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Pathology

ROLE OF FLOWCYTOMETRIC IMMUNOPHENOTYPING IN CHRONIC LYMPHOPROLIFERATIVE DISORDERS: A 6-YEAR STUDY.

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ABSTRACT **INTRODUCTION:** Chronic B-cell lymphoproliferative disorders (B-CLPD) are a biologically heterogeneous group of malignant diseases most often diagnosed by flow cytometric immunophenotyping that identifies a clonal light-chain restricted population expressing B-cell markers in the blood or BM. Flowcytometric studies not only confirm a malignant diagnosis but, in most cases, allow accurate categorization into distinct pathologic entities.

AIMS & OBJECTIVE: The present study is undertaken to study the spectrum and pattern of antigen expression in chronic lymphoproliferative disorders (CLPD) encountered at a tertiary care center of North India.

MATERIALS & METHODS: This is a retrospective study done in the Department of Pathology over a period of 6 years. Basic clinical and hematological details were collected from the reports. The panel of monoclonal antibodies used for analysis of CLPD included: CD45, CD10, CD5, CD19, CD20, CD23, FMC, CD79b, CD3, CD22, CD25, CD103, CD38, kappa and lambda.

RESULTS: A total of 52 cases of CLPD were analysed. The age of the patients ranged from 49 to 80 years with a mean age of 65.5 years and male preponderance. There was male preponderance with M:F:: 2.07:1. The common presenting features were fever and weight loss, lymphadenopathy, splenomegaly, peripheral lymphocytosis along with various other signs & symptoms like fatigue, recurrent infections, severe neutropenia, peripheral neuropathy. Among 52 cases of CLPD, on detailed flowcytometric immunophenotyping, majority of cases were found to be of Chronic lymphocytic leukemia (CLL, 77%) followed by Mantle cell lymphoma (12%), Splenic marginal zone lymphoma (SMZL, 5%), Prolymphocytic leukemia (2%), Hairy cell leukemia (2%), Burkitt lymphoma (2%). The classical immunophenotype profile of each entity in our study has been summarized in a tabular form.

CONCLUSION: The current study highlights the importance of a systematic approach encompassing of clinicohematological profile, bone marrow examination and FCM immunophenotyping in arriving at a diagnosis of various subtypes of CLPDs. This multipronged approach is important for an accurate diagnosis of CLPDs, thereby providing better management for patients.

KEYWORDS : Flowcytometry, CLPDs, Bone marrow, SMZL, HCL, MCL

INTRODUCTION:

Flow cytometry is a standard technique to diagnose suspected chronic lymphoproliferative disorders (CLPD) in patients with lymphocytosis, lymphadenopathy, or other findings suspicious for a CLPD[1-5]. Flowcytometric studies not only confirm a malignant diagnosis but, in most cases, allow accurate categorization into distinct pathologic entities[2-7]. A definitive diagnosis of chronic lymphocytic leukemia (CLL) is essential to distinguish it from other lymphoproliferative disorders like hairy cell leukemia or mantle cell lymphoma, as well as risk stratification of the neoplasm, which is crucial in determining the therapeutic approaches which must be followed.

The presence of the aggressive disease forms in CLL lends special importance to the prognostic tests which are performed to assess the expression of the markers like CD38 and ZAP70 on the tumor cells, as these have been shown to have an impact on the disease response[3-7]. The present study is undertaken to study the spectrum and pattern of antigen expression in chronic lymphoproliferative disorders (CLPD) encountered at a tertiary care center of North India.

MATERIALS & METHODS:

We retrospectively reviewed the records of the flow cytometry reports in the Department of Pathology over a period of 6 years (May 2012 to April 2018). In each case, we reviewed the flowcytometric data and report to collect the following information: pertinent clinical information, hematological profile, panel used and diagnosis. The panel of monoclonal antibodies used for analysis of chronic lymphoproliferative disorders included: CD45, CD10, CD5, CD19, CD20, CD23, FMC, CD79b, CD3, CD22, CD25, CD103, CD38, kappa and lambda.

RESULTS:

A total of 52 cases of CLPD were analysed. The age of the patients ranged from 49 to 80 years with a mean age of 65.5 years. There was male preponderance with M:F::2.07:1 [Figure 1]. The common presenting features were fever and weight loss, lymphadenopathy, splenomegaly, peripheral lymphocytosis along with various other signs & symptoms like fatigue, recurrent infections, severe neutropenia, peripheral neuropathy [Figure 2]. Among 52 cases of CLPD, on detailed flowcytometric immunophenotyping, majority of cases were found to be of Chronic lymphocytic leukemia (CLL, 77%) followed by Mantle cell lymphoma (12%), Splenic marginal zone lymphoma (SMZL, 5%), Prolymphocytic leukemia (2%), Hairy cell leukemia (2%), Burkitt lymphoma (2%) [Figure 3]. B-cell chronic lymphocytic leukemia (CLL) has a characteristic immunophenotype: CD5+, CD23+, FMC7-, CD20 dim+ and clonal surface immunoglobulin (sIg) dim+. This classical immunophenotype profile distinguishes CLL from other B-CLPD [Table 1].

Table 1: Table showing immunophenotypic profile of various CLPD

- Immunophenotypic profile of CLPD:

	CD19	CD5	CD23	CD20	CD22	FMC7	CD79b	CD10	CD3	CD25	CD103
CLL	+	+	+	+	-	+/-	+/-	-	-	-	-
MCL	+	+	-	+	+	+	+	-	-	-	-
SMZL	-	-	-	+	-	-	+	-	-	-	-
PLL	+	+/-	+/-	+	+	+	+	-	-	-	-
HCL	+	-	-	+	+	+	+	-	-	+	+

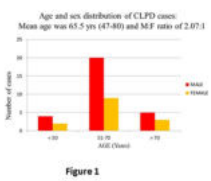

Figure 1

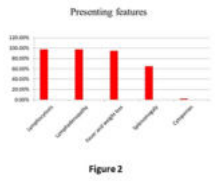

Figure 2

Figure 1: Age & Sex Distribution Of Clpd Cases; Figure 2: Bar Diagram Enumerating Presenting Features In Patient With Clpds.

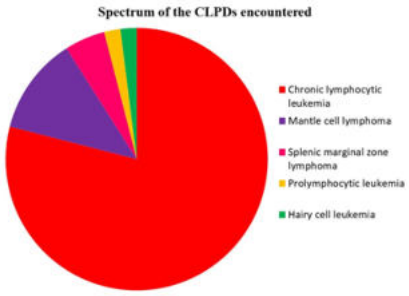

Figure 3: pie-chart showing spectrum of clpds encountered.

In our series, classical CLL as described by Matutes scoring constituted 74% with a dual expression of CD5 and CD 23 seen in 93% cases [Figure 4]. Adverse prognostic markers like CD38 expression was seen in 23%, CD103 positivity and absence of CD 23 was not noticed in this study.

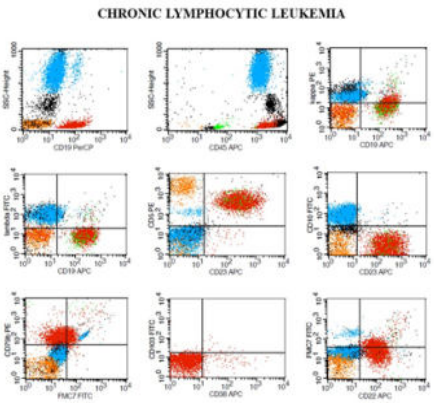

Figure 4: Flowcytometric immunophenotypic analysis in case of CLL.

Mantle cell lymphoma in our study typically displayed the composite phenotype: CD5+, CD23-, FMC7+, CD20 bright +ve, clonal sIg bright positivity [Figure 5].

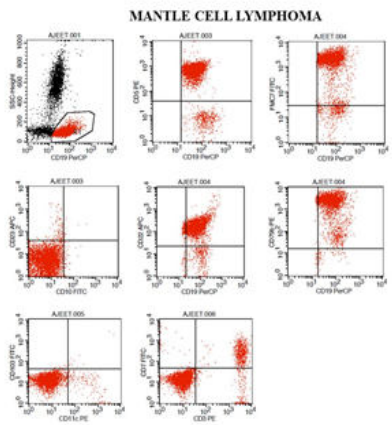

Figure 5: Flowcytometric immunophenotypic analysis in case of Mantle cell lymphoma.

We also noted case of hairy cell leukemia with a characteristic immunophenotype: CD5-, CD11c bright+, CD25+, CD103+ [Figure 6],

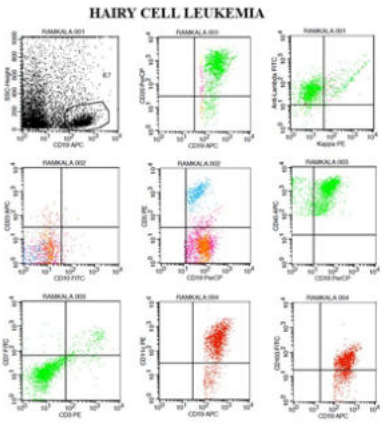

Figure 6: Fcm Immunophenotypic Analysis In Case Of Hairy Cell Leukemia.

which help in distinguishing it from other CD5- B-cell lymphoproliferative disorders, including the morphologically similar splenic lymphoma with circulating villous lymphocytes.

There was a case of SLVL in our series with immunophenotypic analysis showing CD5-, CD79b bright +, CD20 +, CD103 -ve [Figure 7]. Further, peripheral smear, bone marrow biopsy and IHC analysis led us to the diagnosis. On bone marrow biopsy intra-sinusoidal, interstitial and nodular pattern of infiltration by CD20+ve lymphoid cells were found, intra-sinusoidal pattern being highly characteristic of SMZL [Figure 8].

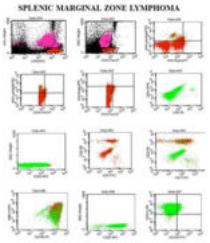

Figure 7

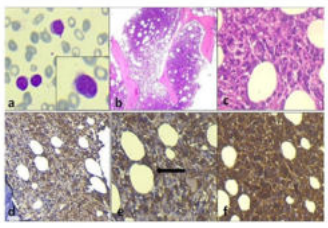

Figure 8

Fig 7- flowcytometric immunophenotypic analysis in case of splenic marginal zone lymphoma.fig 8- on bone marrow biopsy intra-sinusoidal, interstitial and nodular pattern of infiltration by cd20+ve lymphoid cells were found, intra-sinusoidal pattern being highly characteristic of smzl.

In our series, we also describe a case of aggressive Non-Hodgkin lymphoma, i.e., Burkitt lymphoma in a previously healthy 7-year-old male child, where the diagnostic ascitic & pleural fluid cytomorphology in conjunction with flowcytometric immunophenotypic analysis of the effusion helped in clinching an early diagnosis [Figure 9&10].

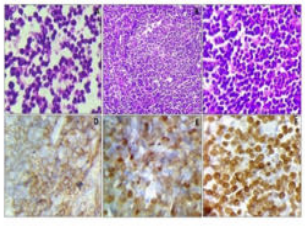

Figure 9

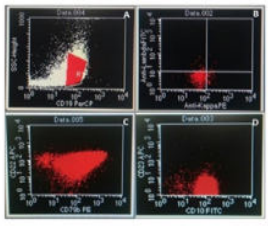

Figure 10

Figure 9-10: Diagnostic ascitic & pleural fluid cytomorphology (Fig-9) in conjunction with flowcytometric immunophenotypic analysis (Fig-10) of the effusion in case of Burkitt Lymphoma.

We noted an another very interesting case of a 25-year-old immunocompromised male patient (retropositive and HCV +ve) who presented with generalised lymphadenopathy and hepatosplenomegaly. Also noted were multiple papular lesion in a generalised manner on

trunk, bilateral upper and lower limbs, measuring 0.5x0.5cms in size. The lesions were non-itchy, mildly erythematous, non-scaly. Peripheral smear showed leukocytosis with lymphocytosis (Absolute lymphocyte count of 24000/microL). 34% of these lymphoid cells were small to intermediate in size with scant cytoplasm, nuclei showing indentation (cerebriform nuclei); few with blastoid chromatin. FCM immunophenotyping of peripheral blood revealed moderately bright lymphoid cluster (56%) of total acquired events. 98% of these cells were found to be of T- lymphoid on CD19/SSC analysis [Figure 11]. These cells showed heterogenous expression for CD5 (dim to moderate), moderate expression of CD3. Majority of these lymphoid cells showed positivity for CD4. These cells were negative for CD7, CD19, CD10, CD22, CD79b, FMC-7, CD-103, CD11c. Presence of the generalized lymphadenopathy, erythroderma, presence of sezary cells in peripheral blood, FCM immunophenotypic profile and characteristic skin biopsy findings were consistent with diagnosis of Sezary syndrome.

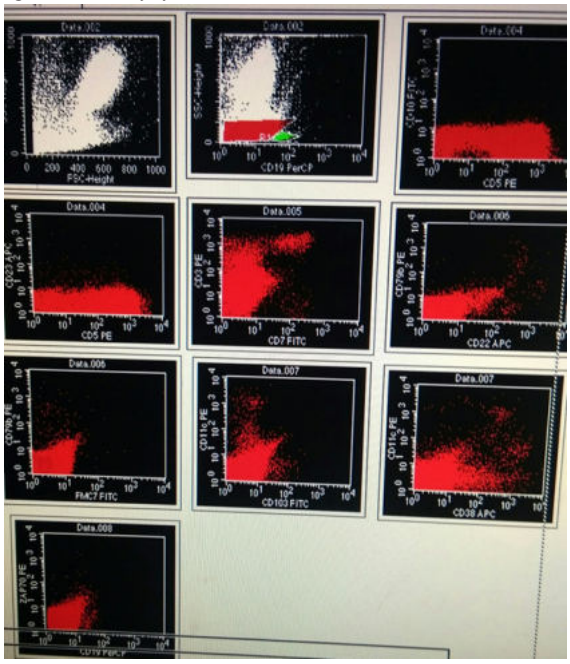

Figure 11: Fcm Immunophenotyping Of Peripheral Blood Revealed Moderately Bright Lymphoid Cluster (56%) Of Total Acquired Events. 98% Of These Cells Were Found To Be Of T-Lymphoid On Cd19/ssc Analysis; Suggestive Of Sezary Syndrome.

DISCUSSION:

Important advances have been achieved in the early diagnosis of chronic lymphoproliferative disorders in the last two decades. Among other factors, this has been related to the introduction of automated analyzers for the routine analysis of different blood cell compartments in distinct disease conditions. From the different WBC-associated flagging alarms provided by these instruments, the presence of an increased absolute number of PB lymphocytes is by far, one of the most frequent ones.

Although in children, absolute lymphocytosis is commonly due to reactive (e.g. infectious) processes, in adults over 50 years, it is frequently related to monoclonal expansions of B-cells due to an underlying neoplastic lymphoproliferative disorder. In recent times, multiparameter flow cytometry immunophenotyping has emerged as the method of choice for the screening of the clonal vs reactive nature of the expanded lymphocytes, leading to a progressively high increase in the rate of early diagnosis of clonal lymphoproliferative disorders, even prior to the onset of disease symptoms.

Nevertheless, recent results indicate that morphological examination of PB samples presenting with absolute lymphocytosis is neither completely specific nor sensitive for the distinction between normal/reactive and clonal processes[6-11]. Accordingly, some neoplastic disorders are classified morphologically as reactive and no definitive cytological diagnosis can be made in a significant proportion of cases, particularly those with mild lymphocytosis. Overall, these results support the notion that multiparameter flow cytometry immunophenotyping should be performed upfront in adult individuals, immediately after an absolute PB lymphocytosis is detected. To speed up and simplify such flow cytometric screening, automated analysis of the flow cytometry list mode data files represents an essential step.

In the present paper we highlight the role of FCM immunophenotypic analysis in early detection of CLPDs including unusual ones. We found a total of 52 cases of CLPD which was almost similar to a study by Okaly et al[12] who reported a series of 66 cases. We reported the median age of diagnosis at 65.5 years while Okaly et al[12] reported it as slightly higher, i.e., 64.5 years. Although, Gogia et al[13]., reported a lower median age of 59 years from a large series study in India. Majority of CLPD cases were noted in the age group of 51-70 years with male preponderance in our as well other study. Lymphadenopathy and peripheral lymphocytosis were the most common presenting features in our study. Various other studies reported lymphadenopathy, fatigue and abdominal pain as common presenting complaints.

We found CLL (77%) to be the most common amongst the total CLPD cases, followed by MCL (12%), SMZL (5%), PLL (2%), HCL (2%) ad BL (2%). A study by Okaly et al also found CLL as the most common leukemia (62.1%) followed by NHL (19.7%), HCL (3%), FL (1.5%) & PLL (1.5%). The overall incidence of various CLPDs appear almost similar in various studies.

CLL is a disease of the elderly and is rarely encountered in individuals under the age of 40. Thereafter the disease incidence increases with age. The incidence rate is about 2-6 cases per 100,000 persons per year, increasing with age reaching 12.8/100,000 at age 65, the mean age at diagnosis. There is a slight male preponderance. The presence of a cytopenia caused by clonal bone marrow involvement establishes the diagnosis of CLL regardless of the peripheral B-lymphocyte count. In bone marrow and peripheral blood smears, CLL cells are small lymphocytes with clumped chromatin and scant cytoplasm which can be clear to slightly basophillic. Nucleoli are indistinct or absent. Smudge cells are typical in blood smears. The CD45 vs SSC/FS gating dot plot shows CLL/SLL cells are CD45 bright and SSC low. It is known that classical CLL cases show dual expression of CD5 and CD23. We found majority of the CLL cases (93%) with double positivity of CD5 & CD23. Okaly et al[12] reported 97.5% cases of CLL with this double expression in their series. CD23 is also known as surrogate marker for CLL as it helps in the differentiation of CLL from other CD5 positive lymphoid leukemias. Even in absence of light chain expression surrogate marker helps. Since assessment of the IgV_H antibody DNA changes is difficult to perform, the presence of either CD38 or ZAP-70 may be surrogate markers of high-risk subtype of CLL Their expression correlates with a more immature cellular state and a more rapid disease course. These cells are CD45 bright and SSC low, express CD19 and CD20 (dim). CD5/CD19 and CD23 are coexpressed and there is a dim monoclonal light chain of either kappa or lambda expression.

FMC7 is of greater diagnostic value than CD20 for distinguishing CLL from other B-cell disorders. CD10 is negative and FMC7 & CD79b are usually negative or weakly expressed in typical CLL.

Mantle cell lymphoma (MCL) is a B-cell neoplasm generally composed of monomorphic small to medium-sized lymphoid cells with irregular nuclear contours and a CCND1 translocation[10,11]. It comprises of 3-5% of NHL. It occurs in middle-aged to older individuals with a median age of about 60 years and a variably marked male predominance. We also noted 12% cases of MCL in our series. The cells express relatively intense surface IgM/lgD, more frequently with lambda than kappa restriction. They are usually positive for CD5, FMC-7 and CD43, but negative for CD10 and BCl6. CD23 is negative or weakly positive. All cases are BCl2 protein positive and almost all express cyclin D1. Cyclin D1 expression in mantle-cell lymphoma reliably distinguishes it from other CLPD's. Of interest is that cyclin D1-positive MCL show significantly worse survival than cyclin D1-negative lymphoma. Cyclin D1 negative cases of MCL show high expression of cyclin D2 & cyclin D3.

Hairy Cell Leukaemia (HCL) is a rare and an indolent form of a small, mature, B-cell leukaemia which is characterized by oval or indented (bean-shaped) nuclei and abundant “hairy” cytoplasm, which involves the Peripheral Blood (PB), Bone Marrow (BM) and the spleen. Hairy

cell leukaemia is a rare disease. comprising 2% of lymphoid leukaemias[17-21]. Patients are predominantly middle-aged to elderly adults with a median age of 50 years. The male to female ratio is 5:1. HCL with its characteristic immunophenotype and positivity for CD11c, CD25, CD103 and CD5 -, helps in distinguishing it from other CD5- B-cell CLPDs. In a report by Chatterjee et al[21], immunophenotypic analysis of HCL and its variants was highlighted. Most of the cases lack expression of CD10 & Cd5.

SMZL is a rare B-cell lymphoid neoplasm involving spleen, bone marrow and frequently blood. SMZL is a rare disorder, comprising less than 2% of lymphoid neoplasms, but it may account for most cases of otherwise unclassifiable chronic lymphoid leukemia that are CD5-. Most patients are over 50 and there is an equal sex incidence. Its diagnosis requires utmost attention of important clues especially in the absence of splenic histopathology for the evaluation. Presence of classical cell morphology (polar villous processes), flowcytometric immunophenotyping and typical nodular, interstitial and intrasinusoidal pattern of infiltration by CD20 positive cells in the bone marrow are important diagnostic clues for the diagnosis of SMZL[20].

Tumour cells express surface IgM and are CD20+, CD79a+, CD5-, CD10-, CD23-, CD43 and Annexin A1-. CD103 is usually negative and cyclin D1 is absent. Staining for Ki67 shows a distinctive targetoid pattern based on the presence of an increased growth fraction in both the germinal centre (if present) and the marginal zone. The absence of cyclin D1 and the infrequent expression of CD5 are useful in excluding mantle cell lymphoma and chronic lymphocytic leukaemia respectively. Absence of annexin A1 excludes hairy cell leukemia and absence of CD10 and BCl6 helps to exclude follicular lymphoma This case also highlights the importance of flowcytometric immunophenotyping and bone marrow biopsy with immunohistochemistry to exclude other possibilities and arrive at a definitive diagnosis.

Burkitt lymphoma is a lymphoma with an extremely short doubling time that often presents in extranodal sites or as an acute leukaemia. Burkitt lymphoma is one of the rare lymphomas to be diagnosed. The tumour cells express moderate to strong levels of membrane IgM with light chain restriction and B-cell-associated antigens (e.g. CD19, CD20, C022), CD10, BCL6, CD38, CD77 and Cd43.

Serous effusions may occur as complications in different types of lymphomas. In a study by Johnston et al, among 584 patients with serous effusion, 15% had underlying lymphoma. [2] Usually, T-cell lymphoma was associated with serous effusion while only rare B-cell lymphoma developed pleural or peritoneal involvement[15-19]. Among these, pleural effusions are commonly observed; however, involvement of peritoneal or pericardial cavity is rare. We noted a case of Burkitt lymphoma in a previously healthy 7-year-old male child, who presented with ascites and pleural effusion. Flowcytometric immunophenotypic analysis of ascitic fluid was done which helped in clinching an early diagnosis. Subsequently, cell block from ascitic and pleural fluid was also made which showed cellular tumor comprising of malignant cells as described above along with interspersed tingible body macrophages. An immediate intervention was made, and specific chemotherapy was started. Subsequently, lymph node biopsy was also sent which showed similar features and IHC panel showed positivity for CD10, CD20, bcl6, Ki67 (98-100%).

Sézary syndrome (SS) and Mycosis Fungoides (MF) are T-cell lymphomas whose primary manifestation is in the skin. It generally affects the skin but may progress internally over time. "Sézary syndrome" is characterised by the triad of erythroderma, generalised lymphadenopathy and the presence of Sezary cells in skin, lymph nodes and peripheral blood. "Sézary's cells" are clonally related neoplastic T-cells with cerebriform nuclei. Tumor cells express CD2, CD3, TCRb, CD5 and CD7 (-/+)[19-22]. Most cases are CD4+, rarely is CD8 expressed. Aberrant T-cell phenotypes are common. In our series, we noted a similar case in a 25-year-old immunocompromised male patient. Klemke C D et al[22] studied 17 cases of Sezary syndrome using FCM. A combination of the generalized lymphadenopathy, erythroderma and presence of sezary cells in peripheral blood as well as characteristic FCM immunophenotypic findings along with skin biopsy help in making a definitive diagnosis.

This study gives a brief description of different aspects of CLPDs with special reference to immunophenotypic profile. Apart from the clinical profile, routine PB and BM examination, FCM immunophenotyping plays a key role in early diagnosis of the disease process. The ability of our panel to accurately detect CLL in the patient samples, led us to believe that a basic minimal panel (which consisted of CD45, CD20, CD22, CD79b, CD19, CD5, CD23, CD10, FMC7, CD3, CD5, CD25, CD103, kappa and lambda) was sufficient for the routine diagnosis of CLL. However, the stratification of CLPD requires the use of more extensive panels. A careful analysis of additional material like serous effusion can also help to clinch the diagnosis.

CONCLUSION:

The present study highlights the significance of multipronged approach for the diagnosis of CLPDs. A systematic approach encompassing analysis of clinicohematological profile, bone marrow examination and FCM immunophenotyping is essential to arrive at a diagnosis of various subtypes of CLPDs. This series also stresses upon the need of examination of additional material (effusion fluid etc) and ancillary techniques (Immunohistochemistry etc) to clinch an accurate diagnosis. An early and accurate diagnosis of CLPDs (including its various subtypes) is pertinent for better management of the patients.

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