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Flow Cytometric DNA Ploidy Analysis in Haemato-Lymphoid Neoplasms: An Analysis of 132 Cases

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ABSTRACT

Background: FxCycle™ Violet (FCV) based flow cytometric (FCM) DNA ploidy analysis is a rapid and simple tool that can substantiate in characterizing the biological behaviour across the spectrum of haematological malignancies and correlates with cytogenetic studies.

Materials and Methods: In this prospective study, we performed simultaneous immunophenotyping with FCV based on ploidy analysis in n=132 consecutive new samples, comprising n=110 samples of haemato-lymphoid neoplasms, including acute leukemias (n=67, 60.9%), CML with myeloid blast crisis (n=1, 0.9%), MDS with excess blasts (n=2, 1.8%), mature B cell/ T cell neoplasms (n=37, 33.7%), multiple myeloma (n=3, 2.7%) along with n=22 normal samples. The FCM DNA data was compared with corresponding conventional karyotyping results, wherever available.

Results: In FCM ploidy analysis (n=110), the overall DNA index (DI) ranged from 0.81 to 2.17 and S-Phase fraction (SPF) from 0.1-31.6%. Diploidy was seen in n = 90 (81.8%), low-hyperdiploidy in n = 10 (9.1%), high-hyperdiploidy in n = 7 (6.4%) with one case each (0.9% each) having near-tetraploidy, high-hypodiploidy and low-hypodiploidy. The DI of all viable cell populations in normal samples ranged from 0.96-1.05. Conventional karyotyping was performed in n=76/110 cases (70%) with n= 11/76 (15%) culture failures. The modal chromosome number ranged from 45 to 63. A concordance of 95.4% (n=62/65) was noted with corresponding FCM DI.

Conclusion: FCV-based ploidy is a sensitive technique that provides complementary information and ascertains a strong correlation with conventional cytogenetics across all haemato-lymphoid neoplasms. It can detect aneuploidy in all B-ALL and myeloma cases, even in hemodiluted samples with cytogenetic culture failure; supplement the diagnoses of erythroleukemia, and provide a useful screen for a higher grade lymph node disease in lymphoma cases with SPF > 3%.

Keywords: DNA ploidy; FxCycle™ violet; S-phase fraction; Cytogenetics; Karyotyping

INTRODUCTION

Risk stratification and prognostication in haemato-lymphoid neoplasms is commonly performed using karyotyping, fluorescence in situ hybridization (FISH) and polymerase chain reaction (PCR). In some countries, single nucleotide polymorphism (SNP) arrays and array comparative genomic hybridization (aCGH) are utilized however these are not cost-effective in limited resources settings[1-5].

Flowcytometric (FCM) DNA content analysis is an age-old technique which has been in existence for decades. It is a rapid and simple tool which ascertains the proportion of cells in various phases of cell cycle and can substantiate in characterizing the biological behaviour of haematological malignancies including acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), myelodysplastic syndromes (MDS), chronic lymphoproliferative disorders (CLPD) and multiple myeloma[2]. However, despite the invaluable information provided by FCM DNA analysis, its utility in clinical flowcytometry was limited in the past by technical issues pertaining to the then available DNA

binding dyes and the flow cytometers themselves. Published literature mentioned several methods for FCM nucleic acid staining utilising Propidium Iodide (PI), DAPI, DRAQ5, Acridine orange, Sytox green, Hoechst, Ethidium Bromide and so on. However, these methods were laborious, as they required prolonged incubation time, involved multiple steps like RNAase treatment, and lacked reproducibility[6-9]. Also, it was difficult to segregate the tumour population from the "dilution effect" of normal hematopoietic elements present in the background, as most of these conventional dyes were excited with blue or red laser and thereby, simultaneous immunophenotypic analysis could not be performed using fluorochromes excited by these lasers due to bleed over into adjacent channels. This becomes even more relevant when dealing with minute tumour populations, as during minimal residual disease estimation. Hence, only DNA analysis is not sufficient on its own in specifically delineating the tumor population and requires concurrent surface immunophenotyping[5,10-12]. Over the last decade, clinical flow cytometers have undergone metamorphosis, with availability of tandem dyes and multiple lasers, permitting up to 8-10 colour flowcytometry along with development of new reagents like FxCycle™ violet (FCV) [1, 5, 13].

FCV (4',6-diamidino-2-phenylindole, dihydrochloride) from Invitrogen, is a cell-impermeant DNA selective dye that is excited with 405/407 nm laser (violet laser) with emission collected in the 450/50 band pass filter or equivalent. This spares the spectrum of colours excited with blue as well as red laser for simultaneous 6-7 colour immunophenotyping, and requires only a single step modification of the routinely used stain → lyse → wash protocol[1, 5, 13].

In the modern risk adapted treatment protocols, ploidy status holds relevance only in B-ALL and multiple myeloma, with high-hyperdiploidy being associated with a superior outcome, and hypodiploidy with a poor outcome[14-27]. While in mature B-cell/ T-cell neoplasms, DNA aneuploidy and high S-phase fraction (SPF) are independent adverse prognostic markers; only limited conflicting data is available about any prognostic relevance of aneuploidy in AML, MDS, T-ALLs and Mixed phenotype acute leukemias (MPAL)[28-35]. Furthermore, there is no consolidated data on FCM DNA ploidy analysis across the entire spectrum of haematological neoplasms.

In this prospective study, all consecutive new cases of haemato-lymphoid neoplasms including acute leukemia, MDS, mature B-cell/ T-cell neoplasms and multiple myeloma were included for simultaneous routine immunophenotyping and FCM DNA ploidy analysis using FCV. The flow cytometric DNA data was then compared with conventional karyotyping results, wherever available, and an attempt was made to address the discrepancies.

MATERIALS AND METHODS

FCV-based DNA ploidy analysis was performed in 132 consecutive samples (Bone marrow: n=92, Peripheral blood: n=38, Cerebrospinal fluid: n=1 and Pleural fluid n=1) received in our flow cytometry laboratory. The DNA analysis was performed simultaneously with routine eight-colour immunophenotyping. The DNA FCM ploidy data was compared with conventional cytogenetic ploidy wherever available.

Written and informed consent was taken from all the patients for the above samples. The study was approved by institutional review board of our hospital.

Nature of Study: Prospective.

Study Duration: April 2019 to December 2019.

Inclusion criteria: All consecutive new cases of haemato-lymphoid neoplasms including acute leukemia, MDS, mature B-cell/ T-cell neoplasms and multiple myeloma were included for simultaneous routine immunophenotyping and FCM DNA ploidy analysis of abnormal population using FCV.

Samples received in FCM laboratory with no flowcytometric or morphological evidence of haemato-lymphoid malignancy were also included for ascertaining FCV based FCM DNA index (DI) in normal cell populations.

Exclusion criteria: None.

Flow cytometric immunophenotyping

Immunophenotyping and DNA ploidy analysis was performed on BD FACS Canto™ II flow cytometer (Becton Dickinson, San Jose, CA). The cytometer stability and sensitivity were checked every day before each acquisition session by running cytometer setup and tracking (CS&T) beads as per manufacturer's instructions. Fluorochromes used for routine immunophenotyping included FITC (Fluorescein isothiocyanate), PE (Phycoerythrin), PerCP-Cy5.5 (Peridinin chlorophyll protein-conjugated to a cyanine dye Cy5.5), PE-Cy7 (tandem conjugate of Phycoerythrin and a cyanine dye, Cy7), APC (Allophycocyanin), APC-H7 (modified tandem conjugate of Allophycocyanin and Cy7) along with violet laser dyes V450 and V500.

Immunophenotypic delineation of blasts, lymphoid cells and plasma cells for categorizing hemato-lymphoid neoplasms was performed as per WHO 2017 criteria[36]. The surface antibodies and cytoplasmic markers (permeabilized with Perm-2 solution, Becton Dickenson, San Jose, CA) used during the study are mentioned in Table 1. For all acute leukemia cases, tubes "ALOT" (acute leukemia orientation tube), "B-ALL1" and "AML1" were used initially and then on cases specific basis, other tubes were used, for instance in erythroleukemia/ megakaryoblastic leukemias, additional immunostaining with "AML2" tube was performed.

In suspected B-CLPDs, tubes "CLPD1", "CLPD2" and "Hairy tube" were run in all cases and other tubes could be performed on case specific basis for ruling out T and NK-CLPDs. For plasma cell neoplasms, tube "Plasma1" was done with cytoplasmic staining for kappa and lambda light chains. All antibodies mentioned were obtained from Becton Dickinson, San Jose, CA. The concentration of these antibodies was pre-determined after standard titration studies. The standard technique of stain, followed by lyse (using BD FACS™ lysing solution), then wash was used. Immunophenotyping data was analysed using BD FACSDiva™ version 8.0.2 software.

Flow cytometric DNA ploidy analysis

FCV was obtained from Invitrogen, life sciences; Catalogue Number F10347[13]. Detailed protocol of FCM ploidy analysis has been published by us previously[37]. In brief, post flow cytometric characterization of the haemato-lymphoid neoplasm, depending on the distinctive position of tumour cells on CD45/side scatter (SSC) plot and the markers expressed by them, in an additional tube, the following markers were incorporated:

1) For CD5 positive CLPDs; CD5-V500, CD19-PE-Cy7, Lambda-FITC and Kappa-PE with CD45-PerCP-Cy5.5 (Figure 1)
2) For CD5 negative CLPDs; CD10 APC or CD103 APC-H7 with Lambda-FITC, Kappa-PE and CD45-PerCP-Cy5.5
3) For plasma cell neoplasms, CD38-APC-H7, CD138-APC were used with CD19-PE-Cy7 and CD45-PerCP-Cy5.5
4) For CD34 positive myeloid blasts; CD34- APC-H7 and CD45-PerCP-Cy5.5
5) For CD34 negative myeloid blasts; CD13-PE or CD33-APC with CD45-PerCP-Cy5.5
6) For Precursor B-ALLs; CD19-PE-Cy7 and CD10-APC with CD45-PerCP-Cy5.5
7) For CD10 Negative Pro B-ALLs; CD19-PE-Cy7 and CD20-V500 with CD45-PerCP-Cy5.5, to distinguish mature normal B-Cells (CD19+CD20+CD45 bright) from Pro B-ALL cells (CD19+ CD20+/- CD45 variable)
8) For T-ALLs; depending upon the marker expressed, CD7-APC and CD45-PerCP-Cy5.5 along with CD34- APC-H7 or CD1a-FITC or CD99-PE

To these surface antibody stained, lysed and fixed cells (formaldehyde is a component of FACS Lyse), 1 µl of freshly reconstituted FCV (1 mg/ml stock solution) was added and incubated for 30 minutes, protected from light. This was followed by acquisition of 10,000 to 30,000 events at low event rate. DNA ploidy data was analysed using BD FACSDiva™ version 8.0.2 software. The voltages for FL7 were adjusted to get the normal G_0G_1 peak of lymphocytes at about the 50th channel of a 256-channel scale on FL7-Area linear scale. DNA index (DI) was calculated as a ratio of geometric mean (GM) of FCV in G_0G_1 peak of specifically gated out tumor cells to the GM of FCV in G_0G_1 peak of normal lymphocytes or granulocytes (Figure 1). FCM ploidy was categorized as near-haploid, low-hypodiploid, high hypodiploid, diploid, low-hyperdiploid, high-

hyperdiploid, near-triploid, near-tetraploid for DI 0.55 to 0.69, 0.70 to 0.88, 0.89 to 0.95, 0.96 to 1.05, 1.06 to 1.15, 1.16 to 1.39, 1.40 to 1.79, and 1.80 to 2.28, respectively based on existing literature and our previously published work (Table 2)[5-27, 37].

For standardization of FCV: 1) Linearity and resolution were established using FCV stained chicken erythrocyte nuclei (CEN) and 2) Doublet discrimination using calf thymocyte nuclei (CTN) (BD DNA QC Particles, BD Catalogue Number 349523). To rule out inter-assay variation, one diploid sample was run 10 times and DNA index for each run was calculated. The mean DI was 0.99 with CV of 0.002%; indicating very minimal intra-assay variation. A serial dilution experiment was also performed to determine the level of sensitivity in the detection of aneuploidy for samples with low cell count. For this assay, a peripheral blood sample from B-ALL case with 20% hyperdiploid (DI: 1.28) blasts was selected. The cells were diluted with cells from cytogenetically diploid bone marrow from a patient with megakaryocytic thrombocytopenia in proportions of 1:1, 1:10, 1:100, and 1:1000. After dilution, these cells were stained for surface makers and FCV as described above. For each dilution, approximately 0.05, 0.1, 0.5 and 1.0 million events were acquired and 10.1% blasts, DI 1.29; 1.2% blasts, DI 1.28; 0.1% blasts, DI 1.29; and 0.01% blasts, DI 1.27 could be detected, respectively. Hence, aneuploidy could be accurately detected in the presence of as low as 0.01% tumor cells.

Conventional Karyotyping

All samples were outsourced to external laboratory (Dr. Lal Path Labs, New Delhi, India) and processed using standard cytogenetic protocols. The results were reported in accordance with the International System for Human Cytogenetic Nomenclature (ISCN) 2016 and patients were classified into: normal karyotype (46 chromosomes), pseudo-diploidy (abnormal karyotype with 46 chromosomes), low-hyperdiploidy (47–50 chromosomes), high-hyperdiploidy (51–65 chromosomes), near-triploidy (66–80 chromosomes), and near-tetraploidy (81–102 chromosomes), hypodiploidy (<46 chromosomes), which was further divided into three categories according to modal chromosome number (MN): near-haploidy (24 to 29 chromosomes), low-hypodiploidy (30–39 chromosomes) and high-hypodiploidy (40–45 chromosomes) (Table 2)[37, 38].

FISH panel and PCR panels

ALL and AML real time PCR based panels were outsourced to external laboratory (Dr. Lal Path Labs, New Delhi, India), as well as multiple myeloma and CLL FISH panels, which were outsourced to Onquest Laboratories (New Delhi, India). ALL PCR panel included BCR-ABL gene rearrangement, t(12;21)(p13;q22)/ ETV6-RUNX1, t(4;11)(q21;q23)/MLL-AF4, t(9;11)(p21-22;q23)/MLL-AF9, t(1;19)(q23;p13.3)/ TCF3-PBX1 and t(11;19)(q23;p13.3)/MLL-ENL. AML PCR panel encompassed BCR-ABL gene rearrangement, t(8;21)(q22;q22)/ RUNX1-RUNX1T1, FLT3 Gene mutation (ITD by fragment analysis), NPM1 gene mutation, PML-RARA gene rearrangement, Inv16(p12q22)/t(16;16)(p13;q22) gene rearrangement. Myeloma FISH was performed using magnetic bead enrichment method and the panel was inclusive of del 17p13 assay (Zytolight, Germany Spectrum orange TP53/ CEN 17 SPEC Green Dual Colour DNA probe); del 13q14.3 assay (Abott, Vysis, Illinois directly labelled LSI D13S25 DNA probe); t(4;14)/ IgH/FGFR3 translocation assay (Abott, Vysis, Illinois LSI IGH/FGFR3 dual colour dual fusion probe); t(11;14)/IgH/CCND1 translocation assay (Zytovision, Germany directly labelled IgH (14q32.3/ CCND1 t(11;14) dual colour dual fusion DNA probe); t(14;16) (Abott, Vysis, Illinois LSI IGH/MAF dual colour dual fusion probe). FISH panel for CLL incorporated t(11;14)/IgH/CCND1 translocation assay (Zytovision, Germany directly labelled IgH (14q32.3/ CCND1 t(11;14) dual colour dual fusion DNA probe); del 11q22 (ATM) assay and Del17p13 assay (LSI 11q22 ATM Spectrum Green/ 17p13 Spectrum orange Dual colour DNA probe, Zytolight, Germany); CEP12 assay/ del13q14 assay/ del13q34 assay (CEP12 Spectrum Green/ 13q14 D13S319 Spectrum Orange/ 13q34 Spectrum Aqua TC DNA probe, Abott, Vysis, Illinois).

Table 1: Immunophenotypic panels used for characterization of hemato-lymphoid neoplasms

Acute Leukemia								
Tube Name	**V450**	**V500c**	**FITC**	**PE**	**PerCP Cy5.5**	**PE Cy7**	**APC**	**APC-H7**
ALOT	cyCD3	CD45	cyMPO	cyCD79a	CD34	CD19	CD7	smCD3
Clone (BD)	UCHT1	2D1	5B8	HM47	8G12	SJ25C1	M-T701	SK7
B-ALL 1	CD20	CD45	CD58	CD66c	CD34	CD19	CD10	CD38
Clone (BD)	L27	2D1	1C3	B6.2	8G12	SJ25C1	HI10A	HB7
B-ALL 2	CD73	CD45	CD123	CD200	CD34	CD19	CD10	CD81
Clone (BD)	AD2	2D1	7G3	MRC OX 104	8G12	SJ25C1	HI10A	JS81
T-ALL 1	CD4	CD45	CD1a	CD99	CD5	CD2	TdT	CD8
Clone (BD)	SK3	2D1	HI149	TU12	L17F12	L303.1	E17-1519	SK1
AML 1	HLADR	CD45	CD64	CD13	CD34	CD117	CD33	CD14
Clone (BD)	L243	2D1	CLB/FcGran1	L138	8G12	104D2	P67.7	MP9
AML 2	CD4	CD45	CD123	CD56	CD34	CD41a	CD15	CD71
Clone (BD)	SK3	2D1	7G3	My31	8G12	HIP8	HI98	M-A712
Chronic lymphoproliferative disorders								
CLPD1	CD20	CD45	Lambda	Kappa	CD5	CD19	CD10	CD38
Clone (BD)	L27	2D1	1-155-2	TB28-2	L17F12	SJ25C1	HI10A	Hb7
CLPD 2	CD20	CD45	CD23	CD200	CD79b	CD19	CD25	CD43
Clone (BD)	L27	2D1	EBVCS-5	MRC OX 104	SN8	SJ25C1	2A3	IG-10
HAIRY	CD4	CD45	CD123	CD103	CD11c	CD19	CD3	CD8
Clone (BD)	SK3	2D1	7G3	BerACt8	B-Ly6	SJ25C1	SK7	SK1
T CELL 1	CD4	CD45	CD30	CD26	CD79b	CD2	CD25	CD3
Clone (BD)	SK3	2D1	BerH83	L272	SN8	L303.1	2A3	SK7
NK CELL		CD45	CD16	CD56			CD57	CD3
Clone (BD)		2D1	3G8	My31			NK1	SK7
T CELL 2	CD4	CD45	CD16	CD8	CD5	CD2	CD7	CD3
Clone (BD)	SK3	2D1	3G8	Sk1	L17F12	L303.1	M-T701	SK7
T-NK CELL		CD45	□□□	□□□	CD56	CD2	CD57	CD3
Clone (BD)		2D1	WT1	11F2	B159	L303.1	NK1	SK7
Plasma cell neoplasms								
Plasma 1	CD20	CD45	cyLambda	cyKappa	CD56	CD19	CD138	CD38
Clone (BD)	L27	2D1	1-155-2	TB28-2	B159	SJ25C1	MI15	Hb7

ALOT: Acute Leukemia orientation tube, BD: Becton Dickinson, San Jose, CA

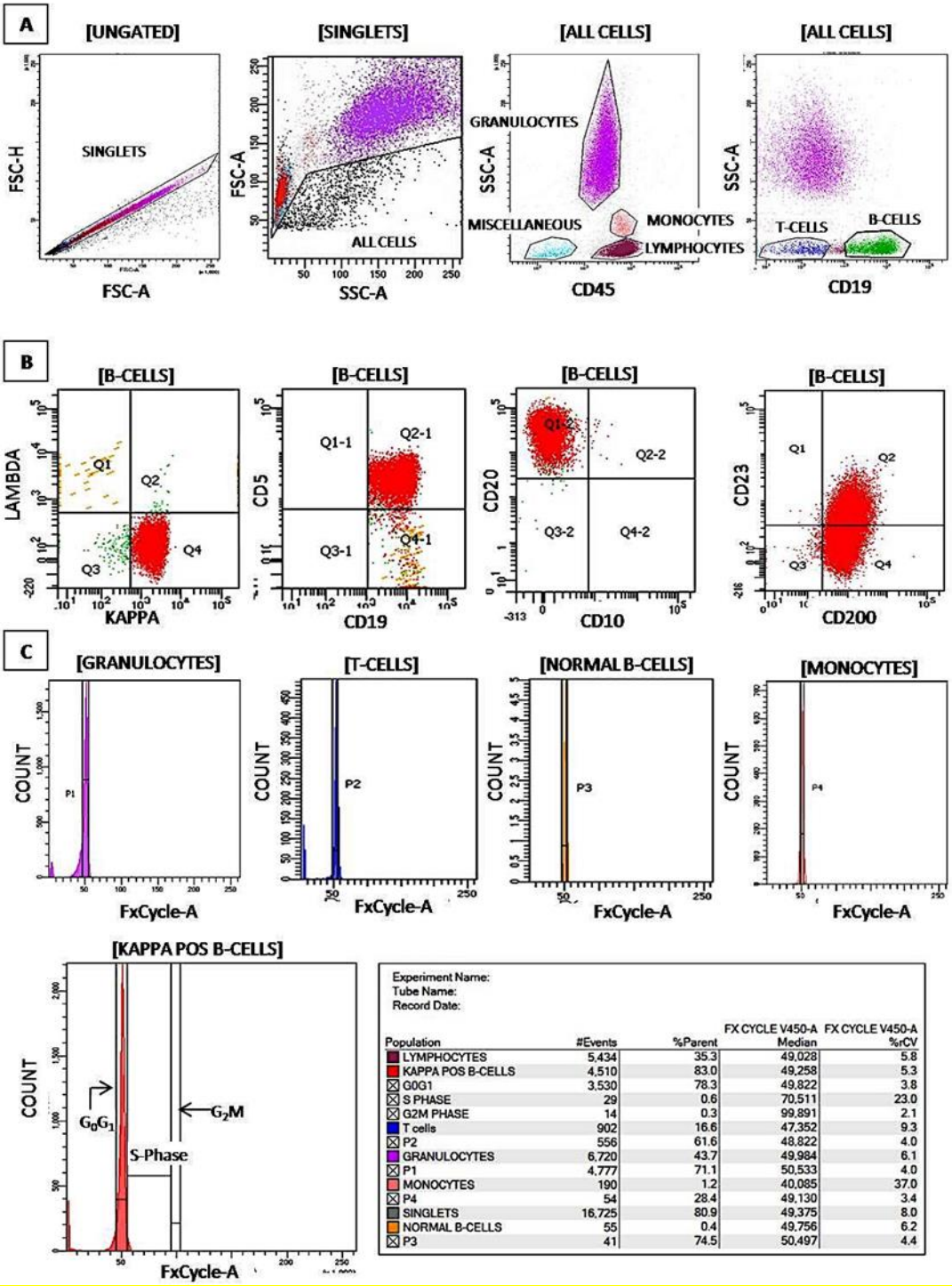

Figure 1: [A] Sequential gating strategy in a case of B-cell chronic lymphoproliferative disorder (B-CLPD) with immunoprofile of chronic lymphocytic leukemia (CLL). Initially "singlets" were selected using forward scatter (FSC)-Area (A) and height (H); "All cells" that are viable, are then selected from singlets using side scatter (SSC)-A and FSC-A; CD45-SSC-A on all viable cells showing granulocytes in magenta, monocytes in orange, lymphocytes in brown; CD19/SSC-A on all viable cells showing all CD19 positive "B-cells" in green and CD19 negative "T-Cells" in blue. [B] Gated B-cells showing kappa restricted clonal abnormal B-Cells in red expressing CD5, CD20, CD23, CD200 (and also CD43, CD79b and negative for CD10, CD11c, CD38, CD103, CD123 not shown here) and normal B-Cells in mustard expressing CD19, CD20, Lambda (and Kappa not shown here) and negative for CD5 (also negative for CD10, CD11c, CD38, CD43, CD79b, CD103 and CD123 not shown here). [C] FxCycle based DNA ploidy analysis showing histograms of FxCycle-A on linear scale for normal diploid granulocytes, monocytes, normal B-cells and monocytes, along with Kappa restricted abnormal B-cells. The DNA index: Median fluorescence intensity (MFI) of G_0G_1 of abnormal kappa restricted B-Cells divided by MFI G_0G_1 normal T-cells= 49,822/48,822= 1.02 with S-Phase fraction of 0.6%.

Table 2: Flow cytometric (FCM) DNA indices and their corresponding modal chromosomal numbers.

Ploidy group	FCM DNA ploidy	Modal chromosome number (MN)
Near-Haploid	0.55 to 0.69	24-29
Low Hypodiploid	0.70 to 0.88	30-39
High-Hypodiploid	0.89 to 0.95	40-45
Diploid	0.96 to 1.05	46
Low-Hyperdiploid	1.06 to 1.15	47-50
High-Hyperdiploid	1.16 to 1.39	51-65
Near-Triploid	1.40 to 1.79	66-80
Near Tetraploid	1.80 to 2.28	81-102

RESULTS

In this study, we performed FCV based DNA Ploidy in 110 consecutive new cases of haemato-lymphoid malignancies (age range 5 months to 82 years, median age 54 years, male: female ratio 1.24: 1). This encompassed acute leukemias (n=67, 60.9%; with n=30 B-ALLs, n=4 T-ALLs, n=32 AMLs and n=1 MPALs), CML with myeloid blast crisis (n=1, 0.9%), MDS with excess blasts (n=2, 1.8%), Mature B cell/ T cell neoplasms (n=37, 33.7%) and multiple myeloma (n=3, 2.7%).

In addition FCV DNA ploidy was also performed on n=22 control samples, immuno-morphologically negative for haemato-lymphoid neoplasms (age range 1 year to 76 years, median age 42 years, male: female ratio 1.1: 1). This included n=12 samples received to exclude any haematological malignancy and n=10 normal samples (n=5 peripheral blood samples from routine health check-up patients, n=3 un-involved staging bone marrow samples from patients with Hodgkin's lymphoma, DLBCL and n=2 marrows from megakaryocytic thrombocytopenia patients. All the normal cell populations from these samples comprising of B-lymphocytes, T-lymphocytes, NK cells, neutrophils, monocytes, eosinophils, hematogones, normal myeloid blasts, normoblastic erythroid cells, megaloblastic erythroid cells in patients with known vitamin B12 and folate deficiency and micro-normoblasts associated with iron deficiency anaemia revealed a DNA index in the diploid range (DI: 0.96-1.05) similar to that in published literature. (10) These populations served as internal normal diploid control denominators for evaluating the DI of abnormal cell populations.

Conventional karyotyping was ordered and available in n=76/110 cases only, most of these (n=67/76) being Acute leukemias, MDS-EB and CML-BC (where karyotyping assists in risk stratification) and not available in most cases of lymphoproliferative disorders and myeloma, where FISH based prognostication is recommended in the recent literature [26,36].

In FCM ploidy analysis (n=110 haemato-lymphoid neoplasms), the overall DI ranged from 0.81 to 2.17 and the mean CV of G0/G1 peak of blasts was 3.0% (range 1.6–4.9%). Diploidy was seen in n = 90 (81.8%), low-hyperdiploidy in n = 10 (9.1%), high-hyperdiploidy in n = 7 (6.4%) and one case each (0.9% each) with near-tetraploidy (DI 2.17), high-hypodiploidy (DI 0.90) and low-hypodiploidy (DI 0.81). The SPF ranged from 0.1-31.6%. Overall, FCM based aneuploidy was noted in n = 20 (18.2%) cases. Conventional karyotyping was performed in n=76/110 cases (70%) with n= 65/76 (85%) successful karyotypes (n= 11, 15% with failed cultures). The modal chromosome number (MN) ranged from 45 to 63. Cytogenetic ploidy analysis in cases with successful karyotypes revealed diploidy in n=54/65 (83.1%) cases, low-hyperdiploidy in n=10 (15.4%) cases and high-hyperdiploidy in n=1/65 (1.6%) cases. A concordance of 95.4% (n=62/65) was noted with their corresponding flowcytometric DI with only three discordant cases.

Acute lymphoblastic Leukemia

Of the total n=34 cases of ALL, n=30 were B-ALLs, while n=4 were T-ALLs. Amongst the B-ALL cases (age range 3 - 81 years, Male: Female ratio 1:1, n=14/30 patients ≤ 18 years), flow ploidy revealed diploidy in n = 17/30 (56.6%) cases with SPF ranging from 2.7-10%, low-hyperdiploidy and high-hyperdiploidy in n =5 (16.6%) cases each with SPFs of 3.9-30% and 2.3-21% respectively, along with n=1 case each (3.4% each) with near-tetraploidy (DI 2.17), high-hypodiploidy (DI 0.90) and low hypodiploidy (DI 0.81), all having SPF <5%. Karyotype analysis was

available in 29/30 B-ALL cases with a culture failure rate of n=9/29 (31%), majority of failed cultures pertained to the cases with aneuploid flow DI n=7/9 (79%) which are otherwise prognostically relevant. (37) Of these, n=9/20 (45%) were classical diploids, n=7/20 were pseudodiploids having additional balanced translocations or gains/losses of genetic material and n=4/20 (20%) were low-hyperdiploid. Overall, 96.5% concordance (n=28/29) was noted between flow ploidy and cases with successful karyotypes. One discordant case (UPN 21) revealed a low hyperdiploid DI of 1.07, while it showed a pseudodiploid karyotype with MN 46,XY,add(7)(p15)[15]/46,XY[5] showing gain of extra chromosomal material probably leading to a low-hyperdiploid flow DI. The ploidy distribution of CD10 positive Precursor B-ALLs and CD10 negative Pro-B ALLs is shown in Table 3 and information pertaining to unique patient number (UPN) in supplementary Table 1.

PCR panel was available in n=27/30 cases of which n=4 cases (all adults >50 years age) revealed major BCR-ABL transcript (high risk rearrangement). (37) Two of these had diploid flow ploidy and karyotype, while n=1 (UPN 10) had a low hyperdiploid DI of 1.09 with modal karyotype 46, XX, t(9;22) (q34;q11.2) [8]/ 50, idem, +2, +14, +16, +der(22) t(9;22)[12] and other case (UPN 19) had a diploid DI of 0.98 with a pseudo-diploid karyotype 45, XX, -7, t(9;22) (q34;11.2)[20]. All these n=4 cases had SPF of <10%. One case each had TCF3-PBX1 and ETV6-RUNX1 rearrangement (both paediatric patients; UPN 17, UPN 20), which are intermediate and standard risk B-ALL translocations respectively (37) had SPF of <5%. Rest n=21/27 cases were negative for B-ALL PCR panel.

There were only n=4/34 cases of T-ALL (age range 2 - 16 years, all males). All of these had a diploid DI with SPF between 3.9-27.3%. In only n=2/4 cases (UPN 31 and UPN 33), karyotyping was available and had a normal diploid karyogram.

Acute myeloid Leukemia

Amongst the n=32 AML cases (Age range 5 months to 82 years, Male: Female ratio 1: 1.3), n=9/32 were AML with gene mutation (n=4/9 only NPM positive, n=2/9 only FLT3 ITD positive, n=3/9 both NPM and FLT3 positive); n=7/32 were AML with monocytic differentiation; n=3/32 Acute Promyelocytic Leukemia (APML); one each case of AML with RUNX1-RUNX1T1, AML with BCR-ABL1 and pure erythroid leukemia and n=10/32 AML not otherwise specified (AML NOS))(Table 3). Overall, diploidy was seen in n=27/32 (84.4%) cases, while n=5/32 (15.6%) were aneuploid. Of these, n=4/32 were low-hyperdiploid while the single erythroleukemia case was high-hyperdiploid.

Cytogenetic analysis was available in all 32 cases with n=2/32 culture failures. Amongst the n=30/32 cases with successful karyotypes, n=15/30 were classic diploids while, n=9/30 were pseudodiploids with additional balanced translocations or gains/losses of genetic material; n=5/30 were low-hyperdiploid and n=1/30 was high hyperdiploid. There was 96.6% concordance (n=29/30) between flow ploidy and corresponding cases with successful karyotypes. The single discordant case (UPN-40) was an APML with diploid flow ploidy (DI 1.05) and MN 47,XX,+8,t(15;17)(q22;21)[15]/46,XX[5].

MPAL, CML in blast crisis & MDS-EB

Only one case (UPN 67) in the cohort was MPAL (T/Myeloid) and had a diploid DI 0.98 with a classical diploid karyotype.

The single case of CML in myeloid blast crisis (UPN 68), flow cytometry could pick up only a single clone with diploid DI 1.0 and SPF of 12.2%, while karyotype revealed multiploidy with two clones: one pseudodiploid and other low hyperdiploid with modal karyotype 46,XX,t(9;22)(q34;q11.2),add(21)(q22)[15]/50,XX,+8 ,t(9;22)(q34;q11.2),+12,+15,add(21)(q22),+der(22)t(9;22)(q34;q11.2)[5].

There were two cases of MDS-EB (Age range 54-78, Male: Female ratio 1:1) both of which had a diploid DI with SPF ranging from 5.4-7.9% and had a classic diploid cytogenetics as well (UPN 69 and UPN 70).

Table 3: Flow cytometric and cytogenetic ploidy distribution in all n=110 cases

		Flow cytometric Ploidy									Cytogenetic Ploidy			
Category	**n**	**Diploid (DI 0.96-1.05)**	**Low Hyperdiploid (DI 1.06 – 1.15)**	**High Hyperdiploid (DI 1.16 – 1.39)**	**Near tetraploid (DI 1.80-1.79)**	**High Hypodiploid (DI 0.89– 0.95)**	**Low Hypodiploid (DI 0.70– 0.88)**	**S-Phase Fraction Range (%)**	**Karyotype available (n=)**	**Culture Failure (n=)**	**Diploid (MN 46)**	**Pseudo-diploid (MN 46)**	**Low hyperdiploid (MN 47-50)**	**High-hyperdiploid (MN 51-65)**
B-ALL														
Pre B-ALL	28	15	5	5	1	1	1	2.3-30	27	9	8	6	4	-
Pro B-ALL	2	2	-	-	-	-	-	4-6.7	2	-	1	1	-	-
T-ALL														
	4	4	-	-	-	-	-	3.9 – 27.3	2		2	-	-	-
AML														
AML with RUNX1-RUNX1T1	1	1	-	-	-	-	-	3.0	1	-	-	1	-	-
AML with BCR-ABL1	1	1	-	-	-	-	-	14.6	1	-	1	-	-	-
APML	3	3	-	-	-	-	-	4.1-5.3	3	1	-	1	1	-
AML with gene mutation	9	8	1	-	-	-	-	0.5-8.9	9	1	5	2	1	-
AML with monocytic differentiation	7	6	1	-	-	-	-	0.7-10.7	7	-	4	2	1	-
Pure Erythroid Leukemia	1	-	-	1	-	-	-	9.8	1	-	-	-	-	1
AML NOS	10	8	2	-	-	-	-	1.2-25.9	10	-	5	3	2	-
MPAL														
T/Myeloid	1	1	-	-	-	-	-	10.7	1	-	1	-	-	-
CML-BC														
CML Myeloid BC	1	1	-	-	-	-	-	12.2	1	-	-	-	1	-
MDS-EB														
MDS-EB-2	2	2	-	-	-	-	-	5.4-7.9	2	-	2	-	-	-
Mature B Cell/ T Cell neoplasms														
CLL	17	17	-	-	-	-	-	0.3-3.9	2	-	2	-	-	-
Mantle	2	2	-	-	-	-	-	2.2-4.1	1	-	1	-	-	-
Follicular	3	3	-	-	-	-	-	0.1-9.5	-	-	-	-	-	-
HCL	3	3	-	-	-	-	-	0.4-0.7	-	-	-	-	-	-
LPL	2	2	-	-	-	-	-	0.4-1.8	1	-	1	-	-	-
MZL	8	8	-	-	-	-	-	0.1-7.7	3	-	3	-	-	-
Burkitt's	1	1	-	-	-	-	-	31.6	1	-	-	1	-	-
PTCL	1	1	-	-	-	-	-	5	-	-	-	-	-	-
Myeloma														
	3	1	1	1	-	-	-	2.3-6.2	1	-	1	-	-	-
TOTAL	110	90	10	7	1	1	1	0.1-31.6	76	11	37	17	10	1

Mature B-Cell/ T-Cell neoplasms

Of the total 37 cases, n=17/37 were Chronic Lymphocytic Leukemias (CLL), n=8/17 marginal zone lymphomas (MZL), n=3 cases each of follicular lymphoma (FL) and hairy cell leukemia (HCL), n=2 each of mantle cell lymphoma (MCL) and lymphoplasmacytic lymphoma (LPL) and one case each of Burkitts lymphoma (BL) and Peripheral T cell lymphoma (PTCL). All these cases had a diploid flow ploidy with SPF ranging from 0.1 -31.6% and conventional karyotyping was available in n=8/37 cases of which 7/8 were classic diploids and one was pseudodiploid.

Most of the CLPDs excluding MCL had a low SPF <2.5%, while MCLs had a higher SPF 2.6%-4.1%. The highest SPF of 31.6% was seen in BL patient (UPN 76). In n=11/37 cases, corresponding lymph node histopathology was available. Amongst these, there were n=4 CLL cases by FCM of which, the one with SPF 0.7% (UPN 80) was consistent with CLL/SLL (small lymphocytic lymphoma) on histopathology as well with low Ki67 proliferation index (ki) <5%, while all n=3/4 with SPF >3% (UPN 77, 91, 101) had a corresponding aggressive CLL histo-morphology with expanded proliferation centres and a high Ki 40-50%. Of the n=2 MZL with available histology, one with SPF 2.5% (UPN 83) was diagnostic of nodal MZL on lymph node histology with Ki 10-12% and other case with SPF 7.7% (UPN 104) had a large cell transformation in lymph node to high grade B-NHL with Ki 60-70%. Both the FLs with high SPF of 9.4% (UPN 73) and 9.5% (UPN 105) had a Grade IIIB FL in lymph node with Ki 40-45%. There was one case each of MCL (UPN 93), LPL (UPN 89) and PTCL (UPN 72) with FCM SPF of 4.1%, 1.8% and 5.0%, in which corresponding histopathology revealed MCL (Ki 40%), LPL (Ki <5%) and PTCL (Ki 50%) respectively. Thus SPF >3% in mature B Cell/ T Cell lymphoma is highly likely of a higher grade lymph node disease.

FISH panel for CLL was available in n=3/17 cases of which one case revealed trisomy 12 and other case revealed del11q and del17p while other case was negative. However, FISH did not reveal any other gains or losses of signals that could potentially alter overall ploidy status.

Multiple myeloma

Of the n=3 total myeloma cases analysed, (Age range 42-64, Male: Female ratio 1:2), there was one case each with diploidy, low-hyperdiploidy and high-hyperdiploidy. Karyotyping was available only in case with diploid flow DI and correspondingly revealed classic diploidy. FISH panel for myeloma was negative in all three cases analysed.

DISCUSSION

In this prospective study, we tried to assess the ploidy status and SPF in n=110 consecutive new cases of haemato-lymphoid neoplasms by FCV based ploidy and compare it with corresponding karyotyping data wherever available. To the best of our knowledge, this is the first such study from Indian sub-continent, which documents and comprehensively elucidates the spectrum of DNA flow ploidy across various haemato-lymphoid neoplasms.

Ploidy is commonly assessed using karyotyping, FISH and conventional FCM DNA dyes. FCV based FCM DNA ploidy can be easily incorporated at institutions equipped with a 3 laser (blue, red and violet) flow cytometer. It is a convenient, inexpensive (additional cost per test < 2$), simple to perform, rapid (just half hour additional incubation) assay, which permits simultaneous 6-7 colour immunophenotyping, thereby permitting analysis of only specifically gated tumor cells in background rich in normal counterparts. It provides results in virtually all cases, even in haemodilute sample (high assay sensitivity 0.01% cells). Karyotyping on the other hand is technically challenging, has a steep learning curve, is not available at most centres and has a longer turn-around time. The frequency distribution of ploidy groups in our study based on FCV DI closely correlated with corresponding karyotypes (overall concordance rate of n=95.4% with only n=3/110 discordant cases) and with the published Asian and Western literature using either cytogenetics or FCV/PI based flow cytometric technique [5-10, 19, 22-25, 28, 30, 33, 35, 37].

In B-ALL and multiple myeloma cases, low quality metaphases and increased culture failure rate, has been widely reported, especially in aneuploid

patients having high-hyperdiploidy and hypodiploidy, both of these being prognostically significant in B-ALL and myeloma cases[37]. FCM ploidy is instrumental in this situation, particularly in B-ALL and myeloma cases were they would be classified as intermediate risk in the event of culture failure and negative translocations/ re-arrangements or otherwise, by flow ploidy, into standard risk high-hyperdiploids or poor risk hypodiploids. In our series, maximum culture failures were present in the B-ALL cases (31.1%, 9/29), FCM analysis could detect aneuploidy in 77.7% (n=7/9) of these cases, which is similar to the findings by other researchers in B-ALLs[5-10, 19, 22-25, 28, 30, 33, 35, 37.]

In AMLs, aneuploidy, though not incorporated in risk stratification protocols [32] was detected in 15% of our cases, similar to that in published literature. The indomitable utility of FCV ploidy was noticed in erythroleukemia cases, which 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, almost all the cases of which have complex cytogenetics with chromosomal aneuploidy, as published by us earlier[39]. Flow ploidy can ascertain aneuploidy in all cases even in the event of haemodilution which complicates morphological counting of total erythroid precursors and pro-erythroblasts, and also distinguishing from erythroid hyperplasia of megaloblastic anaemia and other reactive conditions, which show diploid erythroid precursors only.

Aneuploidy is associated with high risk in NHLs[28], however we did not find aneuploidy amongst the NHL cases in our cohort. A finding with tremendous utility in diagnosis and workup of NHL patients in our study was with respect to FCM SPF based stratification, which can hint towards a higher grade disease in the lymph nodes. In our series, most of the low grade NHLs had an SPF < 3% and in n=3 of these cases, corresponding lymph node histology was also low grade with Ki < 10%. However all cases where the SPF was > 3% and lymph node histo-pathology was available, a higher grade lymph node disease was present with Ki > 40%.

Though FCM ploidy is a very robust tool, one of the important drawbacks is its inability to distinguish diploids from pseudodiploid cases bearing prognostically relevant translocations and rearrangements (which are commonly seen in this ploidy subgroup only) as they do not alter the net DNA content of cells and hence fall within the diploid group only. However, FCV DI was sensitive to the variation in DNA content (gain or loss) resulting from un-balanced translocation/deletions, as seen in our n=3/110 discordant cases (UPN 21, 40, 68). Cases with MN 45 having unbalanced translocations resulting in loss of additional genetic material showed a DI of 0.96 in the lower side of normal diploid range (UPN 47), while patients with > 46 chromosomes having deletions on karyotype (UPN 44), again resulted in a net diploid DI content.

Another drawback of the present study was that PCR panels were used in B-ALL risk stratification, which cannot give similar information about ploidy status as can FISH and karyotyping [37.]

CONCLUSION

FCV based ploidy is an easy to perform technique which can aid in risk stratification of hemato-lymphoid neoplasms in limited resources settings. It is highly sensitive (assay sensitivity 0.01%) in ascertaining DNA content and proliferative index, even in haemodilute samples with low tumor cell count. There is a strong correlation between FCV based DNA ploidy and conventional cytogenetics across all hemato-lymphoid neoplasms, and the information provided is complementary to each other. It has great prospective potential in picking up abnormal aneuploid populations even during high throughput minimal residual disease analysis for haemato-lymphoid neoplasms.

In conclusion, FCM ploidy can detect aneuploidy in all B-ALL and myeloma cases, even in cases with culture failure; can supplement the diagnosis of erythroleukemia; and provide a useful screen for a higher grade lymph node disease in NHL cases with SPF > 3%.

CONFLICTS OF INTEREST

All authors declare no conflicts of interest.

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