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Features of immunophenotypic finding B-cell lymphoproliferative diseases by flow cytometry

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Abstract

Aim. To assess the information content of conventional and additional immunophenotypic markers (CD200, CD305) in the differential diagnosis B-cell lymphoproliferative diseases by flow cytometry.

Methods. An immunophenotypic study using 4-color flow cytometry was performed in 204 patients with different variants of B-cell non-Hodgkin's lymphomas. The study material included peripheral blood and bone marrow. The expression of CD45, CD19, CD20, CD22, CD79b, CD79a, CD5, CD10, CD23, FMC7, CD43, CD38, CD11c, CD103, CD25, CD 200, CD 305, light chains of immunoglobulins (kappa/lambda) using monoclonal antibodies (Becton Dickinson, USA) was evaluated. The intensity of antigen expression was assessed using mean fluorescence intensity (y. e.).

Results. Conventional FMC7-positive expression revealed only half patients with different variants of leukemization of non-Hodgkin's lymphomas, whereas atypical positive expression of CD23 was observed in patients with marginal spleen lymphoma and follicular lymphoma in 27.3 and 28.6% of cases, respectively. In mantle cell lymphoma, expression of CD200 in B-cell was detected in a significantly smaller number of observations, accompanied by a significant decrease in the average intensity of CD200 fluorescence compared to B-cell chronic lymphocytic leukemia (B-CLL) cells. The mean fluorescence intensity (MFI) of CD305 in hairy cell leukemia is significantly higher than in splenic marginal zone lymphoma (SMZL) with "villous" lymphocytes.

Conclusion. Different levels of the information content of some conventional markers were revealed in differential immunophenotypic diagnosis of B-cell lymphoproliferative diseases by flow cytometry; the use of additional markers CD200 and CD305 was highly informative in differential diagnostics between different variants of B-cell lymphoproliferative diseases with similar immunophenotypic and morphological characteristics of lymphoid elements.

Keywords: B-cell lymphoproliferative diseases, flow cytometry, CD200, CD305 markers.

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The advantages of multicolor flow cytometry translate into wide possibilities for its use, primarily in the diagnosis and classification of lymphoproliferative diseases (LPD) [1], monitoring of residual tumor clone, assessment of prognosis factors, and resistance to therapy. A traditional morphological study of LPD does not enable us to determine the differentiation stage and understand if the tumor lymphoid cells belong to any line (T-, B-, or NK). Flow cytometry enables rapid identification of the clonality of tumor B-lymphocytes, co-expression of the surface, and/or cytoplasmic markers of a particular tumor. However, the immunophenotypic characteristics of the tumor lymphocytes of individual patients may not always be consistent with the classical presentation of a variant of B-LDL; this makes it challenging to interpret the data and may lead to an inaccurate diagnosis.

Few studies [2–5] have reported on the use of relatively new monoclonal antibodies in the diagnostic panel for immunophenotyping of B-LPD. We analyzed the information content of traditional and additional immunophenotypic markers (CD200, CD305) in various variants of B-cell LPD.

CD200 (OX2) is a transmembrane glycoprotein that belongs to the immunoglobulin superfamily and is expressed on thymocytes, resting and activated T-lymphocytes, B-lymphocytes, dendritic,

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endothelial cells, and neurons; however, it is absent on NK-cells, monocytes, granulocytes, and thrombocytes. Tumor B-cells are characterized by bright homogeneous expression of CD200 in B-cell chronic lymphocytic leukemia (CLL) and hairy-cell leukemia (HCL); the severity is much weaker in mantle cell lymphoma (MCL) [4, 5]. The expression of CD200 is considered to be an unfavorable prognostic sign in multiple myeloma [6–8].

CD305, or leukocyte-associated Ig-like receptor-1 (LAIR-1), is a transmembrane glycoprotein, that belongs to the superfamily of immunoglobulins and is expressed on a significant part of T-, B-, and NK-lymphocytes as well as monocytes, dendritic cells, and thymocytes. LAIR-1 expression is noted in the early stages of B-lymphocyte differentiation; however, it is absent on plasmoblasts and plasmocytes. In addition, LAIR-1 can function as an inhibitory receptor on T- and NK-lymphocytes [9, 10].

According to the recommendations of the European Consortium for Flow Cytometry (EuroFlow), the LAIR-1 marker is included in the B-LPD differential diagnostic panel for HCL diagnosis (Leukemia, 2012).

This study was designed to evaluate the information content of traditional and additional immunophenotypic markers (CD200, CD305) in the differential diagnostics of B-LPD using flow cytometry.

An immunophenotypic study was conducted in 204 patients who were suspected to have B-LPD in the research laboratory of M. F. Vladimirsky Moscow Regional Scientific and Research Clinical Institute. Total 20 patients with reactive lymphocytosis in whom B-LPD was ruled out were examined. The patients' peripheral blood and bone marrow samples were used for evaluations.

The present study was approved at a meeting of the independent ethics committee of the M. F. Vladimirsky Moscow Regional Scientific and Research Clinical Institute (protocol No. 10 dated 15.10.2015) and was started with patients who were willing to participate in the study and provided informed written consent.

The patient age ranged from 30–80 years, and the average age of the study population was 56 ± 15 years.

Immunophenotypic studies were performed using laser flow cytometry with a FACSCalibur four-color flow cytofluorometer (Becton Dickinson, USA) using CellQwest software and monoclonal antibodies conjugated with fluorescent stains (manufactured by BD Biosciences, USA). The following panel of monoclonal antibodies was used: Anti-CD45-FITC, Per-CP or Per-CP-Cy5.5, APC; anti-CD3-FITC, anti-CD19-APC, Per-CP or Per-CP-Cy5.5; anti-CD20-FITC, Per-CP or Per-CP-Cy5.5; anti-CD5, anti-FMC7, anti-CD38, anti-CD103, anti-CD43, anti-sIg kappa labeled with FITC; anti-CD(16+ CD56), anti-CD10, anti-CD23, anti-CD56, anti-CD22, anti-CD79b, anti-CD25, anti-CD11c, anti-sIg lambda labeled with PE. Moreover, monoclonal antibodies anti-CD200 — PE (Clone MRC OX-104 BD Pharmingen) and anti-CD305-PE (Clone DX26 BD Pharmingen) were included in the panel.

Immunophenotypic studies were performed using a standard sample-preparation technique. The presence of antigen expression on the surface or in the cytoplasm of > 20% of the tumor cells was considered to indicate positivity. The intensity of antigen expression was assessed as per the parameter of the average fluorescence intensity (MFI, Mean Fluorescence Intensity), expressed in relative units (RU).

Statistical processing of the results was performed using the statistical program Statistica 8.0 with calculation of the average value and mean square error (M ± m). To determine the significance of the differences in the average values of quantitative indicators, Student's *t*-test was used. The frequency of the antigens in the studied patient cohorts was compared using Fisher's test.

Among the 204 enrolled patients, 104 were diagnosed with CLL, 18 with HCL, 41 with MCL, 34 with splenic marginal zone lymphoma (SMZL), and 7 with follicular lymphoma leukemization. The MCL diagnosis was confirmed with cytogenetic studies of t(11; 14) and/or detection of expression of cyclin D1 by immunohistochemical methods.

The data on the assessment of traditional immunophenotypic markers in B-cell lymphoproliferative disease (B-LPD) are presented in Table 1.

A common immunophenotypic sign of all B-LPDs is the restriction of membrane light chains of immunoglobulins (kappa- or lambda type) that confirm the clonality of B-lymphocytes in tumor transformation. Assessing the CD20 expression intensity using the MFI parameter enables the differentiation of B-CLL cells with dim expression from other B-LPD tumor cells that are characterized by mod or high/bright expression intensity of this molecule; this is one of the main traditional differential diagnostic criteria used to distinguish CLL from mature cell lymphomas and HCL. The generally accepted traditional immunophenotypic sign of leukemia of non-Hodgkin lymphomas and HCL is the positivity of FMC7 that is usually not expressed in CLL and serves as a differential diagnostic criterion.

The immunophenotype of lymphocytes in CLL was characterized by classical aspects, namely positive expression of antigens CD5, CD23, CD43,

Table 1. Frequency of detection of positive differential diagnostic antigens for various variants of B-cell lymphoproliferative diseases (proportion of cases, %)

Variant	Chronic lymphocytic leukemia/ lymphocytic lymphoma	Mantle cell lymphoma	Follicular lymphoma	Splenic marginal zone lymphoma	Hairy cell leukemia
AG	N=104	N=41	N=7	N=34	N=18
$CD19^+$	100.0	100.0	100.0	100.0	100.0
$CD20^+$	100.0 dim*	80.0 bright; 20.0 dim	100.0 bright	85% bright; 15% dim	100.0 bright
$CD22^+$	95.5 dim; 4.5 bright*	85.0 bright; 15.0 dim	100.0 bright	85% bright; 15% dim	100.0 bright
$CD79b^+$	66.2 dim	88.6 bright	100.0 bright	92% bright; 7.5% dim	100.0 bright
$CD10^+$	0.0	5.7	100.0	0.0	5.5
$CD5^+$	98.6	100	0.0	0.0	22.2
$CD23^+$	92.3	11.4	28.6	27.3	11.1
$FMC7^+$	4.8	57.1	57.1	54.5	88.9
$CD43^+$	98.6	14.3	33.3	12.1	0.0
$CD103^+$	0.0	0.0	0.0	0.0	77.7
$CD25^+$	73.3	70.4	14.3	50.0	81.3
$CD38^+$	26.0	45.2	43.0	0.0	12.5
Restriction of membrane light chains of immunoglobulin (SIg k/L)	86.6	97.4	57.1	100.0	82.4

*Note: dim — low fluorescence intensity; bright — high fluorescence intensity.

and lack of expression of CD10 and FMC7. All CLL patients showed dim expression of the CD20 molecule, some patients (4.5%) had atypical bright expression of CD22, and 33.8% of patients showed a lack of the membrane expression of CD79b. In 14.4% of the patients, there was no restriction on the membrane light chains of immunoglobulins. The absence of the CD79b molecule on the surface of B-CLL cells can indicate deletion (13) (q14.3) or impaired formation of the B-cell receptor complex [11]. Total 26% of the patients showed positive expression of CD38 on B-CLL cells; this is regarded as an immunophenotypic indicator of poor prognosis and therapy resistance [12]. Total 73% of CLL patients had positive expression of the receptor for interleukin-2 (CD25) on tumor B-lymphocytes, ranging from 20%–89% of positive cells. Previous studies have shown a more aggressive disease course in such patients; further, there is a connection with chromosomal abnormalities and stimulation of Toll-like receptors [13, 14]. Our data showed that the marked expression of CD25 on B-CLL cells already at the stage of primary immunophenotypic diagnostics serves as a marker of a high likelihood of treatment refractivity and inefficient disease remission and may indicate the progression or development of disease recurrence [15].

The immunophenotypic profile of tumor B-lymphocytes in leukemization of MCL was characterized by the positive expression of CD5 and the lack of CD23, CD43, and CD10 expression. In 20% and 15% of MCL patients, dim expression of CD20 and CD22, respectively, was detected; 11.4% of the patients showed no CD79b expression. Positive expression of FMC7 was revealed only in 57.1% of the patients.

A particular problem in immunophenotyping is the differential diagnostics between CD5-positive CLL and MCL with variability of antigens CD23, less often CD43, as well as variability in the intensity of expression of CD20. The use of a CD200 marker can help resolve this problem. Evaluation of CD200 expression in the patients under study is presented in Tables 2 and 3.

In all CLL cases and most reactive lymphocytosis cases, the positive expression of CD200 on

Table 2. Frequency of detection of CD200 expression-positive B cells in B-cell lymphoproliferative diseases and reactive lymphocytosis (percentage of cases, %)

Type of pathology	Chronic lymphocytic leukemia/lymphocytic lymphoma N = 104	Mantle cell lymphoma N = 26	Follicular lymphoma N = 7	Splenic marginal zone lymphoma N = 29	Hairy cell leukemia N = 15	Reactive lymphocytosis N = 20
	1	2	3	4	5	6
Indicator	104/104* (100.0%)	3/26 (11.5%)	4/7 (66.7%)	19/29 (65.5%)	13/15 (85.7%)	19/20 (98.3%)

Note: *statistically significant differences between chronic lymphocytic leukemia and mantle cell lymphoma: $p_{1-2} = 0.000$; $p_{1-3} = 0.00016$; $p_{1-4} = 0.000$; $p_{1-5} = 0.01496$; $p_{1-6} = 0.16129$.

Table 3. Average fluorescence intensity (MFI) of CD200 on the tumor cells in different variants of B-cell lymphoproliferative diseases and reactive lymphocytosis (M ± m)

Nosological forms	Chronic lymphocytic leukemia/lymphocytic lymphoma N = 30	Mantle cell lymphoma N = 3	Follicular lymphoma N = 4	Splenic marginal zone lymphoma N = 15	Hairy cell leukemia N = 9	Reactive lymphocytosis N = 15
	1	2	3	4	5	6
MFI CD200, RU	370.5±14.6*	131.7±17.7	95.1±18.1	63.8±16.1	326.8±14.0	102.4±6.6

* Note: statistically significant differences between chronic lymphocytic leukemia and mantle cell lymphoma: $p_{1-2} = 0.0000$; $p_{1-3} = 0.0000$; $p_{1-4} = 0.0000$; $p_{1-5} = 0.016$; $p_{1-6} = 0.0000$.

B-lymphocytes was detected. Positive expression of CD200 was found in significantly fewer MCL patients ($p = 0.000$) than in those with CLL, consistent with previous findings, although the proportion of MCL patients positive for CD200 was slightly higher than reported previously [2, 3, 5]. Other variants of B-LPD were characterized by greater detectability of CD200 than that of MCL. However, in 11.5% of the MCL patients, CD200-positive tumor B cells were detected; thus, we estimated the expression intensity of CD200 on B-lymphocytes using the MFI parameter (see Table 3).

The highest MFI CD200 was in CLL and HCL patients, with a significant increase over that in MCL, follicular lymphoma, SMZL, and reactive lymphocytosis patients. Determination of MFI CD200 is essential for the differential diagnostics of CD5-positive CLL and MCL with the presence of a high content of $CD200^+$ of B-cells. Figure 1 presents the cytograms of patients with CLL, MCL, and reactive lymphocytosis; the different options and intensity of the CD200 antigen expression are shown (explanatory note is below the figure).

A special group of mantle cell lymphomas is made up by the so-called blastoid sub-variant of the disease, wherein tumor cells have blast morphology. Immunophenotypically, such patients (4 cases) had positive expression of CD10, weak expression of CD45 and CD19, marked positive expression of activation antigens CD38 and CD25, in some cases, there was no expression of CD79b, but CD79a expression was present. There was no CD200 expression in any case. Figure 2 presents the variants of positive expression of the CD10 antigen detected in follicular lymphoma (A) and the blastoid variant of MCL (B).

Table 4. Indicators of LAIR-1 (CD305) expression on the tumor cells with lymphoma of the splenic marginal zone and hairy cell leukemia

Type of B-cell lymphoproliferative disease	Splenic marginal zone lymphoma N = 9	Hairy cell leukemia N = 9
Content of CD305-positive cells, M ± m (%)	44.02±11.98	65.9±8.5
Fluorescence intensity, M ± m (CU)	325.1±12.7*	1404.9±14.2

Note: * $p = 0.0000$.

The phenotype of tumor cells of follicular lymphoma was characterized by bright expression of all pan B-cell markers in 100% of cases, CD10 positivity, and variability of the markers CD23, CD43, and CD38. In 43% of cases, restriction of immunoglobulin light chains was detected only with intracytoplasmic staining. Positive expression of FMC7 was revealed in only half of the patients examined (57.1%), and positive expression of CD23 was detected in 28.6% of cases. In the latest classification of the World Health Organization (2016), a new variant of the predominantly diffuse CD23-positive follicular lymphoma with the phenotype $CD10^+$b-cl-2^+bcl-6^+, characterized by a more aggressive course and del 1p36, was identified [16].

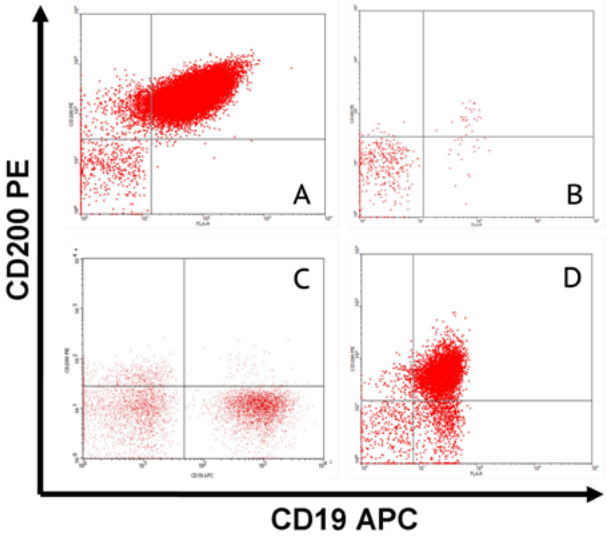

Fig. 1. Expression of CD200 on B-cells in chronic lymphocytic leukemia, reactive lymphocytosis, and mantle cell lymphoma (MCL): A — chronic lymphocytic leukemia, expression of CD200 on 94% of B-chronic lymphocytic leukemia cells, fluorescence intensity (MFI) 350 RU; B — reactive lymphocytosis, expression of CD200 on 0.12% of B cells, MFI = 112.1 RU; C — MCL, expression of CD200 on 1.22% B-lymphocytes (negative), MFI = 57.9 RU; D — MCL, expression of CD200 on 82.9% of B-lymphocytes (positive), MFI = 115.4 RU.

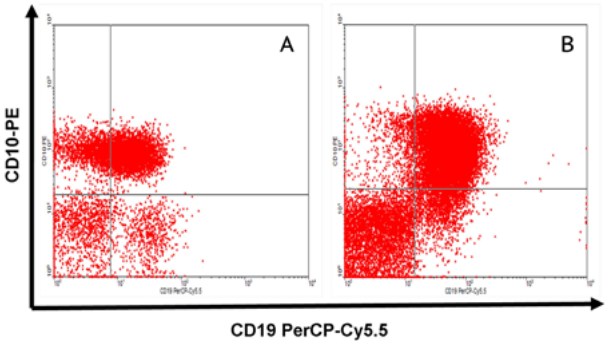

Fig. 2. Expression of CD10 on B-lymphoid elements in follicular lymphoma and blastoid variant of mantle cell lymphoma: A — follicular lymphoma, expression of CD10 on 68.12% of B-cells, fluorescence intensity is 105.4 RU; B — mantle cell lymphoma, blastoid variant, CD10 expression on 55.5% of B-cells, fluorescence intensity was 88.1 RU.

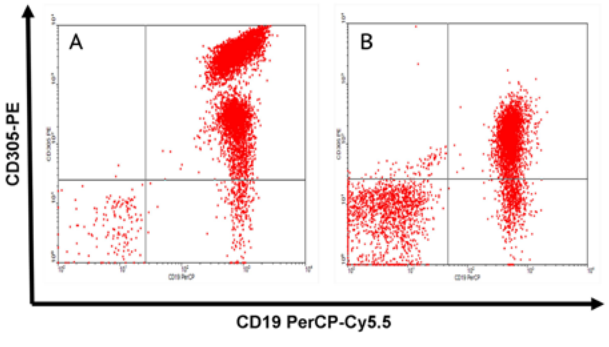

Fig. 3. Expression of CD305 (LAIR-1) on B-lymphocytes with hairy cell leukemia (HCL) and splenic marginal zone lymphoma (SMZL): A — HCL, heterogeneous expression of CD305 on 94.5% of B cells, MFI = 1404 RU; B — SMZL, expression of CD305 on 59.3% of B cells, fluorescence intensity of 320.5 RU.

Immunophenotype of B-lymphocytes in SMZL was characterized by predominantly bright expression of all pan B-cell markers, lack of expression of CD5, CD10, CD43, CD103 and CD38, variability of CD23, CD25 and FMC7. In particular, positive expression of the CD23 antigen, which is not characteristic of this group of patients, was revealed in almost a third (27.3%) of patients, and positive expression of the classic traditional marker FMC7 was detected in only half (54.5%) of patients.

Tumor cells in case of the classic form of HCL showed bright expression of all pan B-cell markers, the absence of CD5, and the presence of positive expression of the FMC7, CD11c, CD103, and CD25 antigens. Total 27.7% of the patients out of 18 patients with immunophenotypically verified diagnosis of HCL had a variant form with a positive expression of either CD5 or CD10, with no expression of CD103, CD25, CD11c, or CD305. In some cases, expression of CD79b was absent, but expression of CD79a was detected. Only in 48% of cases of immunophenotypically verified diagnosis of HCL, typical "hairy cells" were morphologically identified or a descriptive characteristic of lymphoid elements with cytoplasmic outgrowths was given.

Certain difficulties arise in the diagnostics of SMZL and HCL, which is associated with the presence of dendritic or villous cells and "hairy" cells, respectively, for these diseases. In addition to the traditional antigens CD25, CD11c, CD103, we studied the expression of CD305 (LAIR-1) in SMZL and HCL, which is presented in Table 4.

There were no significant differences in the content of CD305-positive cells between these groups of patients; however, the average fluorescence intensity of CD305 with HCL was significantly higher than with leukemization of SMZL (Figure 3). This fact can be used in immunophenotypic differential diagnostics of these variants of B-LPD with a similar morphological characteristic of lymphoid elements.

CONCLUSIONS

1. There were some aspects of the immunophenotypic characteristics of tumor cells in patients with B-cell lymphoproliferative diseases.

2. As per the study findings, 7.5%–20% of the patients with leukemization of mantle cell lympho-

ma and spleen marginal zone lymphoma can exhibit atypical dim expression intensity of CD20, CD22, and CD79b; this hinders the differential diagnostics with chronic lymphocytic leukemia.

3. The positive expression of the traditional marker FMC7, typical for leukemization of mature cell lymphomas and hairy cell leukemia, was detected in about 50% of the patients with mantle cell lymphoma, follicular lymphoma, and splenic marginal zone lymphoma.

4. Several patients had atypical positive expression of CD23 with follicular lymphoma and splenic marginal zone lymphoma (28.6% and 27.3%, respectively).

5. Significantly fewer patients with mantle cell lymphoma had positive expression of CD200 on the tumor cells ($p < 0.0001$) than those with chronic lymphocytic leukemia and other variants of B-cell lymphoproliferative diseases.

6. There was significantly greater reduction ($p < 0.001$) in CD200 fluorescence intensity in mantle cell lymphoma as compared to that in chronic lymphocytic leukemia; therefore, we could use this criterion for the differential diagnostics of these variants of B-cell lymphoproliferative diseases in patients with a high content of CD200-positive tumor B-cells.

7. The average fluorescence intensity indicator CD305 in hairy cell leukemia was significantly higher than in leukemization of splenic marginal zone lymphoma; this could be used in the differential diagnostics of these variants of B-cell lymphoproliferative diseases with similar morphological characteristics of lymphoid elements.

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