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Evaluation of CD307a Expression Patterns during Normal B-Cell Maturation and in B-Cell Malignancies by Flow Cytometry

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Background: Flow cytometric immunophenotyping is deemed a fundamental tool for the diagnosis of B-cell neoplasms. Currently, the investigation of novel immunophenotypic markers has gained importance, as they can assist in the precise subclassification of B-cell malignancies by flow cytometry. Therefore, the purpose of the present study was to evaluate the expression of CD307a during normal B-cell maturation and in B-cell malignancies as well as to investigate its potential role in the differential diagnosis of these entities.

Methods: CD307a expression was assessed by flow cytometry in normal precursor and mature B cells and in 115 samples collected from patients diagnosed with precursor and mature B-cell neoplasms. CD307a expression was compared between neoplastic and normal B cells.

Results: B-acute lymphoblastic leukemia cases exhibited minimal expression of CD307a, displaying a similar expression pattern to that of normal B-cell precursors. Mantle cell lymphoma (MCL) cases showed the lowest levels of CD307a among mature B-cell neoplasms. CD307a expression was statistically lower in MCL cases than in chronic B lymphocytic leukemia (CLL) and marginal zone lymphoma (MZL) cases. No statistical differences were observed between CD307a expression in neoplastic and normal plasma cells.

Conclusion: These results indicate that the assessment of CD307a expression by flow cytometry could be helpful to distinguish CLL from MCL, and the latter from MZL. Although these results are not entirely conclusive, they provide a basis for further studies in a larger cohort of patients. © 2018 International Clinical Cytometry Society

Key terms: CD307a; B-cell maturation; B-cell malignancies; flow cytometry; immunophenotyping

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Additional supporting information may be found online in the Supporting Information section at the end of the article.

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INTRODUCTION

The use of flow cytometric immunophenotyping (FCI) in the study of B-cell lymphoid neoplasms is of utmost importance. This technique assists in the diagnosis and characterization of B-acute lymphoblastic leukemia (B-ALL), lymphomas, and plasma cell disorders (precursor and mature B-cell malignancies). FCI enables the simultaneous evaluation of several antigens, which increases the sensitivity and specificity of immunophenotypic classification (1). This method provides a detailed immunophenotypic characterization of cell populations, allowing the comparison of the obtained information with specific phenotypic profiles of B-cell malignancies defined by the WHO classification (2). Currently, diagnosis of B-cell malignancies is based on WHO criteria, which combine immunophenotypic, morphological, clinical, molecular, and cytogenetic information for the correct subclassification of these disorders. In the study of mature B-cell neoplasms (MBCN), FCI has proved useful as a sole tool to distinguish most MBCN entities (3), but in some cases, due to immunophenotypic overlap, the correct diagnosis cannot be determined. Histopathological analysis is necessary in such instances, as it provides key diagnostic information. Moreover, ancillary exams such as cytogenetic and molecular studies can be used to establish a definite diagnosis (4). Additional investigations may be unfeasible due to economic limitations or the absence of accessible pathological tissue. In such cases, the investigation of novel immunophenotypic markers, which has been the subject of previous studies (5–7), takes on importance, as it might provide more precise panels to improve the diagnostic accuracy of B-cell lymphomas by flow cytometry.

Antigen CD307a, also known as FcRL1, IRTA 5, and FcRH1, is a promising marker in this context. This antigen belongs to the Fc receptor-like (FcRL) family, whose genes are located at the 1q21–23 chromosome locus (8). The CD307a receptor is exclusively expressed in B cells (9) and in some MBCN (10). Therefore, the objective of this study was to investigate the expression of CD307a during normal B-cell differentiation and in B-cell lymphoid malignancies by flow cytometry to determine if this marker has a consistent differential expression among these diseases, with emphasis on MBCN.

PATIENTS, MATERIALS, AND METHODS

To assess the expression of CD307a in normal mature B-lymphocytes, peripheral blood (PB) samples collected from ten (5 male and 5 female) healthy subjects with a median age of 29 years (range 21–59 years) were included in the study. Additionally, seven bone marrow (BM) aspirates obtained from subjects (4 male and 3 female) with no evidence of hematological diseases were analyzed (median age of 23 years; range 2–78 years) to assess CD307a expression in normal BM B-cell progenitors. Samples obtained for diagnostic and follow-up purposes from 115 patients were included in the study and comprised 52 BM aspirates, 44 PB samples, 15 lymph node and other tissue biopsies, three lymph node aspirates, and one ascitic fluid sample. All samples were collected at the Clinical Analysis Division, University Hospital of the Federal University of Santa Catarina or at the Hematology and Hemotherapy Center of Santa Catarina after subjects and patients had signed an informed consent form, according to the local Ethics Committee (CEPSH-UFSC 1.691.983/2016).

The distribution of patients according to the WHO 2008 classification and its 2016 revisions (2,11) was as follows: B-cell chronic lymphocytic leukemia (CLL; $n = 39$), mantle cell lymphoma (MCL; $n = 9$), hairy cell leukemia (HCL; $n = 6$), Burkitt lymphoma (BL; $n = 4$), follicular lymphoma (FL; $n = 11$), diffuse large B-cell lymphoma CD10+ (DLBCL; $n = 4$), DLBCL CD10− ($n = 5$), marginal zone lymphoma (MZL; $n = 3$), lymphoplasmacytic lymphoma (LPL; $n = 4$), unclassified CD5−/CD10− B-cell lymphomas (BCL; $n = 7$), B-acute lymphoblastic leukemia (B-ALL; $n = 13$), and plasma cell neoplasms (PCN; $n = 10$). Of the PCN cases, seven were diagnosed as plasma cell myeloma (PCM) and one as plasma cell leukemia (PCL), whereas two cases had >5% of residual normal plasma cells (PC) within the plasmacytic compartment, suggesting a Monoclonal Gammopathy of Undetermined Significance (MGUS) diagnosis (12). One CLL case showed plasmacytic differentiation and the presence of serum monoclonal IgM.

MCL diagnosis was established by using the immunophenotypic algorithm described by Sandes et al. (6), detection of cyclin D1 in tissue biopsies, and/or detection of t(11;14) by PCR (13). Diagnosis of the other MBCN entities was confirmed by histopathological and immunohistochemistry analyses. Molecular studies were performed in some cases: two FL cases were confirmed by the presence of t(14;18) by PCR (Identiclone™ BCL2/JH translocation assay, Invivoscribe), and BL diagnosis was confirmed by detection of t(8;14) by FISH (IGH/MYC Translocation Dual Fusion Probe, Cytocell) in three of the four cases analyzed. The unclassified CD5−/CD10− BCL cases corresponded to chronic B-cell lymphoproliferative disorders in leukemic phase with no lymph node or tissue biopsy available for histopathological evaluation. According to a previous report (14), these cases are most likely LPL or MZL. Nevertheless, due to the limited data available, we cannot exclude the possibility of other MBCN entities.

Immunophenotypic Analysis

For immunophenotypic diagnosis and classification of B-cell malignancies, samples were assessed using EuroFlow antibody panels as part of local routine procedures (3) (Supporting Information Table S1). In addition, a four-color tube containing CD45 pacific orange (PacO), CD3 pacific blue (PacB), CD307a phycoerythrin (PE), and CD19 phycoerythrin/Cy7 (PE-Cy7) was included in the study (Supporting Information Table S2, Tube 1). For PCN cases, where it was necessary to assess PC, CD38 allophycocyanin/Cyanine tandem 7 (APC-H7) was added to the tube (Supporting Information Table S2, Tube 2). To evaluate CD307a expression in normal B-cell precursors, a different antibody combination was

Table 1
Technical Information on Reagents

Marker	Fluorochrome	Clone	Source	Catalogue Number	μL/test
CD3	APC	UCHT1	BD Biosciences	555335	5
CD3	PacB	UCHT1	BD Biosciences	558117	1
CD4	PacB	MEM-241	Exbio	PB-359-T100	1
CD5	PerCPCy5.5	L17F12	BD Biosciences	341089	7
CD8	FITC	B9.11	Beckman Coulter	A07756	5
CD10	PE	ALB1	Beckman Coulter	IM1915U	10
CD10	APC	HI10a	BD Biosciences	340923	3
CD11c	PerCPCy5.5	3.9	Biolegend	301624	3
CD19	PECy7	J3–119	Beckman Coulter	IM3628U	3
CD20	PacB	B9E9 (HRC20)	Beckman Coulter	IM3607U	3
CD22	PerCPCy5.5	HIB22	BD Biosciences	555425	5
CD23	FITC	9P25	Beckman Coulter	IM0529U	7
CD27	APC	LT27	Exbio	1A-308-T100	5
CD31	FITC	WM59	BD Biosciences	555445	10
CD34	PerCPCy5.5	8G12	BD Biosciences	347203	5
CD38	APCH7	HB7	BD Biosciences	653314	3
CD39	PE	TÜ66	BD Biosciences	555464	10
CD43	APCH7	IG10	BD Biosciences	646787	5
CD45	PacO	HI30	Exbio	PO-684	1
CD49d	APCH7	9F10	BD Biosciences	646788	2
CD56	PE	N901 (NKH-1)	Beckman Coulter	A07788	5
CD62L	FITC	LT-TD180	Exbio	1F-449-T100	7
CD79b	PerCPCy5.5	3A2-2E7	BD Biosciences	656644	5
CD81	APCH7	JS-81	BD Biosciences	646791	5
CD95	PE	DX2	BD Biosciences	555674	20
CD103	FITC	Ber-ACT8	BD Biosciences	550259	7
CD185	APC	51505	R&D Systems	FAB190A	10
CD200	APC	MRC OX-104	BD Biosciences	655406	5
CD305	PE	DX26	BD Biosciences	550811	10
CD307a	PE	282415	R&D Systems	FAB2049P	5
HLA-DR	PerCPCy5.5	L243	BD Biosciences	347364	5
Igκ	PE	G20–193	BD Biosciences	555792	15
Igλ	FITC	JDC-12	BD Biosciences	555796	7
IgM	APC	G20–127	BD Biosciences	551062	10

PacB: pacific blue; APC: allophycocyanin; PE-Cy7: phycoerythrin/Cy7; PerCP-Cy5.5: peridinin chlorophyll protein; APC-H7: allophycocyanin/cyanine tandem 7; PacO: pacific orange; PE: phycoerythrin; FITC: fluorescein isothiocyanate. Ig: immunoglobulin.

used: CD45 PacO, CD20 PacB, CD307a PE, CD34 peridinin chlorophyll protein (PerCP-Cy5.5), CD19 PE-Cy7, CD10 allophycocyanin (APC), and CD38 APC-H7 (Supporting Information Table S2, Tube 3). Technical information on the employed reagents is shown in Table 1. FMO controls were used for validating the new panels developed for this study (15,16).

Staining of surface markers followed by erythrocyte lysis was performed. Leukocyte concentration was adjusted to a maximum of 10×10^6 cells/mL. Subsequently, a 100 μL aliquot from each specimen was incubated with appropriate amounts of antibodies (previously titrated) for 15 minutes in the dark at room temperature. Then, 2 mL of lyse buffer (BD FACS™ Lysing solution) was added, and after 15 minutes of incubation in the dark at room temperature, samples were centrifuged for 5 minutes at 0.6 rcf. The pellet was resuspended in 500 μL of PBS (phosphate buffered saline) solution. Immediately after sample preparation, a minimum of 50,000 events was acquired using a FACSCanto II flow cytometer with FACSDiva™ software (BD Biosciences). Data were analyzed using Infinicyt™ software version 1.7 (Cytognos SL, Salamanca, Spain).

For all samples, B cells were gated based on CD19 expression. Normal and neoplastic PC were identified by their high CD38 expression. Whenever identified, residual normal B cells were excluded from analysis. In control BM specimens, three populations of normal B-cell precursors were recognized (pre-B I, pre-B II, and immature B cells), as previously reported (17,18). Then, the mean fluorescence intensity (MFI) values of the CD307a marker for each sample and for each specific cell population under analysis were obtained. For each case, an unstained control (with no PE-conjugated antibody) was used to determine a possible autofluorescence interference (19) and the staining index (SI) was calculated to normalize signal over background fluorescence (20). Then, the median SI value of each group (same neoplasm and cell population) was calculated.

Intensity of expression was categorized as dim, moderate, or bright by comparison with an internal negative control (T cells) (21). Dim intensity was defined as a slightly increased intensity when compared to the negative control. Moderate intensity was defined as within the second and third log decades of fluorescence, having no overlap with the negative population. Bright

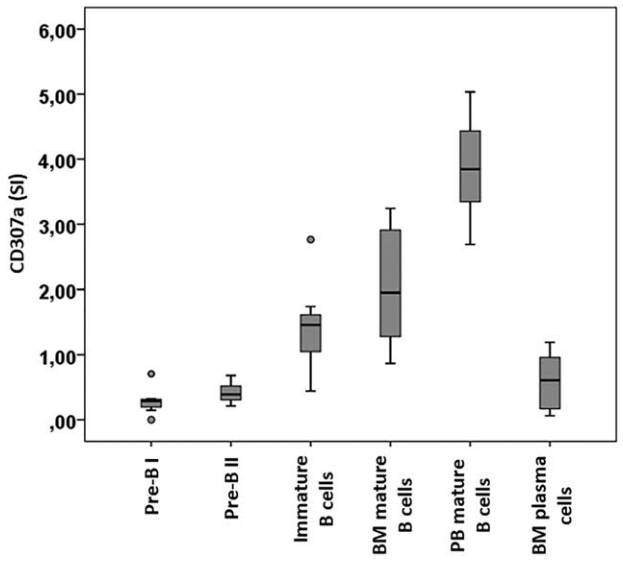

FIG. 1. CD307a expression in normal B-cell subsets. Box plots illustrate the staining index (SI) of CD307a in a linear scale. Notched boxes represent 25th and 75th percentile values; the line in the middle corresponds to the median; and the vertical lines, to the 10th and the 90th percentiles. Circles represent outliers.

intensity was defined as above the third log decade. Expression was considered negative when cells exhibited staining characteristics with virtual complete overlap with the internal negative reference cell population.

Data files corresponding to each individual sample were merged using the Infinicyt™ software to evaluate and compare CD307a expression among B-cell malignancies.

Statistical Analysis

Comparison of CD307a expression (SI values) among groups was performed using the Kruskal–Wallis test with the level of significance set at $P < 0.05$. When a P values of 0.05 or less was found in the Kruskal–Wallis test, the Dunn post-test was applied to analyze pairs of groups. SPSS software version 22 was used for statistical analysis.

RESULTS

CD307a Expression in Normal B-Cell Subsets

An increase in CD307a expression was observed during normal B-cell development, varying from scarcely detectable amounts of this antigen in the earliest B-cell precursor stage (pre-B I population) to moderate expression in mature B-cell populations (Fig. 1). PC showed a downregulation of CD307a and had statistically lower levels of this antigen than mature PB B cells ($P = 0.001$). Although SI values were higher in PB than in BM mature B-lymphocytes, no statistical differences were detected between these groups ($P = 0.148$).

CD307a Expression in Neoplastic B Cells According to Maturation Stage

CD307a was minimally expressed in precursor B-ALL cells. B-ALL cases were uniformly dim to negative, accounting for 85% ($n = 11$) of the negative cases (Table 2). A statistical difference ($P = 0.001$) in CD307a expression was observed between B-ALL cells and normal immature B cells (maturation stage 3) (Fig. 2A).

As a detailed subsetting of normal mature B cells was not available, comparison of CD307a expression between maturation stages and their neoplastic counterpart was not performed. However, it was possible to assess the differential expression of CD307a within MBCN subcategories. Thus, CD307a expression was compared among various subtypes of MBCN using the Kruskal–Wallis test. Statistical differences in SI values were observed between CLL and MCL ($P = 0.010$) (Fig. 2B). Expression of CD307a in CLL was heterogeneous from case to case, whereas expression of this

Table 2
Number of Cases According to the Expression Pattern of CD307a

Disease	Total number of cases	CD307a expression pattern				Median SI	SI range
		Neg (–)	Dim (+)	Mod (++)	Bright (+++)		
CLL	38	3	16	16	3	2.55	0.14–10.93
MCL	9	6	3	0	0	0.64	0.00–1.39
HCL	6	0	4	2	0	1.96	1.18–3.59
BL	4	1	3	0	0	1.46	0.19–2.75
FL	11	3	3	4	1	2.23	0.03–20.10
DLBCL CD10+	4	2	0	2	0	0.68	0.54–2.63
DLBCL CD10−	5	3	0	2	0	1.13	0.18–3.00
LPL	4	2	1	0	1	1.14	0.09–7.10
BCL CD5−CD10−	7	0	1	6	0	3.33	1.92–5.92
MZL	3	0	0	3	0	5.59	4.32–6.78
B-ALL	13	11	2	0	0	0.17	0.06–1.11
PCN	10	10	0	0	0	0.55	0.16–0.82
LDP	4	4	0	0	0	0.65	0.31–1.16

(–): neg = negative expression, +: positive expression (dim intensity), ++: positive expression (mod = moderate intensity), and +++: positive expression (bright intensity). SI: staining index; CLL: B-cell chronic lymphocytic leukemia; MCL: mantle cell lymphoma; HCL: hairy cell leukemia; BL: Burkitt lymphoma; FL: follicular lymphoma; DLBCL: diffuse large B-cell lymphoma; LPL: lymphoplasmacytic lymphoma; BCL: B-cell lymphomas; MZL: marginal zone lymphoma; B-ALL: B-acute lymphoblastic leukemia; PCN: plasma cell neoplasms; LDP: plasmacytic component of lymphomas with plasmacytic differentiation.

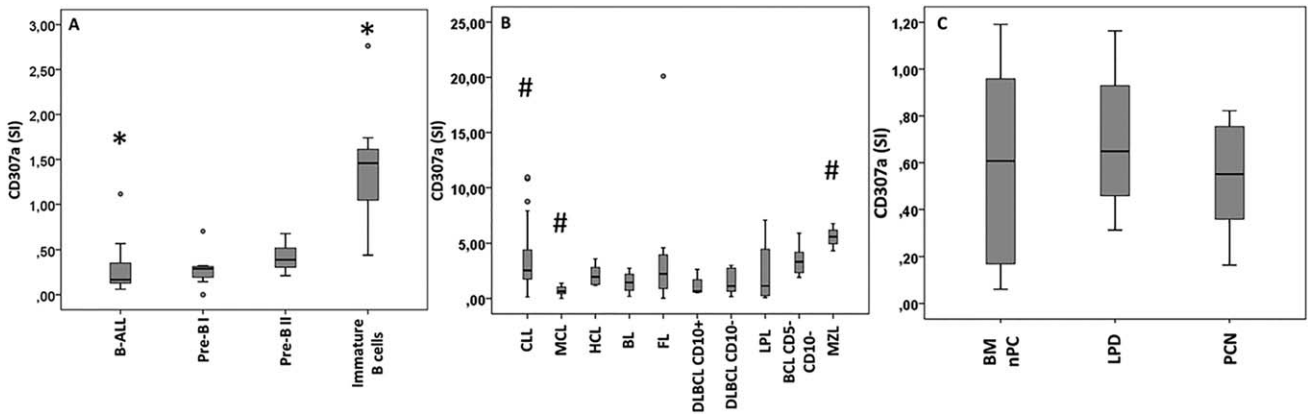

FIG. 2. CD307a expression in neoplastic B-cell. **Panel A**: Comparison of CD307a expression among B-cell precursors and B-ALL.*$P < 0.05$ with respect to normal bone marrow immature B-cells. **Panel B**: Comparison of CD307a expression among MBCN subcategories. #$P < 0.05$ with respect to MCL. *P*-values were calculated using the Dunn post-test. **Panel C**: CD307a expression in neoplastic and normal plasma cell (PC) populations. CD307a expression was not statistically different among these PC populations ($P > 0.05$, Kruskal–Wallis test).

antigen in MCL was more uniform; six out of nine MCL cases were negative for this molecule (Table 2, Fig. 2B). The remaining three MCL cases showed a dim expression of CD307a and were classified based on their typical immunophenotypic profile. Molecular and histopathological data were not available. Three CLL cases showed a negative CD307a expression, overlapping with MCL neoplastic B cells (Fig. 3A). In addition, MZL cases ($n = 3$) showed significantly brighter expression of CD307a in comparison with MCL cases ($P = 0.026$) (Figs. 2B and 3B). No significant difference in CD307a levels was observed among the other MBCN subgroups (Fig. 2B). We highlight that two cases showed an over-expression of CD307a: one FL case (MFI = 1758; SI = 20.1) and one LPL case (MFI = 1286; SI = 7.1) (Table 2).

Expression of CD307a was measured in two different malignant PC populations: neoplastic PC populations from different PCN (7 PCM, 1 PCL, and 2 MGUS) and the malignant plasmacytic compartment of lymphomas with plasmacytic differentiation (LPD). This last group included three LPL cases and one CLL case with plasmacytic differentiation. Results were compared between the two malignant PC groups and normal PC cells using the Kruskal–Wallis test. As shown in Figure 2C, no statistical differences ($P = 0.905$) in CD307a expression were found among these groups. All three populations showed the same position in the bivariate dot-plot regarding CD307a expression (Fig. 3C). PCN and LPD SI values were similar (median SI 0.55 and 0.65, respectively) (Table 2).

DISCUSSION

In the present study, CD307a expression was analyzed in normal B cells at different stages of differentiation and in various B-cell neoplasms. The aim of these

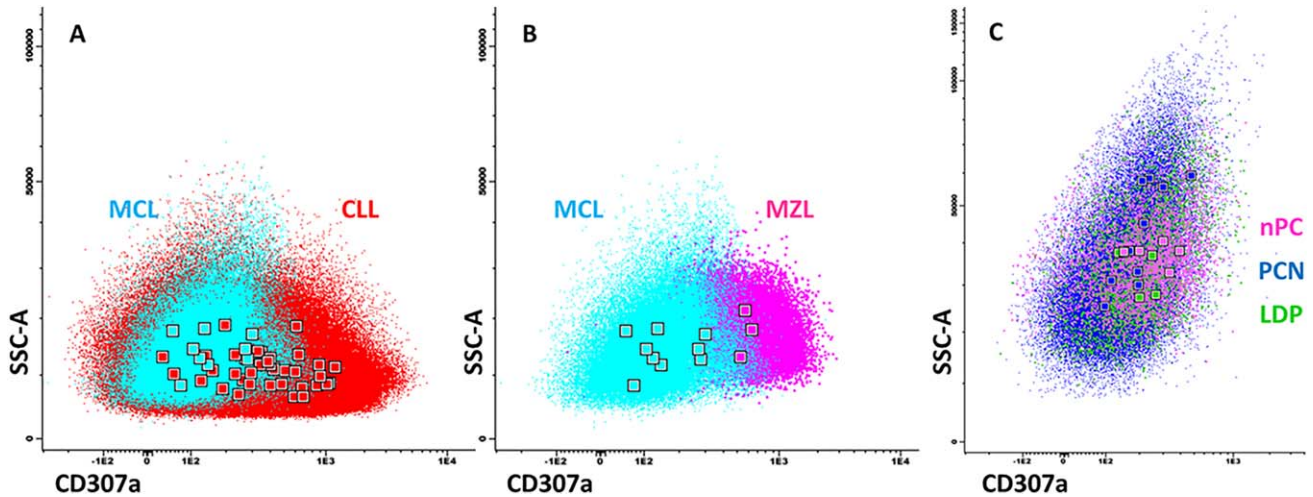

FIG. 3. Bivariate dot plots showing CD307a expression. **Panel A**: CLL (red) vs.MCL (light blue). **Panel B**: MCL (light blue) vs.MZL (pink). **Panel C**: bone marrow (BM) normal plasma cells (nPC; pink), plasma cell neoplasms (PCN; blue), and plasmacytic component of lymphomas with plasmacytic differentiation (LPD; green). Squares correspond to the CD307a mean value from B-cells or plasma cells of each individual patient. [Color figure can be viewed at wileyonlinelibrary.com]

analyses was to expand the understanding of CD307a expression in normal and malignant B cells and determine whether this novel immunophenotypic marker could improve the flow cytometric classification of B-cell lymphoid malignancies, especially of MBCN.

CD307a expression is found at low levels in BM B-cell precursors and increases during B-cell differentiation, reaching peak levels in mature B cells. BM PC showed a downregulation of this marker in comparison to mature B-lymphocytes. These changes in CD307a expression during normal B-cell development have already been reported in other studies (9,22,23).

Most B-ALL cases were negative for CD307a and showed statistically lower levels of this molecule than normal B-cell precursors in their last stage of development (immature B cells). The absence of this receptor in leukemic lymphoblasts may be related to their premature differentiation stage, as 12 B-ALL were classified as common B-ALL and one as pro-B ALL; both categories correspond to B-ALL derived from the first stages of B-cell development (2). Similarly, in a previous study, lack of CD307a expression was observed in all the 46 B-ALL cases tested (24). Thus, some degree of correlation concerning CD307a expression between normal and neoplastic cells can be ascertained. However, no pre B-ALL cases could be included in the present study to allow comparison with the most mature precursor B cells.

MCL showed the weakest CD307a expression among the evaluated MBCN. It has been postulated that MCL cells derive from a naive pre-germinal center B cell at the inner mantle zone (2). Although in the present study CD307a expression was not assessed in this population, the scientific literature indicates that naive B-lymphocytes are the B-cell subset that expresses the highest levels of CD307a (22). In normal human lymphoid tissues, moreover, the mantle zone was shown to express CD307a mRNA with the highest intensity when examined by Northern blot analysis (25). Thus, it could be inferred that CD307a was aberrantly underexpressed in virtually all MCL cases. Although the exact significance of such phenotypic aberrations still needs to be established, they could be used to differentiate MCL populations from residual normal B cells.

Unlike in MCL, CD307a expression in CLL was heterogeneous from case to case. This could be explained by the fact that CD307a expression was strongly associated with IGHV mutation status in CLL, and cases with low antigen expression levels were associated with patients with a more aggressive disease (26). In fact, in the present study, cases with lower CD307a expression exhibited immunophenotypic markers of unfavorable prognosis, such as CD38, CD49d, and low expression of CD305 (LAIR-1) (data not shown).

In the flow cytometric evaluation, surface antigen CD5 was proved useful for classifying MBCN into two main groups. B-cell lymphoid neoplasms positive for CD5 mainly include classical and atypical CLL and MCL (4,14). It is well known that MCL and CLL share phenotypic features, and sometimes a differential diagnosis between these entities can be challenging. A significant difference was observed in CD307a SI values between CLL and MCL groups, but ranges of expression were largely overlapping. Yet, results indicate that moderate and bright CD307a expression (frequently observed in CLL malignant B cells) could exclude MCL diagnosis. This finding amplifies and reinforces the results of a previous report, in which only four MCL cases were analyzed and an absent CD307a expression in most neoplastic B cells was observed (27). It has already been proved that current routine immunophenotypic panels can accurately discriminate between these entities, being CD79b, CD200, sIgM, CD23, and CD20 the most significant parameters for this task (3). Nonetheless, atypical antigen expression involving these markers has been reported. It has been shown that MCL can express CD23 or dim sIgM, as does CLL. Moreover, CLL cases with partial or absent CD23 expression and bright CD20 have been observed, overlapping considerably with MCL (28). Several studies have demonstrated that positive expression of CD200 is useful for distinguishing CLL from MCL (29–31), even in atypical CLL cases (6). However, it has been reported that CD200 expression could be positive in MCL. Furthermore, these CD200-positive MCL cases have also exhibited an atypically bright expression of CD23 (29,31). It is because of the existence of such atypical cases that it could be of great value to further investigate CD307a expression in larger groups of patients and particularly in CLL and MCL cases with atypical phenotypic features.

MCL also showed significantly dimmer CD307a expression than MZL. MZL usually has a CD5− phenotype, but CD5 expression can be frequently observed (4,28,32).The immunophenotypic pattern seen in MZL, such as bright CD20 expression and absence of CD23, overlaps with MCL; CD307a could therefore be an important marker in these cases. It is important to highlight that, in the present study, a small number of MZL cases were analyzed, and all of them showed absence of CD5 expression. Incidentally, a novel immunophenotypic marker for MZL has been recently discovered: CD307d expression was significantly associated with nodal and extranodal MZL with high specificity, being a good candidate marker for MZL diagnosis by flow cytometry (7,33).

An isolated strong expression of CD307a was observed in two MBCN cases (FL and LPL). Previous studies have reported an upregulation of CD307e (also a member of the FcRL family) in BL cell lines with chromosome 1q21 abnormalities, which is a frequent genetic lesion in some BCL (25,34). Accordingly, overexpression of the CD307a antigen might reflect specific genetic abnormalities. Unfortunately, karyotype and cytogenetic data were not available for both cases.

Concerning CD307a expression in the plasmacytic compartment, there was no statistical difference in the expression levels of this molecule between neoplastic (LPD and PCN) and normal PC. The data presented here revealed the limitations of assessing CD307a expression

in PC populations, as it seems to serve no purpose either in the discrimination between neoplastic and normal PC or between LPD and PCN. Similar results were observed in a previous study (35), but these findings could be further analyzed in a larger patient cohort. Although CD307a expression was evaluated in a small number of LPD cases ($n = 4$), to our knowledge, this is the first study to assess the expression of this marker in the malignant PC population of this entity.

In conclusion, our results expand the knowledge on CD307a expression in B-cell malignancies, as this is the first study to evaluate the expression pattern of this antigen in a broad range of B-cell neoplasms. However, more extensive research is needed to determine if the differential CD307a expression observed among MBCN (MCL vs. CLL and MCL vs. MZL) is truly useful in the differential diagnosis of these entities.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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