revealed 16 metaphases with a complex karyotype showing high hyperdiploidy (58~63 chromosomes) with gains and losses of multiple chromosomes. The image of the four diploid metaphases is not shown *(Image courtesy: Dr. Lal Path Labs, Delhi)*

Pure Erythroid leukemia (PEL) is defined as a neoplastic proliferation of immature undifferentiated/ proerythroblastic in appearance committed exclusively to the erythroid lineage (> 80% of all the bone marrow nucleated cells are erythroid, with ≥ 30% proerythroblasts), with no evidence of a significant myeloblastic component. It accounts for <5% of all AMLs and can present at any age with a male preponderance. It may arise de- novo or secondary to prior chemotherapy with alkylating agents, toxic exposure (e.g. alcohol) and occupational exposure to mutagens like benzene, evolving from underlying myeloid neoplasm like myelodysplastic syndrome (MDS), myeloproliferative neoplasm (MPN) or MDS/MPN [9]. The propensity for leukemic transformation in MPN varies according to the MPN variant and is highest in primary myelofibrosis (incidence of 10% to 20% in the first 10 years of the disease), lower in PV (incidence 2.3% at 10 years and 7.9% at 20 years) and lowest in essential thrombocythaemia (<1% in the first decade of the disease). PEL and megakaryoblastic leukemia are the most common variants reported in MPN blast phase [13,14]. Our patient was a known case of PV, which got transformed to PEL. The best known FCM markers for defining erythroleukemia include glycophorin A (CD235), transferrin receptor (CD71), thrombospondin receptor (CD36) along with negativity for CD34 and myeloid associated markers like MPO, CD13, CD33, HLA-DR, though KIT (CD117) may be positive [1-3]. Clonal chromosomal abnormalities with complex karyotype and aneuploidy are seen in 75-100% of all cases of PEL with chromosomes 5 and 7 being the most frequently involved followed by chromosomes 8, 16, and 21 [3,8].

Despite being discretely defined, PEL often poses a diagnostic challenge to the pathologists and morphological categorization is arduous. The left-shifted erythroid maturation in the marrow can occur in various non-neoplastic (reactive erythroid hyperplasia with dyserythropoiesis in megaloblastic anaemia, haemolytic anaemia, erythropoietin administration) or neoplastic conditions (associated with >50% erythroid cells in the marrow with dyserythropoiesis and increased blasts like MDS with excess blasts, AML with myelodysplasia related changes and therapy related MDS/AML) [5-7].

Moreover, the strict WHO definition for PEL requiring >80% erythroid cells in the marrow may not always be met in the event of bone marrow sample hemodilution [9]. The various FCM markers used to delineate immature erythroblasts in PEL lack specificity as well as

consistency. Glycophorin A has been reported to be negative in some PELs, as it is a late erythroid marker. Expression of CD71 is also not restricted to erythroleukemia and can be expressed as a non-specific activation marker in most of AML and few ALL cases as well. CD36, again, is a non-specific marker and detects erythroid precursors at earlier stages of differentiation but is also expressed by monocytes and megakaryocytes. Antigens associated with megakaryoblasts like CD41 and CD61 may also at times be expressed in leukemic proerythroblasts [1-3,15,16].

Although none of the cytogenetic abnormalities are disease defining for PEL, almost all the cases exhibit complex karyotype with multiple structural and numerical chromosomal abnormalities [3,8,9]. Therefore karyotype analysis or FCM DNA analysis is vital for supplementing and establishing a diagnosis of PEL and to distinguish it from reactive mimics, especially due to lack of specific FCM/ IHC markers. Karyotype, however, has a turnaround time of 7-10 days and may be associated with culture failure in approximately one fourth cases analysed [17]. FCM DNA ploidy analysis is a simple and rapid method (results obtained within three hours) to study the DNA content of the cells and to estimate proportion of cells in different phases of the cell cycle. It exhibits strong correlation with cytogenetic ploidy. Literature describes several different methods for FCM DNA ploidy analysis utilizing various nucleic acid binding dyes (DRAQ5, Propidium Iodide, Ethidium Bromide Acridine orange, and Hoechst etc.). However, these methods involve multiple processing steps like RNAase treatment with prolonged incubation times and results lack reproducibility. Also, most of these conventional dyes are excited with blue or red laser, thereby limiting simultaneous immunophenotypic analysis performed using fluorochromes excited by them [10]. FCV is a DNA selective dye (Invitrogen, California, USA) that is excited by violet laser, and spares the spectrum of colours excited with blue and red laser for simultaneous immunophenotypic analysis. The ploidy results can be obtained in almost all cases, on immunophenotypically selected and gated cells, even in hemodiluted samples, sample with low neoplastic cell population, as well as cases associated with cytogenetic culture failures [10,18]. It is a simple, inexpensive (additional cost per test is approximately INR 400) assay to establish, where only 1 µl of reconstituted FCV needs to be incubated with fixed cells stained for routine surface antibodies and only half an hour incubation.

FCM DNA index (DI) of all the normal hematopoietic elements (including lymphocytes, granulocytic cells, monocytes, hematogones, stem cells and erythroid cells) is diploid [18,19]. Even erythroid cells from patients with megaloblastic anaemia and erythroid hyperplasia secondary to haemolytic anaemia demonstrate a diploid DI, though due to increased erythroid proliferation, S-Phase fraction may be higher [20-22]. In our laboratory, FCV based FCM ploidy is routinely performed in all consecutive haematolymphoid neoplasms (including chronic lymphoproliferative disorders, AML, ALL, and multiple myeloma) during diagnosis and follow up. In the present case, FCM DNA ploidy revealed the presence of two clones in the erythroid cells as well as myeloid blasts with a major high hyperdiploid (DI 1.35) peak along with a minor diploid peak (DI 1.01) as shown in Fig. 3. The corresponding clones were also subsequently confirmed by conventional karyotyping as well.

4. CONCLUSION

FCM DNA ploidy has a potential role in picking up neoplastic erythroblasts of PEL (which are usually aneuploid) from a background rich in normal or reactive cellular counterparts. PEL is a clinically aggressive disease and an early and correct diagnosis is imperative. FCV based FCM ploidy is a simple assay, which can provide rapid and strong supportive evidence of a neoplastic process without having to wait for karyotype report which has a longer turnaround time, is technically challenging and not routinely available at many centres.

CONSENT

As per international standard, patient's written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

As per international standard, written ethical approval has been collected and preserved by the author(s).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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