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Research Article

FLOW CYTOMETRY IN DIAGNOSIS OF CHRONIC LYMPHOPROLIFERATIVE DISORDERS – EXPERIENCES OF A TERTIARY CARE CENTRE IN SOUTH INDIA

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A B S T R A C T

Background: Chronic lymphoproliferative disorders (CLPDs) encompass a wide range of neoplasms with distinct clinical and laboratory features. The overlap between these entities is not uncommon as is variability within each. Hence the diagnosis of CLPDs requires a multi-parameter approach starting with the clinical profile and morphological evaluation along with the integration of bits of information from various investigations to direct therapeutic decisions. Flow cytometry(FCM) holds a pivotal position in the diagnostic algorithm.There are not many studies on CLPDs among Asians. Here, we have looked into the profile of suspected CLPD cases received for immunophenotyping at our institution over one year.

Methods: The list of samples subjected to flow cytometry in view of suspicion of chronic lymphoproliferative disorder (CLPD), over a year, was obtained and the details of the patients retrieved from the electronic medical records. Morphology of their lymphoid cells in peripheral blood or bone marrow aspirates and immunophenotype as inferred from FCM were carefully studied in the light of clinical profile.

Results: 74% of the samples revealed clonal lymphoid populations, the majority being of B lineage and chronic lymphocytic leukaemia (CLL) was the most frequent subtype(45%). B-CLPDs other than classical CLL and hairy cell leukaemia posed difficulties in subtyping. Two cases of Hodgkin lymphoma were not evident on FCM. However small neoplastic clones could be picked up in marrow samples sent for the staging of lymphomas.

Conclusion: We found FCM to be a useful tool in delineating and categorising neoplastic lymphoid proliferations so as to guide management decisions especially when tissue biopsy was not available. The scope of FCM has expanded to yield much more therapeutic and prognostic information. Newer modalities like molecular testing are also entering the scene though many are yet to be approved for routine practice.

INTRODUCTION

Chronic lymphoproliferative disorders (CLPDs) are a heterogeneous group of diseases with highly variable clinical profile, morphological features, immunophenotype as well as cytogenetic and molecular changes. Over the past few years, much progress has been made in the classification, biology including the molecular basis and treatment objectives of these disorders, making a correct diagnosis inevitable. In conjunction with clinical, hematologic and cytomorphologic features, multiparametric flow cytometry (FCM) has evolved as a powerful tool for diagnosis and immunophenotypic characterisation of chronic lymphoproliferative disorders, especially in cases where a tissue diagnosis is not available.(1) The scope of FCM is ever-expanding with applications in the prognostic and therapeutic assessment. We have tried to look into our experiences with flow cytometry, over a year, in the evaluation of suspected CLPD cases, including its utility in the screening of such samples as well as their categorisation.

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MATERIALS AND METHODS

The list of samples processed for immunophenotyping by flow cytometry, because of clinical suspicion of chronic lymphoproliferative disorder (CLPD), during the period from January 2018 to January 2019, was collected from the laboratory register. The demographic, clinical and investigation details of these patients were retrieved from the electronic medical records. Morphology of the lymphoid cells

in their peripheral blood or bone marrow aspirates was carefully studied by light microscopy. Subsequently, flow cytometry (FCM) was performed on BD FACS Canto II using peripheral blood or bone marrow samples of these patients by the stain—lyse– wash method wherein sample was added to fluorochrome-conjugated monoclonal antibodies that bound specifically to cell surface antigens. The stained sample was treated with a lysing solution to lyse erythrocytes while preserving the leucocytes. The sample was then washed to remove excess antibody and debris before analysis. In our routine FCM practice for CLPD, we utilise four tubes comprising two tubes with B lymphoid markers, one with T lymphoid markers and a fourth tube dedicated to hairy cell leukaemia (HCL) markers. Our antibody panel thus comprises of CD5, CD23, CD19, CD20, kappa and lambda in tube 1; FMC7, CD200, CD10, CD38, CD19 and CD43 in tube 2; CD2, CD3, CD4, CD5, CD7 and CD8 in tube 3 and CD103, CD123, CD25, CD11c,CD19 and CD20 in tube 4. Gating strategy: From all events, gating on CD19 positive cells was done for B marker tubes and CD3positive cells for the T tube following which the expression of other antigens was evaluated.

RESULTS

A total of 43 samples of suspected CLPD, including 18 bone marrow aspirates and 25 peripheral blood samples were analysed by flow cytometry in our laboratory during one year from January 2018 to January 2019. Of these, 32 had clonal lymphoid populations (74%) while 11 tested negative. 31 of these 32 cases had B lymphoid clones while only one case of an 11-year-old boy showed a T cell proliferation which turned out to be a T lymphoblastic lymphoma on lymph node biopsy (figure 1). Among the patients diagnosed with clonal B-cell proliferation, the majority were in the 6th to 8th decades of life with a mean age of 64.7 years. There were 23 males and eight females with a male to female ratio of 2.87. By FCM, 14(45%) were diagnosed as chronic lymphocytic leukaemias (CLL). In 5 cases, the possibility of hairy cell leukaemia variant (HCL-v) or splenic marginal zone lymphoma (SMZL) was suggested. Five others had neoplastic clones with a non-CLL, non HCL profile. 2 cases were diagnosed as monoclonal B lymphocytosis (MBL), and three samples showed small clonal populations of B cells. Of the remaining 2, one showed a kappa clonal B cell population in a known case of multiple myeloma, and the other had two separate clonal B cell populations raising the possibility of a composite lymphoma (figure1, 2).

For our CLL cases, the median age was 69 years with a male to female ratio of 11:3. 6/14(43%) had lymphadenopathy, 2 of whom had lymph node biopsy showing low-grade B lymphoproliferative disorder while only one patient (7%) had splenomegaly. The total leucocyte count ranged from 15200/mm3 to 272000/mm3 with a mean of 91400/mm3 with good mean haemoglobin (Hb) of 12.5g/dL and mean platelet count of 212300/mm3. Only four patients (28.6%) had Hb level below 11g/dL, and only one had platelet count below 100000/mm3. Mean lactate dehydrogenase level was 256U/L. Morphologically, the lymphoid cells appeared small and resembled mature lymphocytes. On FCM, all CLL cases were positive for CD20 with coexpression of CD5/CD23 and CD200/CD43 and negative for FMC7. Exactly half of our cases were weakly positive for surface kappa light chains, the other half being lambda clonal. All but one case had CD11c expression while dim to moderate CD25 was noted in 50% cases and aberrant CD123 in one case. Positive findings indicated on cytogenetic studies included deletion13q14 and ATM deletion in 2 cases, numerical aberrations of chromosome12 in 2 and 17p deletion in two.

Based on the expression of the four core HCL antigens, CD25, CD11c, CD103 and CD123, along with other B cell markers, differential diagnoses of HCL-v/ SMZL was suggested in 5 cases with atypical lymphocytes having cytoplasmic villous projections. This group had a mean age of 58 years with a M: F ratio of 3:2. 4/5 had splenomegaly with a mean LDH level of 228.6 U/L, mean total WBC count of 26700/mm3 and pancytopenia in only one case. Five cases exhibiting clonal B cell proliferations with non CLL, non HCL profile could not be categorised further due to expression of varied combinations of B markers. Two other cases were reported as monoclonal B lymphocytosis owing to monoclonal B cell count less than 5000/mm 3 in peripheral blood in the absence of lymphadenopathy and splenomegaly. While one of these had a CLL type phenotype, the other had a CD5 negative non CLL phenotype. We picked up small clonal populations of B cells ranging from 3% to 8% of gated cells in bone marrow samples from 3 patients with lymphadenopathy and normal peripheral WBC counts. One of these was a case of mantle cell lymphoma on lymph node biopsy, and another one was a lymphoplasmacytic lymphoma. The remaining two positive cases included kappa clonal lymphoid proliferation in a multiple myeloma patient and another sample showing two separate kappa clonal lymphoid populations from a patient with lymphadenopathy and splenomegaly.

DISCUSSION

Lymphocytes can give rise to neoplasia at any stage of their development. Lymphoid malignancies derived from B/ T lymphocytes or natural killer (NK) cells in their later stages of maturation may infiltrate lymphoid tissues and/ or circulate in the blood and accordingly, are designated as lymphomas or chronic lymphoproliferative disorders (CLPD) respectively though there is no clear cut distinction between the two.(1)

The incidence and distribution of lymphoma subtypes vary among different ethnic and geographic populations. Though B-CLPDs are much more frequent, their frequency is known to be lower in Asians than in Western countries. Conversely, mature T/NK-cell neoplasms are commoner among Asian populations. (2) In India, 79.1% of non-Hodgkin lymphomas (NHL) are of B cell origin, while T-cell lymphomas constitute 16.2% of the total.(3) In our cohort also, all positive cases were B-CLPDs except for the single T lymphoblastic lymphoma case.

The diagnosis of CLPDs requires a host of investigations including complete blood counts, peripheral blood smear, bone marrow aspiration and biopsy. But, gone are the days when lymphoproliferative disorders were characterised solely based on morphologic features, as distinction between low and high-grade neoplasms as well as identification of specific subtypes like hairy cell leukaemia have significant treatment implications. Flow cytometry has evolved as an essential tool in the evaluation of CLPDs since the late 1970s, its two most basic roles being the assignment of lineage and distinction between neoplastic and non-neoplastic lymphoid proliferations. It enables simultaneous evaluation of several antigens to identify deviations from normal patterns, along

with clonality analysis, providing objective and quantitative results even on very small samples. (1,4) The choice of immunophenotypic panels would depend on a variety of factors including the laboratory throughput, technical expertise, number of antibodies and indication for immunophenotyping and they should be tailored to provide maximum information at the least cost.(5,6) As already mentioned, B cell NHLs constitute an overwhelming majority of CLPDs, and hence it is not surprising that most FCM panels are B cell centric. However, phenotypic heterogeneity of B cells in CLPD, and overlapping immunophenotypic profiles often pose challenges in interpretation. (5) It should also be remembered that FCM is only one diagnostic tool in the workup of B-CLPDs, and might be inferior to other techniques like cytogenetic and molecular assays in certain situations.

Chronic lymphocytic leukaemia (CLL) is the most common CLPD of adults and accounts for about 40% of all leukaemias in western countries.(7) The incidence of CLL in India is about 0.41 per 100 000.(8) In our study too, CLL was the most frequently encountered B-CLPD (45%) with a median age slightly higher than that in other Indian studies and a much higher male affliction(6, 9) possibly due to ethnic variations.

In 1994, Matutes *et al* devised a scoring system for the diagnosis of CLL based on its common immunological profile i.e.CD5+, CD23+, FMC7-, and weak expression (+/-) of surface immunoglobulin (SmIg) and CD22 wherein each of these five markers was given a score of 0 or 1 depending upon whether its expression was typical or atypical for CLL. Majority of CLL cases scored 5 or 4 while the score was 0 or 1 for most other B cell leukaemias/ lymphomas.(10) In 1997, Moreau *et al* replaced CD22 by CD79b in the scoring system and claimed that lack of its expression significantly improved the discrimination between CLL and other non-CLL B-CLPDs. (11) Though we do not have CD22 or CD79b on our CLPD panel, all our CLL cases would have scored four on either of these systems owing to their CD20+, CD5+, CD23+, FMC7- and weak SmIg+ profile. Falay *et al* found CD43 and CD200 to be more valuable markers than CD79b, CD22 and FMC7 in differentiating CLL from mantle cell lymphoma (MCL) especially in atypical CLL cases where Matutes scoring system was ineffective.(12) In 2017, Kohnke *et al* came up with a new CLL flow score encompassing CD5/CD23, FMC7, CD79b and CD200 which retained high sensitivity with markedly increased specificity as against the Matutes score.(26) Hence we have used coexpression of CD5 and CD23 as well as CD43 and CD200 with negative FMC7 and weak SmIg as diagnostic of CLL (figure 5). CD11c positivity noted in 13/14 of our cases is frequently reported in CLL/SLL though Nagori *et al* found no relation between its expression level and outcome of the disease.(13) CD25 expression seen in 50% of our cases is also not a prognostic factor in CLL according to Schvidel *et al*.(14) Distribution of kappa and lambda positive cases was similar in our study as reported by Dwivedi *et al*.(3)Wierda *et al* noted that interphase FISH could identify cytogenetic lesions in more than 80% CLL cases.(15) Amongst the limited number of our cytogenetically tested cases, we have come across some of the common abnormalities described in CLL. While isolated deletion in chromosome 13q14 is favourable, ATM deletion portends poor outcome; one of our two patients with these abnormalities was started on chemotherapy while other expired due to ruptured aortic aneurysm. Deletion 17p confers worse clinical course, and both our cases were started on chemotherapeutic regimes with chlorambucil. Hairy Cell Leukemia (HCL) constituting 2% of all lymphoid leukaemias, is characterised by cytopenias, splenomegaly and distinctive cells with cytoplasmic projections. It is one of the best examples for the role of FCM as a diagnostic modality based on surface expression of specific antigens and responds well to appropriate therapy.(16) But, other rarer entities like Hairy Cell Leukemia variant (HCL-v) and Splenic Marginal Zone Lymphoma (SMZL) which mimic HCL clinically and morphologically, do not respond to HCL therapies. Hairy Cell Leukemia variant (HCL-v) is an ill-defined entity which is about one-tenth as common as HCL while SMZL accounts for <2% of lymphoid neoplasms. (17)

As a clinical standard in FCM evaluation, Shao *et al* proposed the use of 4 core antigens- CD25, CD11c, CD103 and CD123, along with common B-cell markers (CD19, CD20 and CD22) in situations where the differential diagnoses included HCL, HCL-v and SMZL. While HCLs express all four antigens brightly, expression of bright CD20, bright CD22, CD11c and CD103, with absent CD25 and dim to absent CD123 would suggest HCL-v. Absent CD103, weak to moderate expression of CD11c, and dim to absent CD123 would favour SMZL or other splenic B-cell lymphomas.(16) However, their rarity, clinical heterogeneity and lack of specific immunophenotypic markers often pose diagnostic dilemmas.(18) We also use the coexpression of these four antigens along with bright clonality in B-CLPDs as diagnostic of HCL. Though we did not have a classical case of hairy cell leukaemia during this period, HCL-v was suggested in 2 cases with lambda clones expressing 2/4 core antigens- CD103/CD123 in one and CD11c/CD103 in the other along with absent CD25 and expression of a few other B markers. The first patient had pancytopenia with splenomegaly prompting BRAF study, which turned out to be BRAF wildtype. In contrast, the second had lymphocytic leucocytosis with preserved Hb levels and platelet count in the absence of splenomegaly. Both of them were lost to follow up.

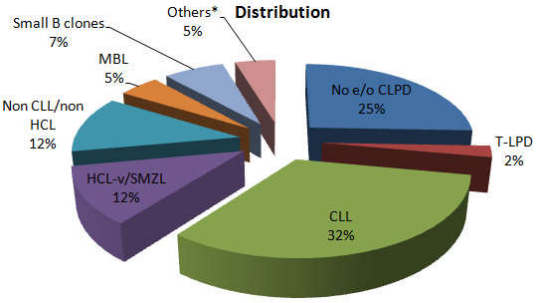

Figure 1 Distribution of cases by FCM.('Others' included a case of myeloma with clonal B cell proliferation and a case with two different B clonal populations)

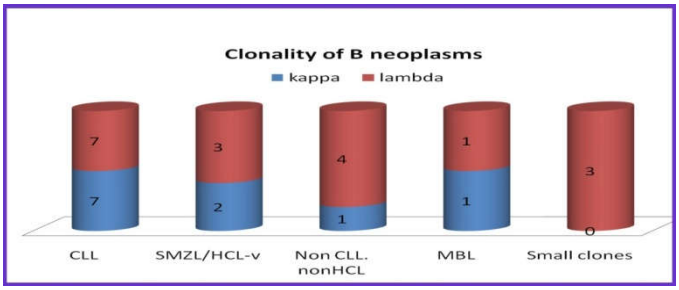

Figure 2 Kappa/ lambda clonality among B cell neoplasms

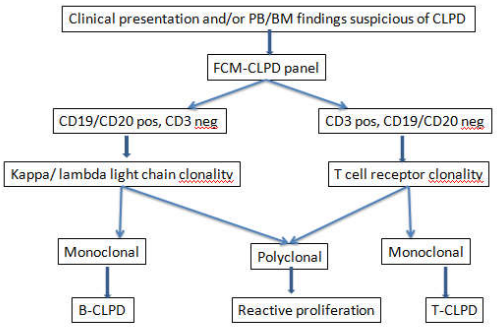

Figure 3 Algorithm for preliminary evaluation

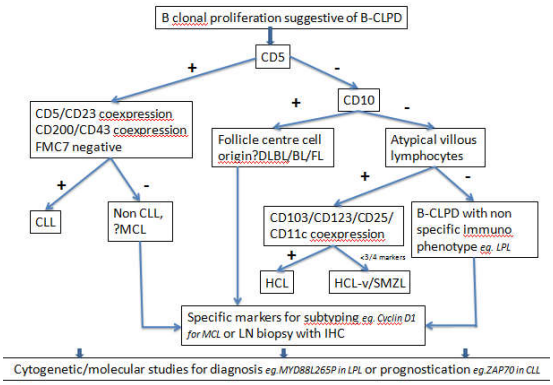

Figure 4 Our algorithm for subtyping of B-CLPD

CLL: Chronic lymphocytic leukemia MCL: Mantle cell lymphoma DLBL: Diffuse large B cell lymphoma BL: Burkitt lymphoma FL: Follicular lymphoma LPL: Lymphoplasmacytic lymphoma HCL: Hairy cell leukemia HCL-v: Hairy cell leukemia variant SMZL: Splenic marginal zone lymphoma LN: Lymph node IHC: Immunohistochemistry

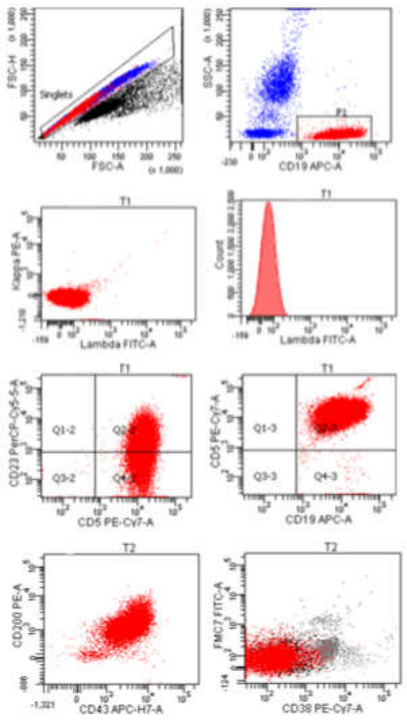

Figure 5 Flow cytometry plot(compiled) in a case of CLL: Gating done on CD19 positive cells which coexpress CD5/ CD23, CD200/ CD43 with dim surface monoclonal Ig and negative FMC7

In 3 cases with splenomegaly, absent lymphadenopathy and bone marrow showing atypical villous lymphocytosis with non CLL immunophenotype and variable expression of markers like CD5, CD38, CD11c, CD43 and FMC7, diagnosis of splenic marginal zone lymphoma was suggested in the context of lack of coexpression of the HCL core antigens. They had a mean Hb of 9.7g/dL, mean WBC count of 20700/mm3 and mean platelet count of 220000/mm3. 2 of these patients had M bands on serum protein electrophoresis which has been reported in about one-third of SMZL cases. (17) As observed by Al-Anizi *et al*, FCM can confirm non CLL non HCL phenotype of circulating neoplastic cells in SMZL, but specific markers are lacking and require clinical correlation.(18) Two of our cases received chemotherapy with bendamustine and rituximab, while one was lost to follow up.

The limitations of FCM in subtyping B-CLPDs are well understood, especially when immunophenotypic profiles are not typical of CLPDs like CLL/HCL, as entity–specific antibodies are very few with limited availability.(19) Also, lymphomas may deviate from typical immunophenotypes with aberrant antigen expression patterns posing diagnostic difficulties. In such cases, a holistic approach integrating all clinicopathologic data with ancillary tests like molecular studies or tissue biopsy with immunohistochemistry become imperative to resolve the diagnostic uncertainty. (4, 20) Of the 5 CLPD cases with non CLL, non-HCL profile which we could not categorise further, due to the expression of a non-specific combination of B markers, two turned out to be follicular lymphomas on lymph node biopsy though their immunophenotype on FCM was not typical. On cytomorphology, one of these cases had small atypical lymphoid cells with nuclear cleaving while other had large cells with high nuclear grade corresponding to grade 3 follicular lymphoma. The remaining three cases showed mature looking atypical lymphocytes, 2 of whom are getting followed up as low-grade B- NHL while one with progressive disease received bendamustine. Although more specific markers might improve the specificity of diagnosis, their practical utilityis said to be debatable.

Monoclonal B cell lymphocytosis defined by monoclonal B cell count </=5x10^9/L in peripheral blood in the absence of lymphadenopathy, organomegaly or other features of B-CLPD is classified as CLL type, atypical CLL type and non CLL type based on immunophenotype. CLL type MBL constitutes 75% of cases and can be subdivided based on the size of the monoclonal population into low count(<500/mm3) and high count (>500/mm3) types with the latter progressing more often to frank CLL.(17, 25) Our case of CLL type MBL was a high count one while the second case had a CD5 negative non CLL phenotype.

Complete and accurate staging of non-Hodgkin lymphoma (NHL) is necessary to determine the extent of disease as it may affect both the prognosis and treatment strategies. Involvement of marrow by NHL might be difficult to confirm by morphological examination alone of aspirate specimens, especially when the number of lymphoma cells is minimal or the specimen is diluted with peripheral blood.(21) FCM is a boon for the detection of such low-level involvement of marrow or peripheral blood by B cell lymphomas/leukaemias in at least another 5% of individuals who would have been understaged by morphologic evaluation alone.(4) We identified small clonal B lymphoid populations in marrow

samples of 3 patients with lymphadenopathy and normal peripheral WBC count, 2 of which were proven lymphoma cases.

We observed a kappa clonal B cell population with non CLL/ non HCL profile in a plasma cell myeloma patient with normal peripheral total leukocyte count and no significant lymphadenopathy. Although most myeloma patients have a dominant myeloma clone producing a single monoclonal protein, Kriangkum *et al* showed evidence for the coexistence of minor but highly expanded unrelated B-cell clones within the marrow and peripheral blood which could explain our observation. However, their clinical impact is not fully established.(22)

The last case with lymphadenopathy, splenomegaly and atypical lymphocytosis had two clonal populations of B cells- the bigger clone was CD5 positive with dim CD23 and FMC7, but lacking CD43/CD200 coexpression while the smaller clone coexpressed CD43/CD200 with negative FMC7. This sample revealed a homozygous deletion of 13q14 locus, which is the most frequent cytogenetic change described in CLL/SLL. The expansion of two or more distinct B-cell clones in B-CLPDs has been reported with an overall frequency of around 5%, mostly in the earlier stages of monoclonal B-cell lymphocytosis, chronic lymphocytic leukaemia and other B-CLPDs. Henriques *et al* attributed this to chronic antigen stimulation.(23) However, a composite lymphoma defined by the presence of two or more morphologically and immunophenotypically distinct lymphoma types involving the same tissue also needs exclusion.

In 11 of the 43 cases processed for CLPD immunophenotyping, we found no evidence of clonal proliferation. Though all these cases had total leucocyte counts within the normal range, 3/11 had lymphadenopathy, and 6/11 had splenomegaly. This group included cases of infectious mononucleosis (IMN), systemic lupus erythematosus (SLE), Evan's syndrome, a case each of myelofibrosis and myelodysplastic syndrome with a complex karyotype. Thus, FCM has been an excellent tool to distinguish such reactive lymphoid proliferations from neoplastic ones. But, within this group were 2 cases of classical Hodgkin lymphoma which we failed to diagnose on FCM possibly due to the rarity of neoplastic cells, their large size and cell lysis during sample preparation. Though isolation of pure, viable tumour cells in classical Hodgkin lymphomas has not been historically possible except in rare cases with markedly increased malignant populations, newer techniques to overcome this problem have now been described in the literature with clinical and research applications.(24)

The scope of flow cytometry in CLPDs has expanded beyond diagnosis and classification. It enables identification of antigenic targets for immunotherapy and measurement of their density to select ideal candidates and to predict response to therapy. It is also useful in the detection of minimal residual disease though many international groups have not endorsed such assays for routine practice. Post-treatment changes in the neoplastic and non-neoplastic cells have to be addressed while selecting FCM panels for this purpose.(4,20) FCM has roles in prognostication by identifying expression of various markers like Zap 70 in CLL as well as DNA ploidy and S-phase fraction analysis.

There are only a few studies on CLPDs among Asian populations.(2) Here, we have compiled the data on clinically suspected CLPD cases submitted for flow cytometric evaluation at our institution over one year. Amongst these, FCM was entirely successful in delineating reactive and neoplastic lymphoid proliferations except in 2 Hodgkin lymphoma cases. All but one of our positive cases were of B-lineage, thereby justifying the use of a B-centric FCM panel. Given the low frequency of T-cell lymphomas, the application of a basic set of markers - CD3 along with CD4 and CD8, for preliminary identification of any suspicious T-cell populations followed by detailed analysis only in required cases, would help reduce unnecessary use of reagents and thereby, bring down the costs. FCM enabled accurate identification of B-CLPDs like CLL and HCL with specific treatment protocols. However, its limitations in subtyping non-CLL/non HCL B-CLPDs like lymphoplasmacytic lymphomas are well known, as was our experience, thereby necessitating procurement of more specific FCM markers or other invasive procedures like tissue biopsy (figures 3,4). We have found FCM useful in identifying marrow infiltration by small populations of neoplastic cells in lymphoma cases. However, we are yet to step into the arena of FCM in plasma cell dyscrasias as well as its prognostic and theranostic applications in CLPDs.

As is well established, diagnosis of CLPDs requires an integrated and holistic approach starting with clinical profile and basic laboratory investigations, after that proceeding to more advanced investigations including flow cytometry, cytogenetic and molecular studies. However, these modalities must always be reconciled with morphology. The relative value of each technique varies in different settings, and this awareness should help the physician decide on appropriate investigation and specimen allocation. Though the armamentarium for CLPD evaluation is evolving, flow cytometry will remain in vogue as its applications get reinvented with the discovery of newer and newer molecules vital for management.

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