

# Identifying Atypical Chronic lymphocytic leukemia Using CD200 by Eight-Color Flow Cytometry

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## ABSTRACT

**Objective:** Flow cytometric immunophenotyping is routinely employed to differentiate between chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL). We investigated the role of cluster of differentiation 200 (CD200) in atypical CLL.

**Materials and Methods:** A total of 90 chronic lymphocytic leukemia cases between January 2018 and December 2019, thirteen (14.4%) out of which were diagnosed as atypical CLL were included in the study. The expression of CD200 using eight-color flow cytometry in combination with the conventional panel of flow cytometry markers was examined. Fluorescence *in situ* hybridization to search for the t(11:14) chromosomal translocation was done to rule out MCL.

**Results:** All 13 (100%) cases of atypical CLL were positive for CD200 and negative for t (11:14).

**Conclusion:** CD200 is considered a useful marker in differentiating between atypical CLL and MCL. It is recommended to be included in the routine investigation of all CLL cases.

**Keywords:** CD200, chronic lymphocytic leukemia, immunophenotyping, mantle cell lymphoma.

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## Introduction

Chronic lymphocytic leukemia (CLL) is the most common form of adult leukemia in Western countries. The incidence increases with age with a median age at diagnosis of about 70 years.<sup>(1)</sup> CLL is small mature B-cell neoplasm that is characterized by proliferation of monoclonal B lymphocytes that co-express CD5 and CD23.<sup>(2)</sup> Most cases are found incidentally on routine blood analysis, but some patients develop lymphadenopathy, splenomegaly, anemia, or thrombocytopenia.<sup>(1)</sup> CLL diagnosis is based mainly on flow cytometry, which helps to differentiate it from other B-cell lymphoproliferative neoplasms in the majority of cases.<sup>(3)</sup> In some cases, there is an overlap between CLL and other mature B-cell neoplasms, mainly mantle cell lymphoma (MCL), which is cluster of differentiation (CD5)-positive as is CLL, but in contrast to CLL, MCL has an aggressive course with a different treatment protocol.

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In these overlapping cases, cytogenetic study for t(11;14) which is the hallmark of MCL, should be performed.<sup>(4)</sup> The CLL cases that do not express the classical immunophenotypic pattern are called atypical CLL; they usually show a more aggressive clinical course and may be misdiagnosed because of lack of a well-defined criteria in the World Health Organization (WHO) classifications.<sup>(5,6)</sup> The modified Matutes scoring system (Table I), which is based on the immunophenotypic analysis of five membrane markers (CD5, CD79b, CD23, FMC7, and surface immunoglobulin [sIg]), is used for differentiating CLL from other chronic B-cell neoplasms.<sup>(7)</sup> Typically CLL shows CD5+, CD23+, FMC7-, and weak expressions of CD79b and sIg.<sup>(1)</sup> By applying this scoring system, we can distinguish the majority of CLL cases; however, atypical CLL cases that present scores  $\leq 3$  are still difficult to distinguish from MCL.<sup>(4)</sup>

**Table I:** The Matutes chronic lymphocytic leukemia (CLL) scoring system

Marker	1 point	0 point
sIg	Weak	Strong
CD5	Positive	Negative
CD23	Positive	Negative
CD79b	Weak	Strong
FMC7	Negative	Positive

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Moreau et al.

CD200 or OX-2 is a transmembrane type Ia glycoprotein that belongs to the immunoglobulin superfamily. CD200 is expressed on dendritic cells, B-cells, and a subset of T-cells, and it plays a role in antitumor activity regulation.<sup>(8, 9)</sup> CD200 is positive in CLL and negative in MCL.<sup>(4,8)</sup> It has been studied as an additional marker for improving the scoring system, mainly with respect to atypical CLL.<sup>(10, 11)</sup>

The aim of our study was to investigate the pattern of CD200 positivity in atypical CLL in order to clarify its role in differentiating between overlapping cases.

## Materials

This is a retrospective study conducted on 90 CLL cases diagnosed by flow cytometry in Princess Iman Research and Laboratory Sciences Center from January 2018 to December 2019.

All of the study patients had lymphocytosis  $\geq 5 \times 10^3/\mu\text{L}$ . The cytomorphology on blood film for all cases showed small mature-looking lymphocytes with high nuclear: cytoplasmic ratio and many smudge cells, which was used to exclude other overlapping B-cell lymphoproliferative disorders, particularly in cases with atypical CLL as shown in figure 1.

Upon applying the Matutes scoring system, 77 cases had a score of 4 or 5 (out of a total of 5) and were labeled as typical CLL, while 13 cases had scores  $\leq 3$ , which were labeled as atypical CLL. Fluorescence *in situ* hybridization (FISH) for t (11; 14) was performed for the 13 atypical cases and revealed negative results.

Patient age range was 45–91 years. The median age was 68 years. The male: female (M: F) ratio was 2:1.

Ethical approval was obtained to conduct this study through the Royal Medical Services Ethical Committee.

## Methods

Peripheral blood and bone marrow specimens in ethylenediaminetetraacetic acid (EDTA) tubes were subject to red blood cell lysis by ammonium chloride and washed using phosphate-buffered saline (PBS). Samples were distributed into test tubes and incubated with fluorochrome-conjugated antibodies for 15 min avoiding light. An adopted large chronic lymphoproliferative disorder panel designed to characterize B-cell lymphomas in our department was applied to all samples. The antibody panel includes CD45 PerCP, CD5 PE, CD19 APC, CD20 APC, CD23 FITC, CD79b PE, CD10 PE, CD103 FITC, CD25 PE, CD11c APC, CD200 FITC, FMC7 FITC, sIg FITC, K FITC, and  $\lambda$  PE. All the antibodies were from Becton, Dickinson company (BD Biosciences) (San Jose, CA).

Immunophenotyping was performed using eight-color flow cytometer FACS-CANTO II (BD Biosciences) with acquisition target of 50,000 leukocyte events. The interpretation of the results regarding the intensity of CD200 expression was performed by quantification of the mean fluorescence intensity (MFI) and sorted into dim, moderate and bright expression in comparison with normal B-cells in the peripheral blood. Negative expression of CD200 was considered when the level of MFI was similar to the level of autofluorescence in a control tube with no conjugated antibody fluorescein isothiocyanate (FITC). Matutes scoring system was applied to all CLL cases to classify them into typical CLL with scores of 4 and 5 and atypical CLL with scores of 2 to 3. Atypical CLL cases underwent FISH study to look for t (11; 14) to differentiate it from MCL.

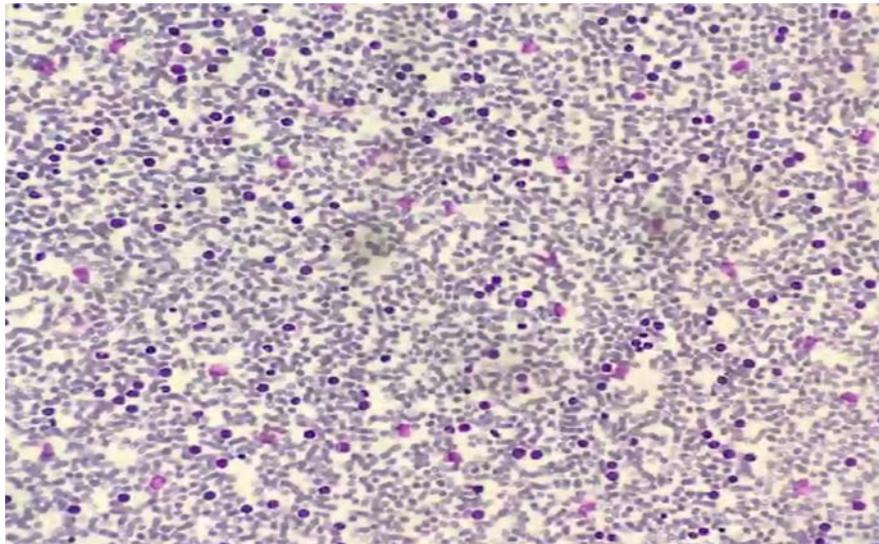
The features of all atypical CLL samples are shown in Table II.

**Table II:** Features of atypical CLL samples

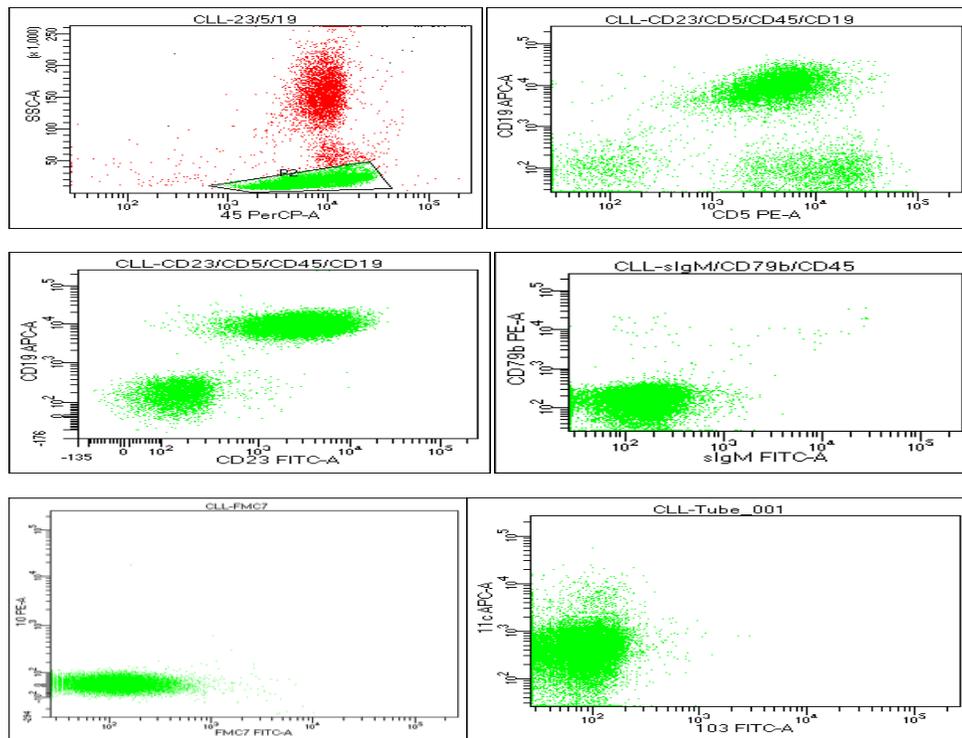
Sample No.	Age (years)	Gender	Sample site	White blood cell count $\times 10^3/\mu\text{L}$	Lymphocytes count $\times 10^3/\mu\text{L}$
1	45	F	PB	23.6	18.36
2	79	M	PB	36.67	25.77
3	60	M	BM	62	45
4	79	M	PB	16	9.3
5	83	M	PB	25.7	22.2
6	64	F	PB	29.3	23.6
7	56	F	PB	15.36	11.7
8	61	F	PB	16.71	10.34
9	83	M	PB	27	20.3
10	61	M	BM	28.5	21.5
11	74	M	PB	31.8	25.9
12	82	F	BM	17.5	13.2
13	72	M	PB	25.5	8.35

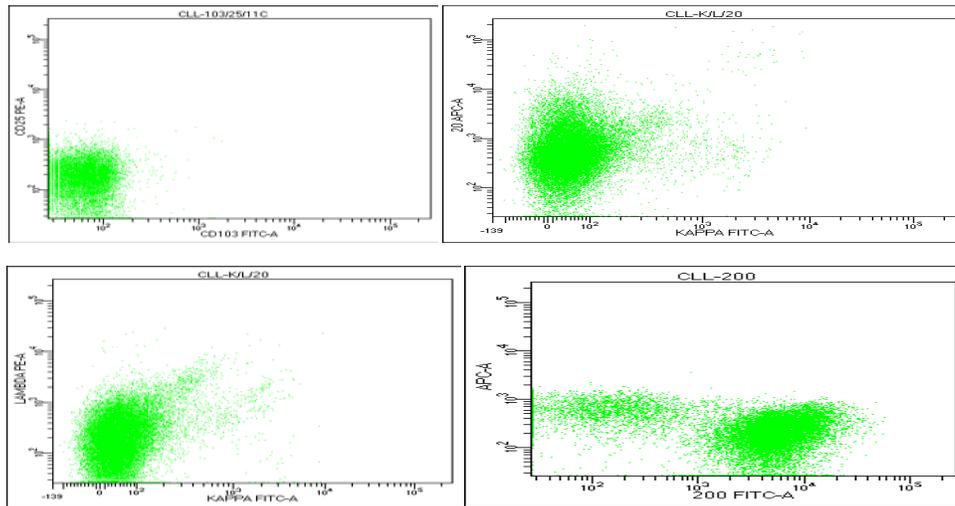
## Results

Using the Matutes scoring system, the cases were divided into two groups: (1) typical CLL with score  $> 3$ , which constitutes 85.5% (77 cases, 60 scores of 5/5, and 17 scores of 4/5) and (2) atypical CLL with score  $\leq 3$  constituting 14.4% (13 cases). Of the typical CLL who scored 4/5, one was CD23-negative, one was FMC7-positive, four were SIG-positive, and 11 were CD79b-positive. All of the cases showed CD19 expression. CD5 was negative only in one atypical CLL case and was labeled as such after exclusion of other lymphoproliferative neoplasms based on cytomorphologic correlation, immunophenotypic findings and FISH study to look for t(11;14) and t(14;18). CD23 was positive in 85 cases (94.4%). CD200 was positive in all CLL cases. We focused on atypical CLL cases with Matutes scores  $\leq 3$  for studying CD200 expression in term of positivity and intensity. These cases were all negative for t(11; 14), thus excluding MCL. All the 13 atypical CLL cases (100%) were positive for CD200 with moderate to high intensity of expression. The Matutes scores of all atypical CLL samples are outlined in (Table III). Representative dot plots of typical and atypical CLLs are shown in Figures 1 and 2, respectively.



**Figure 1:** Chronic lymphocytic leukemia morphology. As shown in the figure there is absolute lymphocytosis with the majority of lymphocytes are small mature-looking with many smudge cells.





**Figure 2:** Typical chronic lymphocytic leukemia (CLL) flow cytometry. The dot plots are showing the gate on lymphocytes population which show bright co-expression of CD5, CD19 and CD23, negative for sIg, CD79b, FMC7, CD103, CD25, and CD11c. CD20 show dim expression. CD200 is brightly positive.

**Table III:** Matutes score of atypical CLL samples

Sample No.	CD5	CD23	FMC7	CD79b	SIG	Matutes score
1	+	+	-	+	+	3
2	+	+	-	+	+	3
3	+	-	-	+	-	3
4	+	+	-	+	+	3
5	+	+	+	+	+	2
6	+	+	-	+	+	3
7	+	+	-	+	+	3
8	+	+	-	+	+	3
9	+	+	-	+	+	3
10	+	+	-	+	+	3
11	+	-	-	+	-	3
12	-	-	-	-	-	3
13	+	-	-	+	+	2

## Discussion

The differentiation between CLL and MCL is very important in overlapping cases as they are both CD5 positive mature B-cell lymphomas but present a major difference in prognosis. CLL has an indolent course in contrast to MCL which is aggressive clinically. The most important and reliable markers used for differentiating CLL from MCL are CD23 and FMC7.<sup>(3)</sup> The Matutes scoring system integrates five membrane markers (CD5, CD23, CD79b, sIg, and FMC7). This scoring system has been recognized as useful for differentiating between CLL and MCL; however, it does not afford 100% differentiation.<sup>(6)</sup>

Typically, the immunophenotypic profile of CLL presents a strong expression of both CD5 and CD23, weak expression of SIG and CD79b, and negative FMC7 (Figure 2).<sup>(6,12)</sup> Among the 90 CLL cases, 77 of these cases were immunophenotypically diagnosed as typical CLL, and 13 cases were labeled as atypical CLL. Deneys et al. studied 77 CLL cases and found results very similar to ours.<sup>(13)</sup> We found that CD5 is the most frequently expressed marker in typical and atypical CLL. It was negative in one case only. We found the same result in a previous study conducted in our center between 2011 and 2015 covering 214 cases.<sup>(14)</sup> CD5 is rarely present in lymphoproliferative neoplasms other than CLL and MCL, but it is not specific for these diseases, and it can be found in lymphoplasmacytic and marginal zone lymphomas that were excluded by cytomorphology.<sup>(4, 15)</sup> The second marker frequently expressed in CLL is CD23, and its positivity is the most representative feature of CLL. Although CD23 is mostly negative in MCL, it is not sufficient to discriminate between CLL and MCL.<sup>(16, 17)</sup> We found that CD23 was positive in 94.4% of cases (five cases were negative with one typical and four atypical CLL). This is in agreement with our previous study and with findings reported by Hulkkonen et al.<sup>(14,18)</sup> CD79b was found in 24.4% of our cases, which is close to the findings reported by Schelette et al. Eleven out of the 12 (91.6%) atypical CLL cases were CD79b positive; this positivity favors the possibility of its association with trisomy 12 and atypicality in CLL.<sup>(19, 20)</sup> In 1997, the Matutes scoring system was modified by Moreau et al. by replacing CD22 with CD79b because of the importance of CD79b in distinguishing CLL, which is mostly CD79b negative when compared with other B-cell neoplasms.<sup>(7)</sup> FMC7 is a powerful discriminator of CLL (which shows negative expression) from other B-cell lymphoproliferative disorders (LPD).<sup>(21)</sup> Atypical CLL shows a higher frequency of FMC7 expression.<sup>(22)</sup> We found that only three of all CLL cases have positive FMC7, one of which is atypical CLL. El-Sewefy reported negative FMC7 in all cases while other studies show higher expression percentages.<sup>(3, 13, 14, 21)</sup>

Surface immunoglobulin is usually weakly expressed in CLL, and it was found positive in 18% of our cases (10 out of which were atypical CLL).

This result was in agreement with what we have reported in Khasawneh et al. and much less than that reported by Geisler et al. and Lewis et al.<sup>(14, 23, 24)</sup>

We noticed that CD79b and SIG are the most frequently expressed markers in atypical CLL; they were the least significant markers in making the differential diagnosis between atypical CLL and MCL because they are expressed in both entities.

CD20 was expressed on all CLL cases but the expression was dim in typical CLL and brighter in atypical CLL. The same result was reported by Delgado et al.<sup>(21)</sup>

By integrating all previously mentioned markers using the Matutes scoring system, our scoring results were close to those reported by Ocier and Moreau et al., who found that 92% and 89.3% of CLL scored 4 and 5, respectively, while 8% and 10.7% scored 3 and <3, respectively. <sup>(7, 12)</sup>

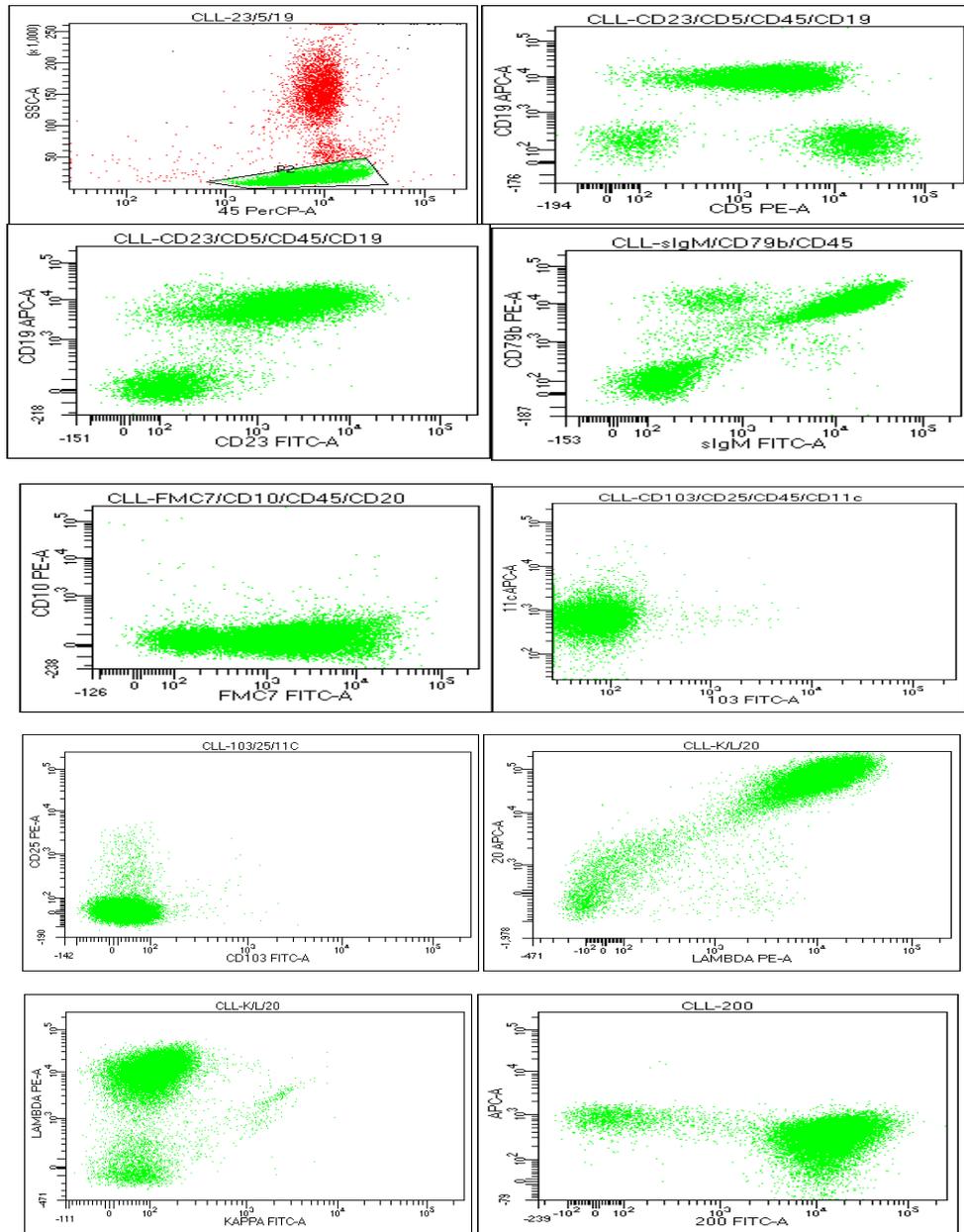
CD200 is normally expressed on B-cells in the peripheral blood and bone marrow. Among lymphoproliferative neoplasms, CLL shows moderate to strong expressions while hairy cell leukemia strongly expresses CD200, which was excluded from atypical CLL cases by its unique cytomorphology (hairy projections) and expression of CD103. Follicular, large B-cell, and marginal zone lymphomas show a range of expressions from moderate to dim, whereas MCL, prolymphocytic leukemia and Burkitt lymphomas show very dim or negative expressions.<sup>(7)</sup> CD200 assessment has been applied according to the EuroFlow and introduced in the World Health Organization (WHO) 2017 guidelines among the strongly positive markers in CLL.<sup>(1, 25)</sup> Many studies were conducted to assess the role of CD200 in differentiating between mature B-cell neoplasms by flow cytometry.<sup>(2-5, 8-11)</sup>

In our center, since 2018 when it was introduced, CD200 has been adopted in the chronic lymphoproliferative disorders panel.

In this study, all the CLL cases showed expression of CD200 with same pattern in typical and atypical CLL. In comparison to other studies Ting et al. Sandes et al. and Lesesve et al. reported 100% expression of CD200 in atypical CLL.<sup>(4, 5, 26)</sup> Regarding the intensity of expression of CD200, all of the studied CLL cases showed moderate to high intensity, which is in agreement with many other previous studies.<sup>(3, 4, 5, 26, 27)</sup> Therefore inclusion of CD200 in the CLL panel could be very helpful in differentiating CLL from other mature B-cell neoplasms. Additionally, CD200 could be used as a targeted therapy with the anti-CD200 antibody.

Some studies suggested modification of the Matutes scoring system by adding CD200 to the system in order to increase the specificity and sensitivity of CLL diagnosis. D'Arena et al. reported that substitution of FMC7 with CD200 increased the specificity and sensitivity of the Matutes scoring system.<sup>(28)</sup> Köhnke et al. omitted SIG because of its low capability of distinguishing CLL from non-CLL and replacing it with CD200.<sup>(2)</sup> The resulting modified score showed an improvement in specificity with high sensitivity in comparison to the modified Matutes score.<sup>(28)</sup> Mora et al. reported that both addition of CD200 to the scoring system and substitution of SIG by CD200 yielded an increase in the accuracy of the scoring system.<sup>(29)</sup>

CD200 is considered a useful marker in differentiating between atypical CLL and MCL. We recommend inclusion of CD200 in the routine CLL investigations.



**Figure 3:** Atypical chronic lymphocytic leukemia flow cytometry. The dot plots are showing the gate on lymphocytes population which show bright co-expression of CD19, CD5 and CD23, negative for CD103, CD25, and CD11c but positive for FMC7, CD79b and sIg . CD20 show bright expression unlike typical CLL. CD200 is brightly positive.

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