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ORIGINAL ARTICLE

Immunophenotyping of Chronic Lymphocytic Leukemia

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SUMMARY

Background: **Chronic Lymphocytic Leukemia (CLL) is one of the most common diagnoses made by flow cytometry laboratories. There is no consensus on which markers need to be used in flow cytometry for accurate immunophenotyping. Herein, we investigated the role of markers used in flow cytometry in the distinction between CLL and MCL.**

Methods: **A total 339 recently diagnosed B lymphoproliferative patient cases were retrospectively studied for their immunophenotypical propoerties using flow cytometry. They included 306 CCL cases and 33 MCL cases.**

Results: **The positivity of CD23 was diagnostic for CLL ($p < 0.001$). CD22, CD79b, and FMC7 expressions were highly positive in CLL cases, but not statistically significant in making differential diagnoses between atypical CLL and MCL ($p = 1.000$, $p = 0.431$ and $p = 1.000$, respectively). Evaluation of CD11c, CD25, CD43, and CD38 expressions, which are included in the LPD panel but not in the matatutes scoring, revealed that CD11c, CD38, and CD43 expressions are statistically significant in the distinction of atypical CLL from MCL ($p < 0.001$, $p < 0.001$, and $p < 0.001$).**

Conclusions: **We can say that CD11c, CD38, and CD43, which have been included in our lymphoproliferative disease panel, were more valuable than CD22, CD79b, and FMC7 in the diagnosis of CLL.**

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KEY WORDS

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INTRODUCTION

The WHO classification of hematolymphoid system neoplasms is based on clinical, morphological, immunophenotypic, and genetic features. Mature B cell lymphoproliferative diseases (LPD) account for more than 80% of hematolymphoid neoplasms [1]. Chronic Lymphocytic Leukemia (CLL) is the most frequent type of LPD [1,2]. Genetics has no role in the diagnosis of CLL although there are numerous genetic abnormalities. Presence of persistent clonal B lymphocytosis (> 5 x 10^9/L) for more than 3 months is needed to make the diagnosis of CLL. It has characteristic morphological features as well as immunophenotypic feature in flow cytometry [1,4]. These include CD5+CD19+, CD23+, weak surface membrane immunoglobulins (sIg), and absence or low expression of CD79b and FMC7 [3,4]. Immunophenotyping has a major role in the diagnosis

of CLL. However, CLL is a quite heterogeneous disease (both morphologically and immunophenotypically), which makes the diagnosis difficult [3-7]. Therefore, a scoring system for the diagnosis of CLL was first defined in 1994 by Matatutes et al. [8]. This scoring system consists of five parameters; CD5, CD22, CD23, FMC7, and sIg. In 1997, Moreau et al. replaced CD22 with CD79b (9). Accordingly, a score of 4 - 5 indicates typical CLL and a score of 3 indicates atypical CLL, whereas 0 - 2 excludes CLL [8,9]. Patients who are classified as atypical CLL have atypical immunophenotypic features (CD5-, CD23-, FMC7+, CD79b+, strong sIg). Atypical CLL is most frequently confused with Mantle Cell Lymphoma (MCL), which expresses CD5 together with CD19 similar to CLL [4,10-14]. Generally, MCL is more aggressive and requires a different therapeutic approach, therefore differential diagnosis between these two diseases should be made precisely. Histochemical or molecular tests [cyclin D1, t(11;14)] can be used for differential diagnosis [4,12]. Molecular tests are not easily available, are time consuming, and more expensive. For this reason, reliable, additional new markers are being investigated in case the Matatutes score is inadequate. There is no consensus on which markers need to be used in flow cytometry for the diagnosis of CLL. Herein, we investigated the role of markers, which are included in our LPD panel in flow cytometry but not in the Matatutes scoring system, in the differential diagnosis between CLL and MCL.

MATERIALS AND METHODS

Patients and samples

This study retrospectively reviewed the medical records of 339 patients (100 females, 239 males) aged 31 - 87 years who were diagnosed with CLL (306 cases: 89 female, 217 male mean age 68.0 ± 10.4) and MCL (33 cases: 11 female, 22 male mean age 65 ± 10.1) at Ankara Numune Training and Research Hospital, Hematology Clinic between January 2009 and December 2015 according to the WHO criteria [18]. All patients had full blood counts performed, and peripheral blood or bone marrow smears were made for morphological assessment under May-Grunwald-Giemsa staining. Peripheral blood or bone marrow was evacuated into a 4 mL K3EDTA tube (BD Vacutainer®, CA, USA) as an anticoagulant and we processed within 2 hours after collection. Diagnosis of MCL was confirmed by immunohistochemical detection of cyclin D1 in bone marrow biopsies or detection of t(11:14) by FISH.

Flow cytometry immunophenotyping

For flow cytometric study, fresh peripheral blood/bone marrow samples were drawn into 4 mL K3 EDTA containing tubes (BD Vacutainer®, CA, USA), and studied without delay within the same working day. Cells in suspension (2 x 10^6 cells in 50 - 100 µL per tube) from the peripheral blood/bone marrow samples were stained with monoclonal antibodies (MoAb) directed against cell surface markers using a stain-lyse-and-then-wash direct immunofluorescence technique, as previously described [17]. The MoAb used for labeling in flow cytometry were obtained from Beckman Coulter, USA: CD45 APC, CD79b ECD, CD3PE, CD5PC5,CD10 ECD, FITC, CD11c FITC, CD19 PE, CD19 ECD, CD20 ECD, CD22 PC5, CD23 FITC, CD25 APC, CD38 PC5, CD103 PE, FMC7 FITC, CD43 APC, Kappa FITC, and Lambda PE. The following panel of 4 - 5 color combinations of MoAbs Fluorescein Isothiocyanate (FITC), Phycoerythrin (PE), Phycoerythrin-Cyanin 5 (PC5), Phycoerythrin-Texas Red (ECD), Allophycocyanin (APC) was used in all cases. Data acquisition was done immediately after completion of sample staining, using a Beckman Coulter (BC) flow cytometer (Navios, BC, USA) and the Kalusa software (BC, USA). For each sample data from at least 10 x 10^4 events per tube were acquired. Briefly CD19+ B cells were selected (at least 2000 events according to the threshold of the isotypic control). Data file using conventional gating strategies (forward and side light scatter and the pattern of CD19 expression). A cutoff limit of 30% of lymphoid cells was used to denote a positive result with a given antibody using computer software Kalusa (BC). The 30% cutoff was selected as recommended by the British Committee for Standards in Haematology (BCSH) guideline [2]. Matatutes scoring was defined as ≥ 30% cell surface expression. Instrument alignment was confirmed daily using an alignment control bead (Flow-Check, Beckman Coulter). Instrument sensitivity was confirmed daily using a fluorescence standard bead (Flow-Set, Beckman Coulter). Sensitivity was maintained within 1%. The accuracy and precision of cell count were tested using international quality controls purchased from United Kingdom National External Quality Assessment Scheme (UK NEQASLI, Sheffield, UK) (z score range -2.0 - 2.0).

The diagnosis of LPD was established according to the WHO classification based on clinical data and on morphologic, immunophenotypic, and genetic criteria. The revised Matatutes score system [9], based on the immunophenotypic analysis of five membrane markers (CD5, CD23, FMC7, surface Ig, CD79b) was employed to classify all cases, this comprises 1 point each for expression of CD5, CD23, and dim sIg, and lack of expression of CD79b and FMC7 (Figure 1). Typical CLL cases were defined by a score ≥ 4: and atypical cases were identified by a score < 4 or lack of CD23. Diagnosis of MCL was confirmed by immunohistochemical detection of cyclin D1 in bone marrow biopsies or detection of t(11:14) by FISH. The study was approved by the local Ethical Committee of the Ankara Numune Training and Research Hospital (Ankara, Turkey Ethics committee number is 2015-20796219). Bone marrow sample is required for the accurate CLL diagnosis. In order to evaluate the degree of progress of CLL in bone marrow and to confirm the diagnosis of MCL by cyclin D1, we performed bone marrow aspiration.

Table 1. Demographic features.

Variables	n = 339
Age (years)	68.0 ± 10.4 (45 - 89)
Gender	
Female %	100 (29.4%)
Male % CLL MCL	239 (70.6%) 306 (90.26%) 33 (9.74%)
White blood cell x 10	19.8 (8 - 274)
Lymphocyte x 10^3	17.3 (6,8 - 240)
Hemoglobin, g/dL	12,7 ± 2,27
PLT x 10^3	202 (19 - 403)

Table 2. Distribution of cases by marker positivity in the differential diagnosis of MCL and atypical CLL score ≤ 3, CLL score ≥ 4.

	MCL (n = 33)	Atypical CLL score ≤ 3 (n = 185) 60%	p	CLL score ≥ 4 (n = 121) 40%	p
CD20	31 (93.9%)	184 (99.5%)	0.061 *	113 (93.4%)	1.000
CD22	32 (97.0%)	177 (96.2%)	1.000 *	90 (74.4%)	1.000 *
CD23	6 (21.2%)	177 (97.0 %)	≤ 0.001 *	118 (96.7%)	≤ 0.001 #
CD79b	25 (75.8%)	151 (81.6%)	0.431 #	47 (38.8%)	≤ 0.001 #
CD25	10 (30.3%)	111 (60.0%)	0.002 *	77 (63.6%)	0.002 #
CD38	31 (93.9%)	46 (24.9%)	≤ 0.001 *	29 (24%)	≤ 0.001 #
CD43	13 (39.4%)	177 (95.7%)	≤ 0.001 *	119 (98.3%)	≤ 0.001 #
CD11C	11 (33.3%)	116 (62.7%)	0.002 #	87 (71.9%)	≤ 0.001 #
FMC7	32 (97%)	180 (97.3 %)	1.000 *	51 (42.1%)	≤ 0.001 #

* - Fisher's exact test, # - Pearson's Chi-square test, MCL - Mantle Cell lymphoma, CLL - Chronic Lymphocytic leukemia.

Statistical analysis

Data analysis was performed using SPSS for Windows, version 15 (SPSS Inc., Chicago, IL, USA). Descriptive statistics were shown as number of cases and percentages. Categorical data were analyzed by Pearson's Chi-square or Fisher's exact test, where appropriate. A p-value less than 0.05 was considered statistically significant.

RESULTS

The evaluation of 339 patients (mean age 68 ± 10.4 years) revealed that median PB lymphocyte count at diagnosis was 19.8 x 10^9 lymphocytes/L (range: 0.8 - 274 x 10^9 lymphocytes/L). Of the patients, 306 (90.3%) had CLL and 33 (9.7%) had MCL. Immunophenotypic scoring of CLL patients was done according to the Matatutes scoring system; the score was ≥ 4 in 121 (40%) patients and 3 in 185 (60%) patients (Table 1). Regarding CD22, CD79b, FMC7, and CD23 expressions, which are found in the Matatutes score, CD23 expression was negative in 11 CLL (3 typical CLL and 8 atypical CLL) patients, whereas it was positive in 21.2% of MCL cases. The positivity of CD23 was diagnostic for CLL ($p < 0.001$), but lack of CD23 does not exclude CLL. CD22, CD79b, and FMC7 expressions were highly positive in CLL cases (74%, 38.8% and 42%, respectively) (Table 2), but not statistically significant in making a differential diagnosis between atypical CLL and MCL ($p = 1.000$, $p = 0.431$, and $p = 1.000$, respectively). Evaluation of CD11c, CD25, CD43, and CD38 expressions, which are included in the LPD panel but not in the Matatutes scoring (Table 2), revealed that CD11c,

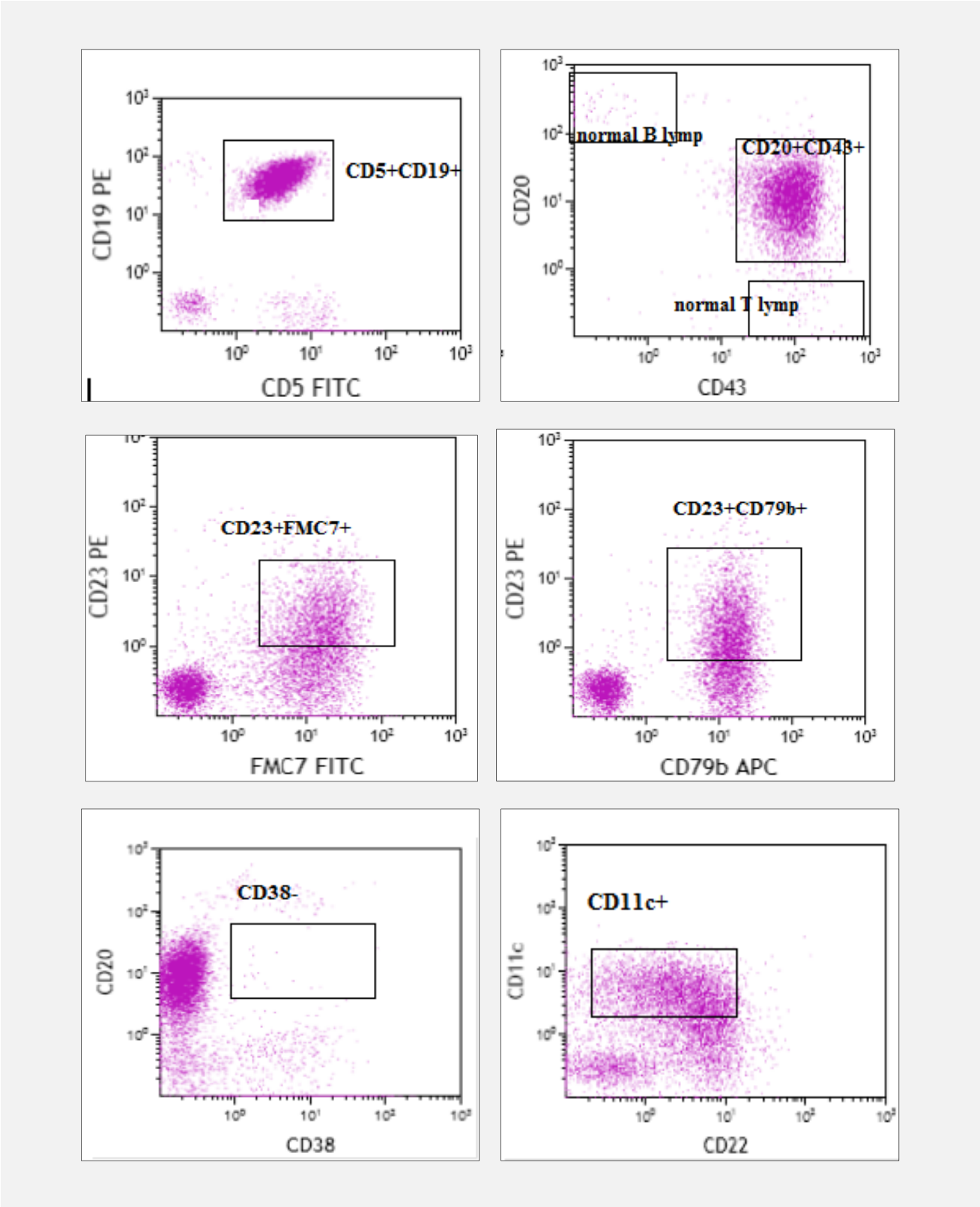

Figure 1. Representative flow cytometric histograms gated on lymphocytes of atypical chronic lymphocytic leukemia (CLL) atypical CLL cases showing CD5+ CD19+, CD23dim+, CD20 dim+, FMC7 and CD79b mod+, CD43+, CD38-.

CD25, CD38, and CD43 expressions are statistically significant in the distinction of atypical CLL from MCL ($p < 0.001$, $p = 0.002$, $p < 0.001$, and $p < 0.001$, respectively).

DISCUSSION

In general, making the diagnosis of CLL is easy in the presence of characteristic immunophenotypic features (CD5+ CD19+ dual positive, CD23+, CD22-/low, CD79b-/low, sIg low, FMC7-, CD20 low). However, it is difficult both to diagnose and to distinguish from MCL in cases where immunophenotypic features are not typical. We found CD11c, CD25, CD38, and CD43, which have been included in our LPD panel but not in the Matatutes scoring system, to be significant in making the differential diagnosis.

CLL and MCL are B-cell LPD and express CD5+ CD19+. MCL usually has a more aggressive clinical course and requires a different therapeutic approach. Immunophenotyping by flow-cytometry is beneficial in the distinction of CLL from MCL and is a frequently used method [3,4,15]. Problems arise when dealing with atypical immunophenotypes including some of the Matatutes score 3 cases. It may be difficult to distinguish some MCL cases in particular from atypical CLL. CD23 positivity is the most characteristic feature of CLL [10,11]. Earlier studies have reported that CD23- is a reliable marker in the distinction between CLL and MCL [28,29]. In the present study, while 2.1% of typical CLL cases were CD23-, 21.2% of the t(11:14)+ MCL cases were CD23+. According to the results of the present study, CD23 alone is not sufficient to make a differential diagnosis between CLL and MCL [12,13, 16]. FMC7, which is an epitope of CD20, has been expressed in 47% of the present CLL cases, whereas CD22 and CD79b have been expressed in 42% and 52% of the cases, respectively. These are high rates for CLL. Normally, CD22, CD79b, and FMC7 expressions are high in MCL; in the present study, however, they were high in both CLL and MCL cases. For this reason, the majority of the present cases (60%) have been classified as atypical CLL when Matatutes scoring was used. Earlier studies stated that FMC7, CD79b, and CD22 are not sufficient to make a differential diagnosis [10,17,28]. At this point, the present study has a limitation; we could obtain different results if we had used MoAbs of different clones from different manufacturers.

With regard to CD11c, CD25, CD38, and CD43, which have not been included in the scoring system, CD11c and CD25 expressions show heterogeneous distribution in both disease groups with higher expression in CLL as compared to MCL ($p < 0.001$ and $p = 0.002$, respectively) [10,18,19]. There is no agreement concerning CD11c and CD25 expressions. Such different results between the studies can be partially explained by individual differences between the patients as well as absence of specific techniques (staining, sample preparation, analysis, interpretation, etc.) and procedures in flow cytometry. We observed that CD38 expression, which in fact is considered a prognostic factor for CLL, is higher in MCL ($p < 0.001$) but heterogeneous in CLL [10,21,22]; thus, it is difficult to standardize. In addition, the disease may have a fluctuating course [20,23]. These factors need to be taken into account while evaluating.

CD43 expression was first defined in 1999 by Harris et al. for the classification of malignant lymphomas [24]. In the present study, it was higher in CLL as compared to MCL and we observed that it is quite effective in accurate classification particularly in the cases with a score of 3 according to the classical classification ($p < 0.001$) [25-27].

One of the restrictions in our study is the inclusion of only cyclin D1 and t(11:14) positive cases among the MCL patients. However it would be possible that some MCL cases have negative values of the above. If we could have studied SOX11 as an immunohistochemical in MCL cases, our MCL case number would have been higher.

CONCLUSION

There is no single marker yet to make a definite diagnosis of CLL by flow-cytometry. Therefore, new markers for differential diagnosis are under investigation. According to the results of the present study, we can say that CD25, CD38, and CD43 in particular are more valuable markers than CD22, CD79b and FMC7, which are within the scope of Matatutes scoring. They can be considered as definitive markers in the atypical CLL cases where the scoring system remains incapable. However, as in the other markers, heterogeneous distribution and different rates of expression may be in question for these markers. For this reason, harmonization studies need to be conducted in larger and various patient groups by defining standardized sample preparation and staining, as well as specific techniques. We think that identification of a new scoring system following these studies would be beneficial.

Declaration of Interest:
The authors declared no conflict of interest.

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