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Immunophenotyping using Flowcytometry for B-Chronic Lymphocytic Leukemia (B-CLL): A Case Study from J&K.

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Abstract:
A case study of 72-yearmale with overweight, diabetic and hypertensive condition, presented with gout, frequent gastric complications. After studying the symptoms, morphology, patient condition, blood test, the patient was doubtful for the presence of B-CLL and immunophenotyping and FISH (Fluorescence in-situ Hybridization) analysis was done to confirm the same. After the immunophenotyping the patient was confirmed for the B-CLL leukemia and thus was precededforward for further treatment therapies. In the present study our main focus is to determine the importance of immunophenotyping using the flowcytometry for determining the disease and be specific in the process of giving the specified treatment to the affected individual.

Keywords: B-CLL, immunophenotyping,flowcytometry, CD- markers,FISH (Fluorescence in-situ Hybridization)

Introduction:

Chronic lymphocytic leukemia (CLL) is a clonal proliferation of mature B lymphocytes characterized by indolent (slow) and heterogeneous clinical course and are more prevalent in western world (Atore*et al.* 2018) and is caused due to altered or impaired apoptosis of the B-cell. The heterogeneity is explained by the deletion of 17p and inactivation of the TP53 gene which is an important factor that controls the treatment effectiveness and the outcome (Puente*et al.* 2015). This is common in the age of 70 years or above and its more common in males (2:1) (Aitken *et al.* 2019).This clonality is characterized biologically by low expression of surface immunoglobulin (sIg) and restriction to a single immunoglobulin light chain associated with high expression of CD5 antigen and positivity to B cell antigen lymphocytes such as CD19, CD20 and CD23 and negativity to FMC7. The immunological profile and morphological analysis of lymphoid cells are the main means for the differential diagnosis of B-CLL from other chronic lymphoproliferativediseases. The use of immunophenotyping is an

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excellent option to confirm the certain lineages from others as it is more authentic and reasonable and is presently main pillar in diagnosing the leukeima (Abdel-Ghafer*et al*.2012). The flocytometry is very common option for examining the bone marrow as well as the peripheral blood but not for the tissue specimens (Martinez*et al*. 2003).Immunophenotyping showed that a prominent presence of several surface and intracellular markers are checked and found positive expressions of CD38 (Haferlach*et al*, 2007), ZAP70 expression (70 kDa zeta-associated protein, CD19,CD20, CD23 and negative for FMC7 and the profile help to identify the B-CLL easily (Atore*et al.* 2018).Campana and Behm (2000) gave detailed information about different cell markers for different types of leukemia. They said that normal pathway of lymphoid cells includes active proliferation and in controlled manner matures, thus lymphocyte activation, expamsion and selection in peripheral organs is a well organised process. But the complexity in the process incteases in the neoplastic cells in case of chronic lymphoproliferative disorders (Caligaris-Cappio, 1996; Catovsky 1995)

OBJECTIVE: The aim of this study was to evaluate the expression pattern of a variety of membrane antigens in leukemic cells originating from patients with B-CLL using multiparametric flow cytometry.

METHODOLGY Peripheral blood samples were collected from the patients with their prior consent. The sample was send to Dr Lal Path lab (Delhi) for immunophenotyping analysis and wasanalyzed by multiparametric flow cytometry(FACSCanttoII ,using Diva software with 99 percent cell viability and used CD19 bright vs. lowSSC gating). Routine hematologic exams, using a panel of monoclonal antibodies (MoAb): T cell markers (CD3, CD5, CD7), B- cell markers (CD19, CD20, CD79b (surface), IgM(surface)Kappa(surface), Lambda (surface), CD5 & CD19 co-expression), Other markers: CD10, CD11c, CD23, CD25, CD38, CD45, CD103, CD123, CD200, FMC-7, CD43). The haematological data were obtained using a haematologicalanalyzer.

A CASE STUDY:CASE REPORT

A 72 year male over weight, diabetic and hypertensive condition, presented with gout, frequent gastric complications like acidity and constipation was admitted to the hospital. Previous medical history showed constant joint pain and a condition of hyperthyroidism were found.Problem in breathing was also reported on mild note. Close physical examination and detailed interview showed that there were bruises that were appearing on the body especially on the chest area and the extremities for the last two years with petechial spots on the back.` The body temperature was on higher side (38.2^{o}), pulse rate 103 per minute, blood pressure 154/69, oxygen saturation 95 %.Abdominal computed tomography (CT) showed hepato-spleenomegaly. The routine hematological tests revealed complications of marrow failure. He was referred to a haematological team and various required tests like Bone marrow aspiration and biopsy, immunophenotyping,cytogenetic studies were done and all these tests confirmed the disease as B-CLL. Life style profile showed the urban lifestyle, non- vegetarian diet, regular smoking and occasional alcohol intake. Complete white blood cell count 42.0 x 10^9/L with lymphocyte count was 5.0 x 10^9/l(normal range 4.00–10.00×10^3/uL), haemoglobin8.1g/dl and platelet count 98 lac/cumm showing thrombocytopenia.

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IMMUNOPHENOTYPING:

Flow cytometry was done using FACS analysis on peripheral blood using the following fluorescent labelled antibody markers. Peripheral blood samples was used for B-CLL analysis by multiparametric flow cytometry and routine hematologic exams, using a panel of monoclonal antibodies (MoAb): T cell markers (CD3, CD5, CD7), B- cell markers (CD19,CD20, CD79b (surface), IgM(surface)Kappa(surface), Lambda (surface), CD5 & CD19 co-expression), Other markers: The hematological data were obtained using a hematological.

Flowcytometric analysis reveals an atypical population of B lymphoid cells showing kappa light chain restriction and co expression of CD5 & CD19 with dim to moderate expression of CD20, CD23, sIgM, CD43,ROR-1,moderate to bright expression of CD45 and CD200. These atypical cells are negative for CD10, CD11c, CD25, CD38 (Haferlach*et al*, 2007), CD79b, FMC-7, CD103 andCD123.

This investigation highlights the importance of immunophenotyping for correct diagnosis of chronic lymphoproliferative syndromes, and the MoAb panel used was sufficient for diagnostic confirmation of B-CLL. Also Overall flow cytometric findings are consistent with B cell Chronic Lymphocytic Leukemia and Correlation with clinical features, peripheral blood findings was seen.

T cell markers			
CD3	0.3	Negative	Negative
CD5	96.5	Dim to Mod	Positive
CD7	0.6	Negative	Negative
B cell markers			
CD19	100	Bright	Positive
CD20	96.9	Dim pos	Positive
CD79b (surface)	0.0	Negative	Negative
IgM (surface)	100	Dim to Mod	Positive
Kappa (surface)	1.4	Negative	Negative
Lambda (surface)	1.2	Negative	Negative
CD5 & CD19 co-expression	96.5	Mod to Bright	Positive
Other markers			
CD10	0.0	Negative	Negative
CD11C	58.3	Dim pos	Positive
CD23	95.0	Dim to Mod	Positive
CD25	0.0	Negative	Negative
CD38	0.2	Negative	Negative

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CD45	100	Bright	Positive
CD103	0.1	Negative	Negative
CD123	0.0	Negative	Negative
CD200	99.9	Bright	Positive
FMC-7	0.2	Negative	Negative
OTHER MARKERS			
CD43	98.9	Dim pos	Positive

Table 1: showing the presence of various CD markers, FMC-7, Lambda, Kappa and Igm surface makers to detect the presence of B-CLL using FACS analysis (FACS CanttoII ,using Diva software with 99 percent cell viability and used CD19 bright vs. lowSSC gating).

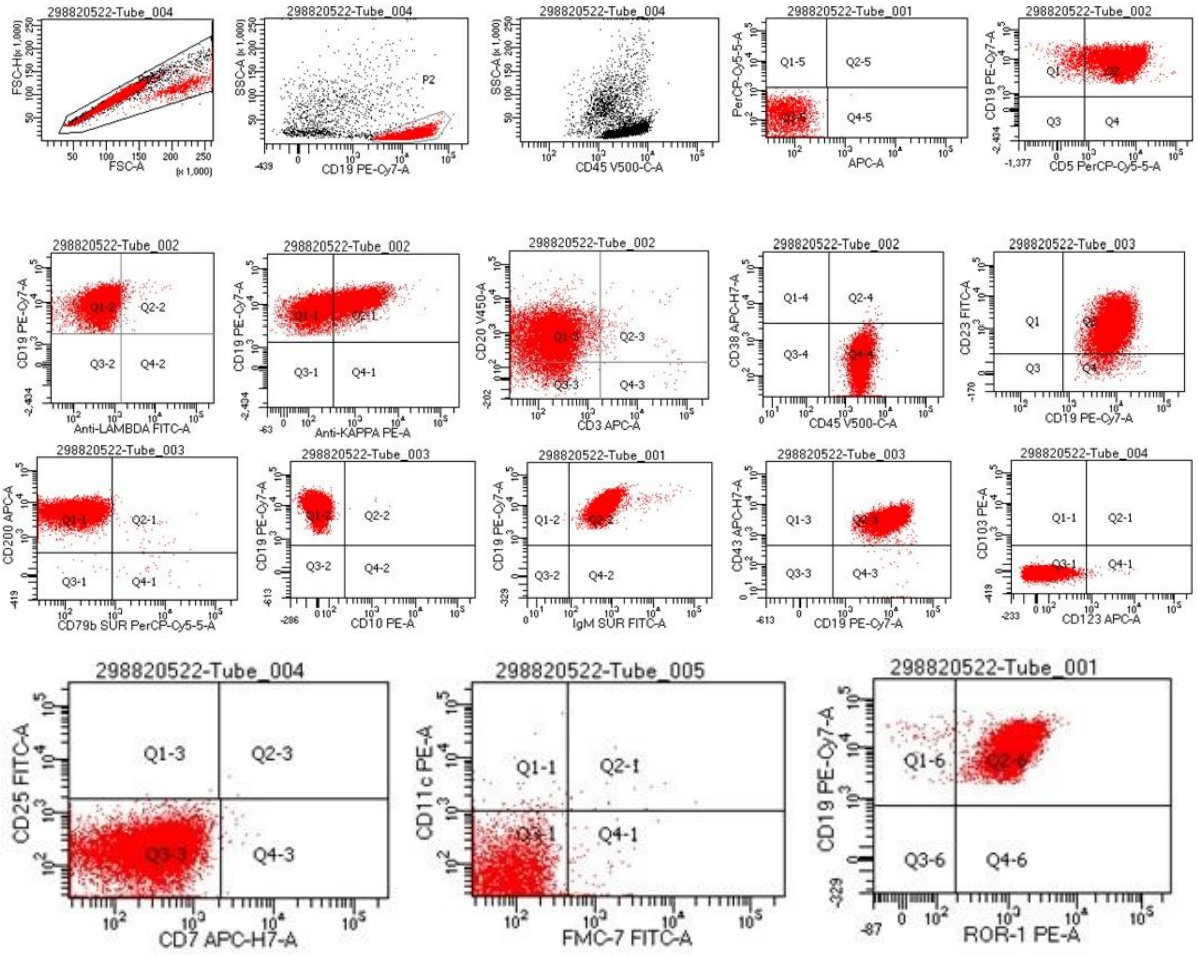

Figure 1: Dot plot showing CD19 bright vs. lowSSC gating stratergy for analysing the Leukemia cells for the B-CLL using multiparametric flowcytometer using various CD markers, FMC-7, Lambda, Kappa and Igm surface makers.

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In order to decide the partical treatment therapy ,FISH (Fluorescence in-situ Hybridization) analysis was also performed on 200 Interphase nuclei for (17p13.1) Spectrum Orange probe representtwo dots showing negative for deletion present in chromosome 17 p 13.1 which is associated by the CLL (Farooqui *et al.* 2015, Hui *et al.* 2019). So this patient was cytogeneticaly normal for 17p and TP53 deletions and thus could have better chances of having good outcomes from treatment.These chromosome 17 deletions arealso associated with several conditions like Lissencephaly (smooth brain), Multiple myeloma, Chronic Lympocyticleukemia& Miller Dieker syndrome. Patients with 17p deletion and karyotypic deletion 13 or hypodiploidy are considered to have high-risk myeloma. Patients with Chronic Lymphocytic Leukaemia (CLL) whose tumour cells harbour a 17p deletion are universally considered to have a poor prognosis as the TP53 has a critical role in the cell cycle, cell arrest and it deletion is common phenomenon in CLL patients (Aitken et al. 2019). The deletion can be detected at diagnosis or during the evolution of the disease and the status regarding the cytogenetical conditions is important for the prognosis and the treatment therapy for a patient. Campo *et al.* 2018 also mentioned the importance of looking for the 17p deletion and TP53 status before selecting the course of treatment as this proves to be avery important factor in the survival outcome. This is being supported by Monti *et al*. 2020 and they also added that the inactivation of the TP53 gene leads to the resistance in chemotherapy treatment and thus adds on to the poor survival outcome in such patients.

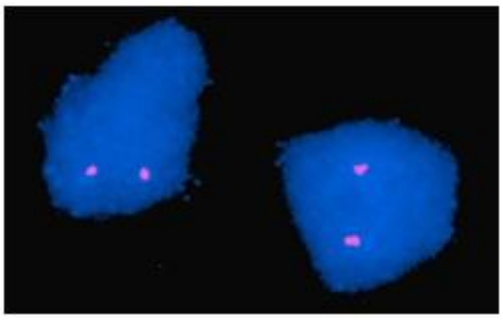

Figure2 : showing the Interphase nuclei for (17p13.1) deletion using FISH (Fluorescence in-situ Hybridization) and the Spectrum Orange probe showed two pots showing negative for deletion present in chromosome 17 p 13.1 associated with the CLL

Discussion:

The diagnosis includes blood counts, blood smears test, and immunophenotyping of circulating B-lymphocytes carrying B-cell markers. Prognosis of the disease depends on the various factors including the physical examination and blood counts certain biological and genetic markers. Deletion of chromosome 17 (*del(17p)*) suggests avoiding harsh chemotherapy treatment and using combination treatment therapy like use of anti-CD20 antibody (obinutuzumab or rituximab or ofatumumab) as this deletion cause resistance to available chemotherapies (Hallek 2015). Blood reports showing the thrombocytopenia, anaemia and high lymphocyte count ensure the symptomatic CLL. The organomegaly with constant high fever also ensured aboutthe CLL (Hallek 2015). The presence of >5,000 B-lymphocytes/mL in the peripheral blood for the duration of at least 3 months is required for CLL while<5000/ml of blasts with absence of organomegaly and cytopenia specifies

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monoclonal B- lymphocytosis (MBL)while presence of lymphadenopathy and absence of cutopenias hallmarks the Small lymphocytic leuekima (SLL)(Marti*et al.* 2005, Hallek2015).

The use of immunophenotypingto ensure the type of lineage in addition to symptoms, morphology of the cells, cytogenetic and molecular is a better option and is quick and more reliable. Since this technique is an emerging as a new and more precise than the earlier existing techniques and is readily being used worldwide and is accepted by various authorities associated with the study of hematopoietic disorders and their treatments. This is also less time consuming which serves a better option when treating the patients who are suffering with such a lethal disease.In several studies showed concordance of histopathology with the immunophenotyping and thus proved to be useful. Abdel Ghafer*et al*. 2012 compared immunophenotyping and immunohistochemistryand found immunohistochemistry little more sensitive than immunophenotyping for the CLL. They found 100% of the CLL cases revealed CD19, CD20 (72.7%,dim intensity), CD5 and CD23, 87.5% expressed CD22, 67.5% were negative for CD79b, 77.5% were negative for FMC7 but 35% were positive for CD38. SmIg light chain restriction showed κ restriction in 54.5% and λ in 45.5% of cases. Geisler *et al*. 1991 had shown the role of low CD23 expressionand IgM in short survival.

DiGiuseppe and Borowitz (2012) suggested being very careful in choosing the immunophenotyping panels of antibodies as very slight variation in the panel setting can be useful differentiate between the different forms of the diseases and go for accurate treatment therapy accordingly. For instance a panel markers (CD5+, CD23+, CD20 dim+, FMC7-, clonal surface immunoglobulin (sIg) dim+) is used for B-CLL while mixed panel of (CD5+, CD23-, FMC7+, CD20 bright+, clonal sIg bright+) distinguishes it from another CD5+ B-cell lymphoproliferative disorder i.e. mantle cell lymphoma. Similarly hairy cell leukemia can be distinguished by using CD5-, CD11c bright+, CD25+ and CD103. According to Gieseler *et al.* 1991, in his study all the cases that were CD5+ expressed IgD, IgM, CD20,CD21,CD22 and low CD23 expressions and high IgM fluorescence intensity, high FMC7 and CD23 had a significant prognostic importance with age and clinical stage of the disease and the short survival on performing univariate analysis. In Cox multiple regression analyses IgM-fluorescence intensity, age, CD23, and clinical stagehad independent prognostic importance.

Campana and Behm (2000) studied the different immunpphenotyping panels for different types of leukemia and classified different sets of cell markers for different lineages.The panel of markers used was CD19, CD79a, CD79b, CD20, CD22, CD37 even though some antigens may express low or not at all. Similar findings were reported by Cabezudo *et al.* 1999 and DiGiuseppe and Borowitz 1998. Brown and Wittwer (2000) reported the use of some makers as the key to identify the B-CLL that included CD5, CD19, CD20, CD23, CD25 and CD11c and stated that CD25 and CD11c was often seen present while CD20 was seen to be positive occasionally.

Fan *et al.* 2015 used the CD200 and CD148and suggested that these markers have the capacity to distinguish between the B-CLL and Mantle cell lymphoma (MBL) which are both B-cell chronic lymphoproliferative disorders. SimilarlyLesesve*et al.* 2014 found the

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Combination of CD160 and CD200 to be a useful set of markers for differential diagnosis between chronic lymphocytic leukemia and other mature B-cell neoplasms. Strati and Shanafelt 2015 also defined monoclonal B –cell lymphoma as if the number of clonal B-cells is < than $5*10^9$/l and if it count increases in the peripheral blood can lead to CLL, also coexpressing CD5, CD19, and CD23 and a weak expression of CD20, CD79b, and surface immunoglobulin (sIg). If such population of cells shows enlarged lymph nodes without peripheral lymphocytes, is termed as small lymphocytic lymphoma (SLL)(Santos*et al.* 2012).

Strati and Shanafelt 2015 referred to study from Northern Italy, of 1779 individuals among whom 138 had low-count MBL on screening (96 CLL-phenotype MBL, 21 atypical CLL-phenotype MBL, 20 CD5- negative–phenotype MBL) (Perz *et al.* 2008). A follow-upof 34 months, a small clonal B-cell population persisted in 90% of those with CLLphenotype MBL and no patients progressed to CLL, SLL, or other lymphoid malignancy. Thusshowing that low count MBL individual has a life expectancy of same as the normal individuals. Falay and Ozet 2017 suggested that CD11c, CD38, and CD43, a lymphoproliferative disease panel, was more specific than CD22, CD79b, and FMC7 in the diagnosis of CLL and proved more significant. Falay *et al.*2018 found that CD200 was positive 97.3% of atypical CLL cases, whereas it was dimly expressed in only 6.1% of MCL cases. CD43 was seen positivein 95.7% in atypical CLL cases and in MCL 39.4%.

Henry et al. 2019 studied immuno-flowFISH for the detection of chromosomal abnormalities in CLL, specifically trisomy 12 and del(17p). Mononuclear cells were isolated and immunophenotyped with fluorescently conjugated CD3, CD5, and CD19 monoclonal antibodies. Deletion of 17p was detected in about 8% of cases to which was phenotypically identified CD5/CD19-positive B-cells. Hoffmann *et al.* 2020 used CD200 and CD43 andconcluded that they have very informative value in diagnostic immunophenotyping and help in distinguishing CLL from other B-NHLs (B-cell non-Hodgkin lymphomas) particularly in ambiguous cases

Mansoor *et al*. 2021 reported a very rare case in India in an adolescent girl with symptoms of fever, weight loss and cervical lymphadenopathy and thus need an awareness regarding these kinds of cases. Gupta et al. 2021 associated the Chronic lymphocytic leukemia with direct antiglobulin test (DAT), autoimmune haemolytic anemia, CD 38 EXPRESSION .Also positive association was seen between advanced Rai and Binet stage and DAT.A positive association was also seen between CD 38 and DAT (P = 0.008). The data regarding DAT test is not enough for the Indian population.

Reddy *et al.* 2012 studied FMC-7 expression that identifies phenotypically atypical chronic lymphocytic leukemia and our study result matches with first group of the reddy's finding that expressed CD5+, CD19+, CD23+ AND FMC-7 negative typically showing CD23+ and FMC-7 negative group. Garcia *et al.* 2001 also documented the similar pattern for accurate identification of atypical B-CLL using CD23 and FMC-7 Antigen Expression Patterns in B-Cell Lymphoma Classification. Similar study was reported by Godke*et al.*2017 in which two cases of B-CLL were confirmed as B-CLL patients with demonstration of CD5+ B-cell

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population with co-expression of CD23, weak expression of CD20, and one type of immunoglobin light chain (either kappa or lambda) as necessary for the diagnosis of CLL.

Authors and year	**Title**	**markers used**	**Type**
Campana and Behm (2000)	Immunophenotyping of leukemia	D19,CD79a,CD79b,CD20, CD22, CD37	B-CLL
Matutes and Polliack (2000)	Morphological and Immunophenotypic Features of Chronic Lymphocytic Leukemia	$CD5^{+}$, $CD23^{+}$, $FMC7^{-}$, weak expression of surface Ig (sIg) and weak or absent expression of membrane CD22 and CD79b.	B-CLL
Subramanian et al. 2010	Differentiating Chronic Lymphocytic Lymphoma From Other of Mature B Cell Non Hodgkin's Lymphoma.	CD19, CD22, CD23, CD25, CD79b, CD200 and Kappa positive, with CD20 dim+ and CD5, CD11c, CD103, CD123	CLL
Falay and Ozet 2017	Immunophenotyping of Chronic Lymphocytic Leukemia	CD11c, CD38, CD43, CD22, CD79b, and FMC7	B-CLL
Arlindo *et al*. 2017	Quantitative flow cytometric evaluation of CD200, CD123, CD43 and CD52 as a tool for the differential diagnosis of mature B-cell neoplasms	CD52, CD200, CD123 and CD43	B cell neoplasms
Ting *et al.* 2018/ Mora *et al.*2018	CD200 is a useful diagnostic marker for identifying atypical chronic lymphocytic leukemia by flow cytometry	CD200	CD200 + for CLL and dim to absent in MCL
Henry et al. 2019	Immuno-flowFISH" for the Assessment of Cytogenetic Abnormalities in Chronic Lymphocytic Leukemia	CD5/CD19-positive B-cells	B-CLL
Salem and, Stetler-Stevenson (2019)	Clinical Flow-Cytometric Testing in Chronic Lymphocytic Leukemia	CD5, CD19, dim CD20, dim CD22, CD23, bright CD43, dim CD45, dim to negative CD79b, dim CD81, CD200, and dim monoclonal surface	CLL

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		immunoglobulin.	
Ibrahim et al. 2020	Composite Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma and Mantle Cell Lymphoma; Small Cell Variant: A Real Diagnostic Challenge. Case Presentation and Review of Literature	CD19, CD79b, CD20, CD5, FMC7, cBCL2, IgD, IgM, and partial CD23 with lambda light chain restriction. and CD19, CD23, CD5, CD43, CD200, CD38, cBCL2, IgD, IgM, dimmer CD79b, and CD20 (partial dim) with kappa light chain restriction.	CLL/SLL

Table 2: showing the comparative study of various authors and use of certain immunophenotypingmarkersused to distinguish the B-CLL from other chronic lymphoproliferative disorders

Conclusions: B-CLL is a disease of heterogenous course and it becomes must to identify the different variants of lymphoid lineages to ensure the right treatment is to be initiated.So clear diagnosis of the diseases and choice of treatment selection decides the survival outcome for the patients. Other factors that decides thesurviaval outcome is the age of the patient, fitness of the patient, less multiple health issues , genetic outcome/ status and the response of the first line treatment. Thus immunophenotyping panel ofCD5 & CD19 with dim to moderate expression of CD20, CD23, sIgM, CD43,ROR-1,moderate to bright expression of CD45 and CD200 used in the present study was enough to decide the type and proceed for the treatment for B-CLL.

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