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ORIGINAL STUDY

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Flow cytometric immunophenotypic characteristics of plasma cell leukemia

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Abstract: The aim of this prospective study was to define the flow cytometric characteristics of simultaneously investigated bone marrow and peripheral blood plasma cells antigens expression in 36 plasma cell leukemia (PCL) patients. The immunophenotypic profile of plasma cells was determined with a panel of monoclonal antibodies. The antigen expression intensity was calculated as relative fluorescence intensity (RFI). Bone marrow plasma cells showed expression of particular antigens in the following proportion of cases: CD49d 100%, CD29 94%, CD54 93%, CD44 83%, CD56 60%, CD18 26%, CD11b 29%, CD11a 19%, CD117 27%, CD71 30%, CD126 100% and CD19 0%, while the expression of those antigens on peripheral blood plasma cells was present in the following percentage of patients: CD49d 100%, CD29 96%, CD54 93%, CD44 95%, CD56 56%, CD18 50%, CD11b 53%, CD11a 29%, CD117 26%, CD71 28%, CD126 100% and CD19 0%. The expression of CD54 was significantly higher than that of adhesion molecules belonging to the integrin $\beta 2$ family: CD11a, CD18 and CD11b, on both bone marrow and peripheral blood cells ($p < 0.01$). Expression of CD18, CD11a and CD11b was differential between two cell compartments: lower on bone marrow and higher on peripheral blood cells. We found that plasma cells in the bone marrow of patients with plasma cell leukaemia showed significantly greater granularity and size than those in the peripheral blood ($p = 0.0001$ and $p = 0.04$, respectively). However, no differences in cell size or granularity were revealed between bone marrow plasma cells from patients with PCL and multiple myeloma. In conclusion, impaired expression of adhesion molecules such as CD11a/CD18 (LFA-1) or CD56 may explain hematogenic dissemination characterizing PCL. The following pattern of adhesion molecule expression according to the proportion of plasma cells expressing a given antigen in peripheral blood and bone marrow and arranged in diminishing order may be established: CD49d > CD44 > CD54 > CD29 > CD56 > CD18 > CD11b > CD11a. Immuno-phenotyping of plasma cells in PCL, as in multiple myeloma, might be useful in detecting minimal residual disease in cases with aberrant antigen expression and for selecting therapeutic agents towards specific membrane targets. (*Folia Histochemica et Cytobiologica 2011; Vol. 49, No. 1, pp. 168–182*)

Key words: plasma cell leukemia, immunophenotype, flow cytometry

Introduction

Recent studies on myeloma cell associated antigens: CD45, CD44, CD56, CD117, CD126 and CD138 have revealed their multiple roles in the biology of this tumor. Besides their immunophenotypic marker function, these antigens contribute relevant processes for cell survival, participate in lytic bone lesion formation, and mediate the action of novel anti-myeloma drugs and resistance to treatment [1–12].

CD38 is a common marker for a variety of different cell populations e.g. T-cells, B-cells, NK-cells. But it has been found that a high-density CD38 expression ($CD38^{++}$) on the cell surface is a characteristic feature of normal and malignant plasma cells [13].

CD138 (syndecan-1), a molecule belonging to the heparan sulfate family, is a marker of normal and malignant plasma cells [14]. Retaining lymphocyte antigen (CD45) on some plasma cells is interesting in terms of plasma cell differentiation. Expression of CD45 seems to be important for myeloma cell sig-

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naling and proliferation processes [15, 16]. Adhesion molecules participate in cell-to-cell interactions, cell migration and homing. Integrins, present on the cell surface, play the role of receptors for particular proteins of the extracellular compartment. They are a family of heterodimeric molecules that share a common β_1 chain (CD29) together with one of six α_1 chains (α_1–α_6) (CD49 a–f). VLA-4, an integrin consisting of α_4 and β_1 chains, is a molecule that enables cell binding to extracellular matrix through fibronectin. Apart from VLA-4 contribution, the direct contact of lymphoid malignant cells with bone marrow stromal cells is mediated by such molecules as a transmembrane glycoprotein CD54 (Intercellular Adhesion Molecule-1, ICAM-1), a multistructural and multifunctional glycoprotein CD44 (Homing Cell Adhesion Molecule, H-CAM), and CD11a/CD18 (Lymphocyte Function Associated Antigen, LFA-1). LFA-1 is a ligand for ICAM-1. An intercellular contact mediated through LFA-1-ICAM-1 molecules is a requisite of malignant plasma cell proliferation. Novel aspects of CD44 expression for myeloma are associated with its prognostic and possibly therapeutic significance [17, 18].

Plasma cell leukemia (PCL) is a rare malignant plasma cell disorder accounting for 2–3% of all plasma cell dyscrasias [19]. It may develop during the course of multiple myeloma (MM) (secondary PCL) or it may be diagnosed as a primary manifestation of the disease. Due to its low incidence, most publications on PCL are based on single case reports. Little is known about the immunophenotype of tumor cells in primary PCL because very few reports have looked at the evaluation of single antigens expression on bone marrow or peripheral blood plasma cells [20–29].

The aim of this prospective study was a parallel assessment of expression of adhesion molecules, receptor for interleukin-6 (CD126), receptor for stem cell factor SCF (CD117), and receptor for transferrin (CD71) on plasma cells of peripheral blood and bone marrow in 36 patients with PCL. Moreover, expression of some of these antigens was compared with their expression on myeloma plasma cells, based on our previous, partly-published, studies in MM patients [30, 31].

Material and methods

Patients

The study included 36 PCL (23 primary, 13 secondary) and 47 MM patients hospitalized in the Department of Hematology of the Institute of Hematology and Transfusion Medicine in Warsaw between 1995 and 2009.

The following parameters were estimated at diagnosis for each patient: age, sex, complete blood examination, percentage of plasma cells in bone marrow (at bone marrow collection for FACS analysis, regular bone marrow smears were performed stained with May–Grünwald–Giemsa method), monoclonal protein isotype (using Beckman Paragon Immunofixation Electrophoresis Kit), urine immunoglobulin/ /24 hours, serum concentrations of monoclonal protein, albumin, calcium, creatinine, LDH and β_2-microglobulin (using Beckman ARRAY 360 autoanalyzer), complete X-ray skeletal survey, and stage of disease according to Durie and Salmon and also the International Staging System for multiple myeloma [32]. PCL was diagnosed where there was an absolute plasma cell count of at least 2.0×10^9/l or 20% of the total white blood cell count in the peripheral blood of patients with other typical symptoms of clonal plasma cell malignancy.

Each patient's informed consent was obtained prior to bone marrow aspiration or biopsy.

Cell preparation and immunofluorescence

Immunophenotyping was performed simultaneously on fresh bone marrow and peripheral blood samples. The samples were incubated with monoclonal antibodies against the analyzed antigens for a time recommended by the manufacturers of these antibodies. Cells were stained with triple labeling with mouse anti-human monoclonal antibodies conjugated to respective fluorochromes. Cell surface antigens were assessed by direct immunofluorescence using fluorescein isothiocyanate (FITC), phycoerythrin (PE), phycoerythrin-cyanide 5 (PE-Cy5) conjugated monoclonal antibodies against antigens: CD138-FITC, CD138-PE (Serotec, UK), CD38-PE-Cy5, CD45-FITC, CD45-PE-Cy5, CD56-PE, CD29-FITC, CD18-FITC, CD71-FITC, CD19-FITC, CD20-PE, CD10-PE, κ/λ/CD19 (Dako, Denmark), CD49d-PE, CD11b-PE, CD117-PE, CD33-PE, CD58-PE (Becton Dickinson, USA), CD54-PE, CD44-FITC, CD11a-PE, and CD126-PE (Beckman Coulter, USA). Positivity levels were determined according to a FITC-, PE-, PE-Cy5-conjugated isotypic Ig control.

Flow cytometry analysis

The samples were analyzed using flow cytometer CytoronAbsolute with ImmunoCount II software (Ortho, Raritan, NJ, USA). Ten thousand cells were analyzed at a time. The subjects of analysis were plasma cells identified on the basis of strong CD38 expression, expression of CD138 and $CD45^{low/-}$ as well as their typical light scatter distribution. Monoclonality was confirmed by immunoglobulin light chain

analysis. All investigated antigens expression was analyzed on $CD38^{++}/CD138^{+}$ cells. The percentage of positive events in plasma cell subset was calculated and compared to the isotype control. The results were presented as a percentage of cells with expression of investigated antigen. The threshold criterion value for antigen positivity was more than 20% positive plasma cells.

The intensity of particular antigen expression was measured by an indirect quantification method previously described and the values of this expression intensity were calculated as Relative Fluorescence Intensity (RFI) indices [3]. To determine CD45 expression intensity on plasma cells, we applied a method of referring RFIs on monoclonal plasma cells to RFIs on lymphocytes, and a score of CD45 RFI values on myeloma cells to CD45 RFI values on lymphocytes was calculated in particular patients. A method of calculating RFI score using antigen expression as a reference point was applied in flow cytometry methodics [33]. Standardization of flow cytometry was performed with Dako fluorospheres calibration and external controls using CEQUAL.

Statistical analysis

To estimate the statistical significance of differences observed between mean values for different analysed group pairs, the Wilcoxon and Student's tests were used with Statistica software. Differences were considered significant for $p < 0.05$.

Results

The data referring to the patients' characteristics is set out in Table 1. Cell size and granularity, as well as CD45 expression intensity on peripheral blood and bone marrow plasma cells, are presented in Tables 2 and 3, respectively.

The results of antigen expression analysis in particular PCL patients are presented in Table 4, while their frequency is summarized in Table 5. The relative numbers of plasma cells with a given antigen expression and values of expression intensity indices (RFI) of those antigens in peripheral blood and bone marrow of PCL and MM patients are compared in Tables 6 and 7, respectively.

Cell size and granularity

Two parameter cytograms based on forward scatter height (FSC) and size scatter height (SSC) of plasma cells were designed. SSC (side scatter channel) is a rough indicator of cellular granularity and number of organelles. The signal collected by the FSC detectors is proportional to cell size [34].

Bone marrow plasma cells in PCL showed significantly greater granularity and greater size than those in peripheral blood ($p = 0.001$ and $p = 0.04$, respectively) (Figure 1). This difference was found in almost all patients. However, no differences in cell size or granularity were revealed between bone marrow plasma cells in PCL and those in MM (Table 2).

Expression of particular antigens

CD45

CD45 RFI on plasma cells in relation to CD45 RFI on lymphocytes in the same patients was analyzed. Intensity of CD45 expression on peripheral blood and bone marrow PCL plasma cells was low and uniform, while the values of CD45 RFIs on lymphocytes were high (Table 3). A score of RFI value on plasma cells/ /RFI value on lymphocytes for CD45 antigen was calculated. The values of this score ranged from 0.0 to 0.8, with a mean of 0.2 ± 0.3. One may assume that the values of the score exceeding this mean value indicate $CD45^{low}$ cells, while those below this mean value correspond to $CD45^{low/-}$ cells.

Although most PCL cases presented with low and relatively homogeneous CD45 expression intensity, in particular cases we also observed such a conspicuous diversity of this expression that two subsets of plasma cells with different values of RFI (R2 and R3) could be distinguished (Figure 2)

No differences in CD45 RFI of PCL plasma cells between peripheral blood and bone marrow compartment were found; similarly, no differences in CD45 RFI of bone marrow plasma cells between PCL and MM patients were revealed (Table 3). This may indicate a similar degree of maturity of plasma cells in bone marrow and peripheral blood in both PCL and MM.

CD138 (Syndecan-1)

Expression of CD138 antigen was found on both bone marrow and peripheral blood leukemic cells in all patients (Tables 4, 5).The expression of this antigen was very strong (Table 6). CD138 expression intensity (CD138 RFI) on bone marrow and peripheral blood PCL plasma cells was similar in both individual patients and the whole group of patients (Table 6). Expression intensity of CD138 on myeloma bone marrow plasma cells showed heterogeneity from weak (RFI = 4.7) to very high (RFI = 26.6); the mean RFI value was 13.5 ± 4.3 (4.7–26.6), and the median RFI value was 14.2 (Table 7). The proportion of CD38 positive myeloma plasma cells was very similar to that of CD138.

In all patients, the expression of CD38 was also revealed on leukemic cells of both cell compartments.

Table 1. Clinical and biological disease characteristics in plasma cell leukemia (PCL) and multiple myeloma (MM) patients

Parameter	Primary PCL n = 23	Secondary PCL n = 13	MM n = 47
Gender			
Female (%)	13 (57)	8 (62)	21 (45)
Male (%)	10 (43)	5 (38)	26 (55)
Age (years) mean ± SD; median	60.4 ± 14.2; 62.5	64 ± 11.4; 63	61.5 ± 9.4; 62
Bone marrow plasma cells (%) mean ± SD; median	70 ± 21; 80	75 ± 17; 75	45 ± 20; 40
Peripheral blood white blood cells × 10^9/L mean ± SD; median	26 ± 25; 15	9 ± 7; 6	4.0 ± 1.7; 4
Peripheral blood plasma cells (%) mean ± SD; median	41 ± 25; 31	24 ± 9; 26	
Serum albumin < 3.5 [g/dL], % cases mean ± SD; median	33 3.7 ± 0.7; 3.9	25 3.4 ± 0.7; 3.6	20 3.5 ± 0.6; 3.7
Serum β_{2M} > 5.5 [mg/L], % cases mean ± SD; median	80 10.8 ± 9.9; 8.4	40 5.6 ± 5.2; 2.9	21 4.8 ± 4.3; 4.4
Serum LDH > 450 [U/L], % cases mean ± SD; median	40 685 ± 1011; 389	66 1356 ± 1312; 720	12 370 ± 174; 328
Serum calcium > 2.75 [mmol/L], % cases	52	30	10
Serum creatinine > 2.0 [mg/L], % cases	33	23	17
Monoclonal protein isotype , % cases			
IgG	73	46	66
IgGκ	46	23	40
IgGλ	27	23	26
IgA	0	46	22
IgAκ	0	23	11
IgAλ	0	23	11
IgEκ	4	0	0
Bence Jones	19	8	12
Kappa	19	0	10
Lambda	0	8	2
Nonsecretory	4	0	0
Monoclonal proteinuria, % cases	52	46	41
Stage of disease, % cases			
Acc. Durie Salmon			
I			15
II	15	22	23
III	85	78	62
Acc. ISS			
I			40
II	20		32
III	80		28
Osteolysis present, % cases	67	85	72
Median survival time (months)	9 (0.5–46)	1.5 (0.2–7.0)	43 (2–132)

CD29/CD49d (VLA-4)

The molecule that showed the highest expression on bone marrow and peripheral blood plasma cells of all the analyzed adhesion molecules was CD49d (α chain of VLA-4 molecule) (Tables 4–6). Positivity of CD49d, as with CD29 positivity, was revealed in all PCL patients. A statistically significant difference in expression intensity of CD29 (CD29 RFI) was found between peripheral blood and marrow compartments ($p = 0.009$) (Table 6). Plasma cells from peripheral blood presented with a higher expression, while those of bone marrow presented with a lower one.

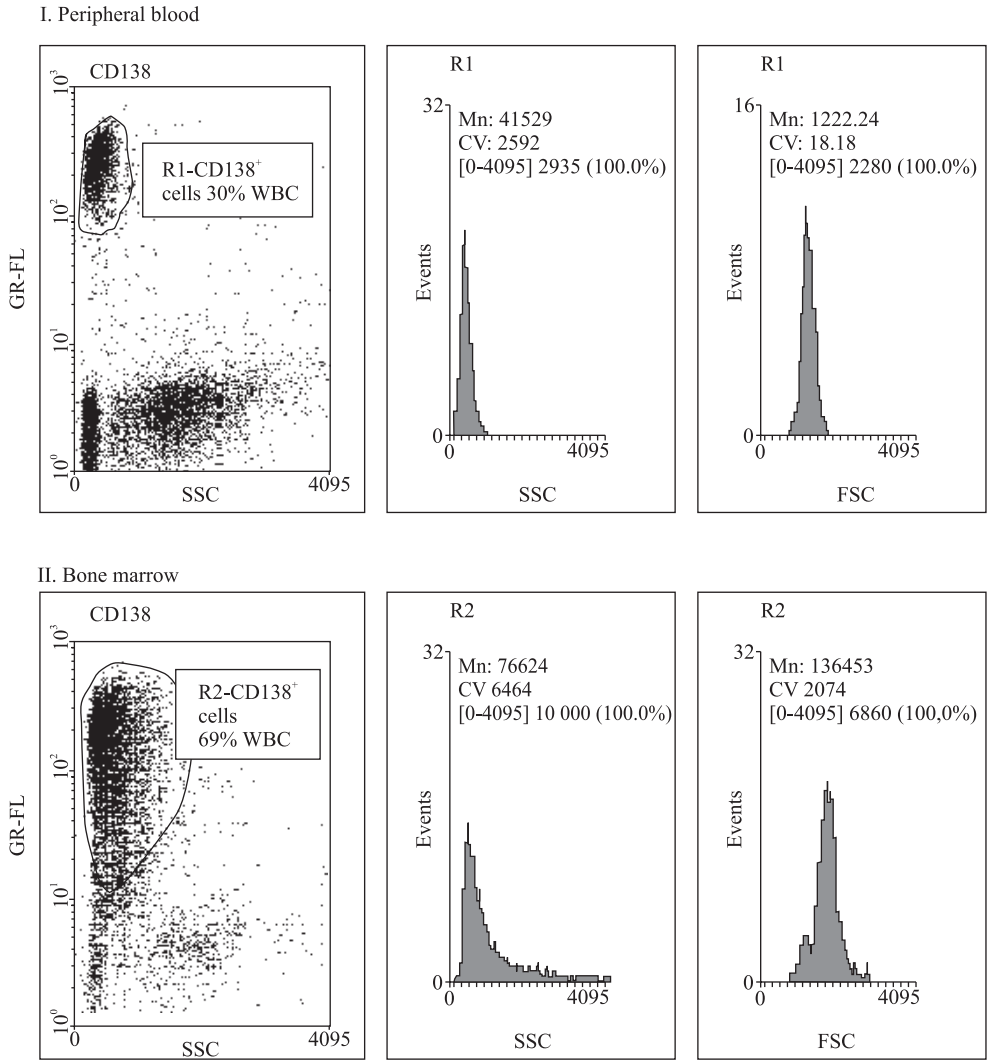

Figure 1. Comparison of CD138+ plasma cells in PCL in peripheral blood and bone marrow: PCL plasma cells in peripheral blood (I) show less granularity (SSC) and smaller size (FSC) than those in bone marrow (II)

Table 2. "Granularity" and size of peripheral blood and bone marrow PCL plasma cells and bone marrow MM plasma cells

Parameter	PCL Peripheral blood mean ± SD median (min.–max.)	p	PCL Bone marrow mean ± SD median (min.–max.)	MM Bone marrow mean ± SD median (min.–max.)	p
Granularity (SSC)	554 ± 191 480 (287–1064)	0.00014	786 ± 253 738 (384–1369)	833 ± 260 803 (436–1449)	0.249
Size (FSC)	1308 ± 186 1273 (1037–1784)	0.041	1439 ± 266 1349 (1085–2257)	1414 ± 240 1405 (926–2232)	0.659

"Granularity" — Side scatter (SSC); Size Forward scatter (FSC) — in cytometry linear scale

CD44 (H-CAM)

Expression of CD44 on peripheral blood and bone marrow plasma cells in PCL was high and uniform (Table 6). This contrasts with the expression of this antigen in MM where heterogeneity of CD44+ expression in bone marrow was found, with the proportion of CD44 positive cells ranging in individual patients from very low to very high. The difference in the rate of CD44+ cells in bone marrow between PCL and MM was statistically significant (p = 0.0062) (Table 7).

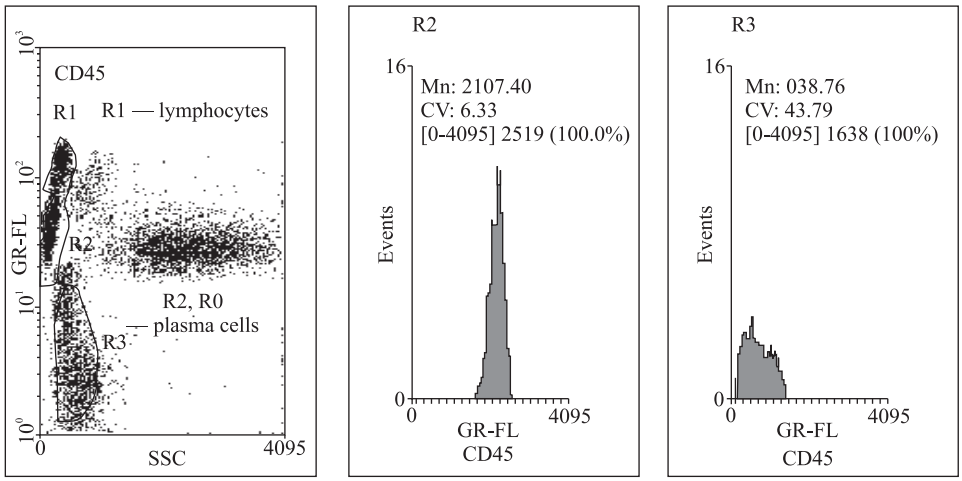

Figure 2. Differential CD45 expression intensity on peripheral blood CD138+ plasma cells in PCL patient: R2-CD45^{low}plasma cells, R3-CD45$^{low/negative}$ plasma cells

Table 3. CD45 expression intensity (RFI) on peripheral blood and bone marrow PCL plasma cells and bone marrow MM plasma cells

Parameter	PCL Peripheral blood CD45^{low} cells mean ± SD median (min.–max.)	p	PCL Bone marrow CD45^{low} cells mean ± SD median (min.–max.)	MM Bone marrow CD45^{low} cells mean ± SD median (min.–max.)	p
RFI	5.0 ± 6.3 2.3 (0.1–22.3)	0.1716	3.1 ± 3.6 1.8 (0.4–16.5)	3.3 ± 2.3 2.7 (0.3–12.0)	0.731
Score RFI*	0.2 ± 0.3 0.1 (0.001–0.8)	0.446	0.2 ± 0.2 0.1 (0.02–0.8)	0.17 ± 0.11 1.5 (0.04–0.59)	0.1176

*Score RFI = RFI CD45^{low} plasma cells/RFI CD45 lymphocytes

CD54 (ICAM-1)

A strong expression of CD54 on PCL peripheral blood and bone marrow plasma cells was found in 93% of patients (Tables 4–6) (Figure 3).

In MM patients, the expression of CD54 on bone marrow plasma cells was also found in 93% of patients. Expression of CD54 was high (Table 7). The mean percentage of cells with CD54 expression was 73 ± 25% (13–98), median 82, while mean CD54RFI was 15.8 ± 4.5 (8.7–25.1), median 15.7. The proportions of CD54+ cells and CD54 antigen intensity (RFI) were positively correlated (r= +0.58565; p = 0.001). This means that a plasma cell subset with a high proportion of CD54+ cells is characterized by high CD54 expression intensity on particular cells.

CD11a/CD18 (LFA-1)

Most PCL patients did not display expression of CD11a/CD18 (LFA-1) molecule on leukemic cells (Figure 3). The proportion of CD18+ plasma cells was higher in the peripheral blood than in bone marrow and the difference in the proportion of CD18+ plasma cells between those two compartments was statistically significant (p = 0.0014). CD18 expression intensity presented by CD18 RFI values behaved similarly (p = 0.0021).This phenomenon may be associated with distinct adhesive properties of these cells (Table 6).

Similarly, positivity of CD18 on plasma cells in peripheral blood was found in a higher proportion of patients than that in bone marrow (50% vs. 26%) (Table 5).

In PCL patients, a mean proportion of CD11a+ cells was higher in the peripheral blood than in bone marrow (25.2 ± 26.7% vs. 13.3 ± 17.9, respectively; p = 0.073) (Table 6). Similarly, slightly more PCL patients showed CD11a positive plasma cells in peripheral blood than in bone marrow (29% vs. 19%). Expression intensity (RFI) of CD11a was higher on plasma cells of peripheral blood and lower on those of bone marrow, and the difference was statistically significant (p = 0.003) (Tables 5, 6).

CD11b

CD11b expression was differential depending on whether the leukemic cells belonged to the bone marrow or the peripheral blood compartment. In PCL patients, the mean proportion of CD11b+ cells in the bone mar-

Table 4. Immunophenotype of bone marrow and peripheral blood plasma cells in particular patients with primary (cases 1–23) and secondary (cases 24–36) PCL

Case	Antigen	CD19	CD20	CD45^{low}	CD38	CD138	CD56	CD44	CD29	CD49d	CD54	CD18	CD11a	CD11b	CD58	CD117	CD71	CD126	CD10	CD33
1.	**BM**	–	+	46%	+	+	–	0	+	+	+	–	–	–	0	0	–	0	0	0
	PB	–	+	25%	+	+	–	0	+	+	+	+	+	–	0	0	–	0	0	0
2.	**BM**	–	+	74%	+	+	+	+	+	+	+	+	+	+	0	0	+	0	–	0
	PB	–	+	11%	+	+	+	+	+	+	+	+	+	+	0	0	+	0	–	0
3.	**BM**	–	0	22%	+	+	–	+	+	+	+	+	+	+	0	0	–	0	0	0
	PB	–	0	12%	+	+	–	+	+	+	+	+	+	+	0	0	+	0	0	0
4.	**BM**	–	0	41%	+	+	–	+		+	+	+	+	–	0	0	–	0	0	0
	PB	–	0	22%	+	+	–	+	+	+	+	+	+	+	0	0	–	0	0	0
5.	**BM**	–	0	38%	+	+	–	+	+	+	+	–	–	+	0	0	–	0	0	0
	PB	–	0	22%	+	+	–	+	+	+	+	–	–	+	0	0	–	0	0	0
6.	**BM**	–	+	74%	+	+	–	+	+	+	+	–	–	–	0	–	+	0	0	0
	PB	–	–	55%	+	+	–	+	+	+	+	–	–	–	0	–	+	0	0	–
7.	**BM**	–	–	57%	+	+	+	0	0	0	+	–	–	–	0	–	–	0	–	0
	PB	–	–	5%	+	+	+	0	0	0	+	–	–	–	–	–	–	0	–	0
8.	**BM**	0	0	27%	+	+	+	0	0	0	–	0	0	+	+	0	0	0	0	0
	PB	0	0	9%	+	+	+	0	0	0	+	0	0	0	–	0	0	0	0	0
9.	**BM**	–	+	59%	+	+	–	+	0	0	+	–	–	–	+	+	–	0	0	–
	PB	–	+	59%	+	+	–	+	0	0	+	–	–	–	–	+	–	0	–	–
10.	**BM**	0	0	37%	+	+	+	0	0	0	+	+	–	–	–	–	–	0	0	0
	PB	0	0	19%	+	+	+	0	0	0	+	+	–	–	–	–	–	0	0	0
11.	**BM**	0	0	42%	+	+	–	0	0	0	+	–	–	0	+	–	0	+	0	0
	PB	–	0	32%	+	+	–	+	+	+	+	–	–	0	+	–	0	+	–	–
12.	**BM**	–	–	59%	+	+	+	+	0	0	+	–	–	0	0	–	–	–	0	–
	PB	–	0	25%	+	+	+	+	0	0	+	–	–	0	0	–	–	0	0	–
13.	**BM**		0	64%	+	+	+	0	0	0	+	0	–	–	+	+	–	0	0	0
	PB	–	0	62%	+	+	+	0	0	0	+	0	–	–	+	+	0	0	0	0
14.	**BM**				+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	PB	–	0	56%	+	+	–	+	+	+	+	0	–	0	0	–	0	0	–	–
15.	**BM**	–	0	58%	+	+	–	+	+	+	+	0	–	0	0	+	0	+	–	–
	PB	–	0	79%	+	+	–	+	+	+	+	0	–	0	0	–	0	+	–	–
16.	**BM**	–	0	45%	+	+	–	+	+	+	+	+	+	–	0	0	–	+	0	0
	PB	–	0	25%	+	+	–	+	+	+	+	+	–	–	0	0	–	+	0	0
17.	**BM**	–	0	61%			–	+		+	+	–	–	–	0	–	0	+	0	–
	PB	0	0	41	+	+	–	0	+	+	+	–	–		0	0	0	+	0	0
18.	**BM**	–	–	54%	+	+	+	–	+	+	+	–	–	0	–	+	0	+	0	0
	PB	–	0	31%	+	+	+	+	+	+	+	–	–	0	0	+	0	+	0	0
19.	**BM**	–	0	42%	+	+	+	0	0	0	0	0	–	0	0	0	0	0	0	0
	PB	–	0	47%	+	+	+	+	+	+	+	–	–	0	0	–	0	+	0	0
20.	**PB**	–	–		+	0	0	0	0	0		–	–	–	0	0	–	0	–	0
21.	**PB**	–	0		+	+	0	0	0	0	0	0	0	0	0	+	0	0	0	–
22.	**BM**	–	0			+	0	0	0	0	0	0	0	0	0	–	0	0	0	0
23.	**BM**	–+	0		+	+	+	+	0	0	+	0	0	0	0	–	0	0	0	0
24.	**BM**	–	0	54%	+	+	+	+	+	+	+	–	–	–	0	0	+	0	0	0
	PB	–	0	10%	+	+	+	+	+	+	+	+	+	+	0	+	–	0	0	+

→

Table 4. Continued

25.	**BM**	0	0	88%	+	+	+	–	+	+	–	–	–	0	0	–	0	0	0	0
	PB	0	0	27%	+	+	+	–	+	+	–	–	–	0	0	–	0	0	0	0
26.	**BM**	–	0	88%	+	+	+	–	+	+	+	–	–	–	0	0	+	0	0	0
	PB	–	0	23%	+	+	+	+	+	+	+	+	+	+	0	0	+	0	0	0
27.	**BM**	0	0	31%	+	+	–	0	+	0	+	+	+	0	0	–	+	0	0	0
	PB	0	0	8%	+	+	–	0	+	0	+	+	+	0	0	–	+	0	0	0
28.	**BM**	–	0	40%	+	+	+	+	+	+	+	–	–	–	0	0	+	0	–	0
	PB	–	0	4%	+	+	+	+	+	+	+	+	+	+	0	0	–	0	–	0
29.	**BM**	–	0	38	+	+	+	0	–	0	+	–	–	0	0	+	–	0	0	0
	PB	–	0	7%	+	+	+	0	–	0	+	+	–	0	0	–	–	0	0	0
30.	**BM**	–	0		+	+	–	+	+	+	+	–	0	+		–	–	0	0	0
	PB	–	0		+	+	–	0	+	+	+	+	0	+	0	–	–	0	0	0
31.	**BM**	–	0	66%	+	+	+	0	0	0	–	–	–	0	–	–	–	0	0	0
	PB	–	0	18%	+	+	+	0	0	0	–	–	–	0	–	–	–	0	0	0
32.	**BM**	–	0	80%	+	+	+	+	+	+	+	0	–	0	0	–	0	+	–	0
	PB	–	0	42%	+	+	+	+	+	+	+	0	–	0	0	–	0	+	–	0
33.	**BM**	–	0	15%	+	+	+	0	0	0	0	0	0	+	0	0	–	0	0	0
	PB	–	0	6%	+	+	+	+	+	+	+	+	+	+	0	0	–	0	–	–
34.	**PB**	–	0	36%	+	+	–	+	+	+	+	0	–	0	0	±	0	+	0	–
35.	**PB**	–	0	42%	–	+	+	0	0	+	+	0	–	0	0	–	0	+	0	0
36.	**BM**	–	0	47%	+	+	+	0	+	+	+	–	–	0	0	0	0	+	0	0
	PB	–	0	23%	+	+	+	0	+	+	+	–	–	0	0	0	0	+	0	0

BMI — bone marrow plasma cells; PB — peripheral blood plasma cells; "+" — expressed; "–" — not expressed; 0 — not determined

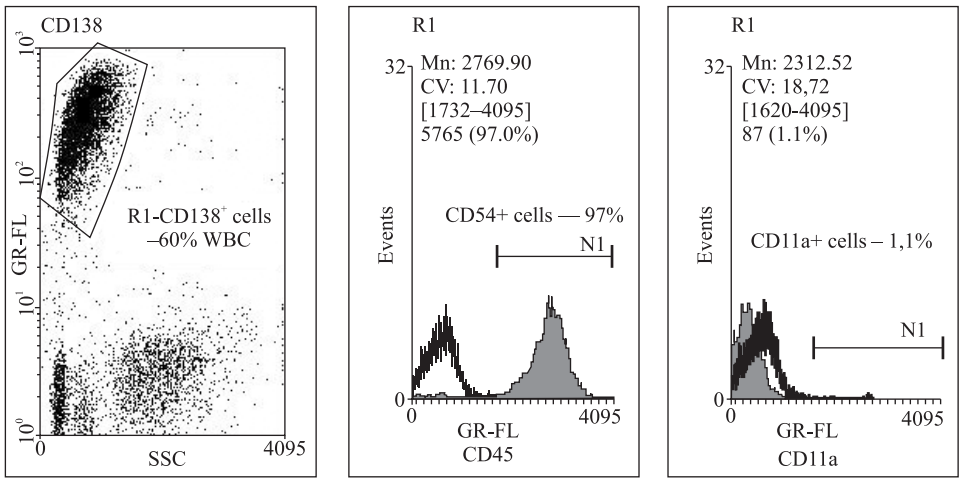

Figure 3. Differential expression of adhesion molecules on peripheral blood CD138+ plasma cells in PCL: high proportion of CD54+cells and low proportion of CD11a+ cells

row was less than half that in the peripheral blood (15.9 ± ± 23.7 vs. 32.7 ± 25.8, respectively; p = 0.0725) (Table 6). Similarly, fewer patients showed positivity of CD11b on plasma cells in the bone marrow than in the peripheral blood (29% vs. 53%) (Table 5).

CD56 (NCAM)

Expression of CD56 in PCL patients did not produce a uniform pattern. Nine of 12 (75%) patients with PCL in terminal stage of MM showed CD56 expression on bone marrow plasma cells at MM diagnosis, and on both bone marrow and peripheral blood plasma cells in the leukemic phase of disease. In the remaining three (27%) cases, CD56 expression was found neither at the time of MM diagnosis nor in the leukemic phase of the disease.

Only nine of 20 (45%) patients with primary PCL displayed CD56 expression on both bone marrow and peripheral blood plasma cells. A less frequent CD56 positivity was found in primary PCL (45% of cases) as

Table 5. Frequencies of analyzed antigens expression on bone marrow and peripheral blood plasma cells in patients with PCL

Antigen	CD 19	CD 20	CD 38	CD 138	CD 56	CD 44	CD 29	CD 49d	CD 54	CD 18	CD 11a	CD 11b	CD 58	CD 117	CD 71	CD 126	CD 10	CD 33
Bone marrow																		
Number of patients analyzed	26	5	32	32	30	18	17	17	28	23	27	17	6	18	20	7	5	4
Number of positive	0 (+1)*	4	32	32	18	15	16	17	26	6	5	5	4	5	6	7	0	0
Percentage positive	0	80	100	100	60	83	94	100	93	26	19	29	66	27	30	100	0	0
Peripheral blood																		
Number of patients analyzed	28	6	34	34	32	20	24	23	32	26	31	17	7	21	20	10	10	10
Number of positive	0	3	33	34	18	19	23	23	30	13	9	9	2	6	5	10	0	1
Percentage positive	0	50	97	100	56	95	96	100	93	50	29	53	28	28	25	100	0	10

*In 1 patient 2 plasma cell populations $CD19^-$ and $CD19^+$ present

Table 6. Relative numbers and relative fluorescence intensity (RFI) of bone marrow and peripheral blood plasma cell adhesion molecules subpopulations in PCL patients

Subpopulation	Percentage of subset cells		p	Relative fluorescence intensity		p
	Bone marrow mean ± SD median (min.–max.)	Peripheral blood mean ± SD median (min.–max.)		Bone marrow mean ± SD median (min.–max.)	Peripheral blood mean ± SD median (min.–max.)	
Plasma cells % WBC	50.3 ± 18.5 45 (16–86)	28.4 ± 20.2 23 (4–79)	0.0001			
CD49d	81.4 ± 23.6 88 (25–100)	85.9 ± 18.9 93.5 (38 –100)	0.5976	12.9 ± 4.4 11.5(7.7–18.3)	14.6 ± 3.3 13.1 (10.8–21.1)	0.4036
CD29	62.5 ± 28.8 64 (1–94)	69.5 ± 31.1 77 (11–99)	0.5168	7.9 ± 0.9 7.7 (6.9–9.6)	10.0 ± 2.4 9.9 (5.5–15.3)	0.0009
CD44	78.8 ± 33.1 97 (5–99)	83.7 ± 23.3 97 (27–100)	0.6470	13.6 ± 2.7 13.4 (8.2–17.7)	15.5 ± 4.8 13.9 (12.3–29.2)	0.2956
CD56	43.7 ± 40.3 50.5 (1–99)	40.4 ± 36.6 23 (1–99)	0.7568	16.5 ± 4.6 15.6 (10.0–25.6)	14.9 ± 3.3 15.8 (9.5–20.6)	0.1728
CD54	70.9 ± 31.1 87 (4–99)	72 ± 30.2 84 (1–99)	0.8962	16.2 ± 4.2 16.3 (8.9–25)	15.6 ± 3.2 14.7 (9.9–21.1)	0.6178
CD18	16.3 ± 16.0 11 (1–75)	36.7 ± 20.5 38.5 (1–82)	0.0014	12.8 ± 3.4 14.1 (4.1–16.4)	16.2 ± 1.5 16.6 (13.7–19.0)	0.0021
CD11a	13.3 ± 17.9 6.5 (1–90)	25.2 ± 26.7 16 (1–86)	0.073	14.4 ± 3.0 14.7 (9.7–18.5)	18.7 ± 3.7 19.9 (11.1–22.3)	0.003
CD11b	15.9 ± 23.7 7.5 (1–96)	32.7 ± 25.8 31 (1–88)	0.0725	17.3 ± 4.3 16.8 (10.8–23.1)	20.3 ± 3.0 19.9 (15.1–25.1)	0.0681
CD138	53.4 ± 18.0 54 (25–86)	30.9 ± 22.3 26 (4–79)	0.002	16.9 ± 4.4 18.2 (7.5–22.2)	17.6 ± 4.1 18.7 (11.3–23.5)	0.5537

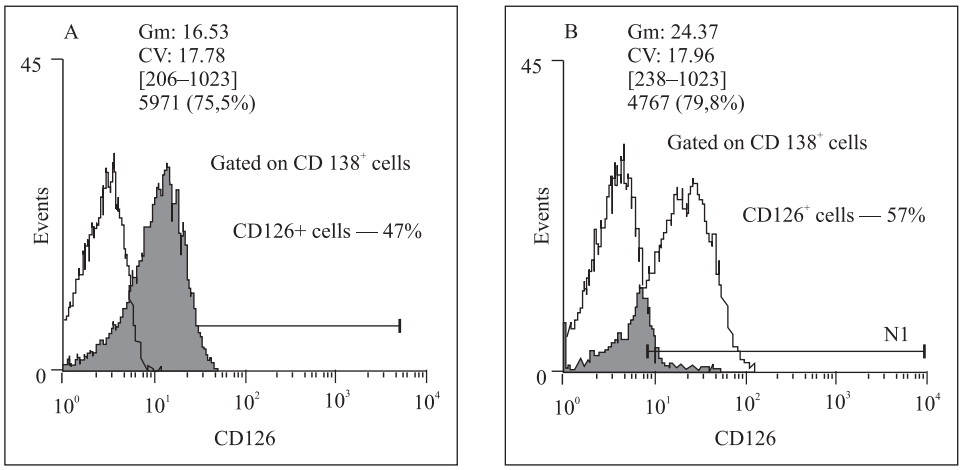

Figure 4. High expression of interleukin-6 receptor (CD126) on CD138$^+$ plasma cells in peripheral blood (A) and bone marrow (B) in PCL

Table 7. Comparison of relative numbers of bone marrow plasma cell CD138, CD44, CD54, CD56, CD71 subpopulations in PCL and MM patients

Subpopulation	Percentage of subset cells		p
	PCL patients mean ± SD median (min.–max.)	MM patients mean ± SD median (min.–max.)	
CD138	53 ± 18 54 (25–86)	21 ± 17 13 (4–68)	0.00001
CD44	79 ± 33 97 (5–99)	46 ± 36 45 (1–99)	0.0062
CD54	71 ± 31 87 (4–99)	73 ± 25 82 (13–98)	0.6207
CD56	44 ± 40 50 (1–99)	67 ± 37 75 (1–99)	0.01
CD71	22 ± 22 14 (1–68)	34 ± 26 29 (2–81)	0.0133

compared to PCL developed in the end phase of MM (75% of cases) (p = 0.04).

CD126 (IL-6R)

All ten analyzed patients with PCL showed expression of CD126 on plasma cells in both peripheral blood (mean: 61% ± 27%, median 53%, range 32–98%) and bone marrow (mean: 58% ± 23%, median 56%) (Figure 4).

CD117 (c-kit)

In seven out of 23 (30%) evaluated PCL patients, plasma cells showed CD117 expression, whereas in the other 16 (70%) patients they did not. CD117 antigen positivity was found in five patients with primary PCL and in two patients with PCL which developed in the end phase of multiple myeloma. In CD117-positive PCL cases, expression of CD117 was detected in bone marrow plasma cells as well as in peripheral blood plasma cells. In only one case, the bone marrow plasma cells expressed CD117, while those of peripheral blood did not.

CD71 (Tr1R)

The proportion of cells with expression of CD71 in the population of plasma cells in peripheral blood was low (mean value of CD71$^+$ cells was 16.5 ± 16.4, median 13.5, range 1–67). The figures representing proportions of CD71$^+$ cells of one quarter of patients were above the threshold value (20% and more). No differences in either proportion of CD71-positive cells or expression intensity of this antigen between peripheral blood and bone marrow compartments were found (p = 0.713 and p = 0.831, respectively). The proportion of CD71$^+$ cells in the bone marrow of MM patients was significantly higher than that of PCL patients (p = 0.013) (Table 7) while expression in-

tensity of CD71 on plasma cells of the bone marrow compartment was similar between those entities (mean: 9.0 ± 3.9, median: 7.7, range 3.6–17.6).

CD19

Expression of CD19 was found in none of the analyzed cases, but in one case of a patient with an unusual rarely occurring monoclonal protein of IgEκ class, two populations of cells were revealed in the bone marrow: large, granular cells displaying plasma cell features with $CD38^{++}$, $CD138^{+}$, $CD56^{+}$, $CD45^{-}$, $CD19^{-}$ immunophenotype constituted approximately 30% of all nucleated cells, while 11% of cells were lymphoplasmocytes with $CD19^{+dim}$, $CD38^{++}$, $CD138^{+}$ immunophenotype.

Results recapitulation

Overall, the expression of analyzed antigens on leukemic cells did not differ significantly between patients with primary PCL plasma cells and those with plasma cell leukemia terminating advanced MM. As previously mentioned, there were slight differences concerning CD56 antigen.

On the basis of our analysis of particular surface antigens expression on leukemic cells, it was possible to establish the immunophenotype of PCL cell as follows: $CD19^{-}$, $CD45^{-}$, $CD38^{++}$, $CD138^{+}$, $CD54^{+}$,$CD49d^{+}$, $CD29^{+}$, $CD44^{+}$, $CD126^{+}$. Expression of CD18, CD11a, and CD11b differed in two cell compartments: lower in the bone marrow and higher in the peripheral blood cells. In most patients, PCL cells did not display expression of CD56, CD71 and CD117 in both cell compartments.

With regard to the expression of adhesion molecules ('adhesion immunophenotype' of plasma cells in PCL), presented as a proportion of plasma cells with expression of a given molecule, one may arrange the following sequence of antigen expression on peripheral blood and bone marrow cells (in diminishing order): CD49d > CD44 > CD54 > CD29 > > CD56 > CD18 > CD11b > CD11a.

The expression of CD54 was significantly higher than that of adhesion molecules from integrins β_2 family. The levels of statistical significance (p) of this difference in relation to particular molecules were as follows: for CD11a in comparison between peripheral blood compartments ($p = 0.01$) and inter-bone marrow compartments ($p = 0.000006$); for CD18 in peripheral blood compartments ($p = 0.01$), and in bone marrow compartments ($p = 0.00007$); and for CD11b in comparison between peripheral blood compartments ($p = 0.02$) and in bone marrow compartments ($p = 0.0001$).

Discussion

Immunophenotypic analysis of leukemic cells showed a strong expression of CD38, and common presence of CD138 expression, in all PCL patients.

It is generally accepted that a strong CD38 expression is characteristic of myelomatous plasma cells. Our study unequivocally demonstrated the presence of CD38 expression on leukemic PCL cells, not only in the bone marrow but also in the peripheral blood, thus on those cells that had lost their communication with the bone marrow environment.

The presence of CD38 expression on myeloma cells beyond the bone marrow i.e. in peripheral blood or body fluids, has been reported [21, 23, 35, 36].

Our study revealed expression of CD138 on PCL leukemic cells. The CD138 antigen is nowadays considered as one of the most specific markers of monoclonal plasma cells. Our findings may be important, especially when taken together with the observation of Jourdan et al. [37] that expression of CD138 is limited exclusively to living, and not apoptotic, myeloma cells. It may importantly impact on the purging of malignant plasma cells from autologous stem cell grafts. A recently-reported regulatory function of CD138 in myeloma proliferation, resulting from involvement of syndecan-1 in both the angiogenesis and apoptosis processes, may explain the relationship between surface cell expression of this molecule and stimulation of plasma cell proliferation [37, 38]. CD138 plays a role of co-receptor for numerous myeloma cells growth factors such as BAFF/APRIL, EGF, and HGF [39, 40]. The demonstration of CD138 expression on PCL leukemic cells in our study aligns with the results of another analysis [23].

We also found that leukemic cells from bone marrow and peripheral blood compartments do not express LFA-1 in most PCL patients, while the expression of ICAM-1, a ligand for LFA-1, is very strong.

A disclosed expression pattern (high ICAM-1 and very low LFA-1 expression) on bone marrow cells in PCL resembles a common expression profile presented by bone marrow myeloma plasma cells. However, we should stress that expression of both ligands on leukemic PCL cells may be affected by the environment in which they reside. Thus, it has been shown that leukemic cells forming a cutaneous tumor show an immunophenotype $CD11a^{+}/CD18^{+}$ and $CD54^{-}$ ($LFA1^{+}$ and $ICAM\text{-}1^{-}$) that is the opposite to the one aforementioned [41]. This finding underscores once again the pivotal role of that ligand pair for migration and homing of tumor cells. A very significant decrease of LFA-1 expression found in both PCL cell compartments may be of particular importance for

PCL pathogenesis. Deficiency of LFA-1 molecule is believed to be a poor prognostic factor in monoclonal gammopathies, and other lymphoproliferative disorders, because it facilitates the escape of cells from immunological surveillance [42, 43]. A lack of expression of another integrin belonging to the LFA family, namely LFA-3 (CD58), on leukemic cells in PCL has also been reported [44]. In most patients in our study, peripheral blood and bone marrow leukemic cells did not display CD58 expression.

Most PCL patients in our study explicitly expressed another integrin molecule, VLA-4, on bone marrow and peripheral blood cells. Opinions on the role of late activation antigens in PCL are divided. Some authors have observed the expression of all these molecules on leukemic cells, while others have found the presence of VLA-4 combined with an absence of VLA-5 expression and stated that this surface antigen constellation was important for leukemic proliferation [45, 46]. The expression of VLA-4 on the leukemic cells is a requisite of their increased invasiveness because contact between this antigen and its ligand in a capillar wall leads into extravasation of those cells from the blood into extravascular space [47].

A similar role may be played by the third of the analyzed integrin molecules — CD11b. The characteristic pattern of CD11b expression observed in our study i.e. decreased bone marrow expression and increased expression on circulating cells, suggests a substantial role for this molecule in the leukemic process.

Acquisition of CD11b expression by peripheral blood leukemic cells facilitates egress of those cells through the capillar wall and leads to the expansion of proliferation which is so characteristic of the leukemic process [48]. It is also believed that among $CD11b^+$ cells, there may be a subset of cells which survive chemotherapy and are responsible for a post--treatment relapse of plasma cell proliferations [49]. Therefore, removal of these cells from the peripheral blood, by means of targeted immunotherapy or application of cell chemosensitivity methods, may be of relevant therapeutic importance [49].

The expression of H-CAM on leukemic cells analyzed in our study was high. H-CAM is a very important molecule for lympho- and plasma cell proliferations and its expression shows differential effects on plasma cell tumors [50, 51].This glycoprotein is involved both in inter-cellular interactions of tumor cells with other bone marrow cells and interactions of tumor cells with bone marrow extracellular matrix via its linkage with hyaluronic acid. CD44 plays a crucial role in the adherence of blood monoclonal cells into fibroblasts and belongs to those adhesion molecules whose direct contact with stromal receptors leads into a release of numerous cytokines [50, 52]. In some lymphoproliferative disorders, increased H-CAM expression has been found, especially in cases of extramedullar tumor expansion [53]. H-CAM may also have a regulatory effect on LFA-3 and CD2 interactions, thus on the relationship between plasma cells and T lymphocytes [44].

In our study, the object of analysis was the so-called 'standard CD44' without determining the expression of particular isoforms of this molecule. Expression of some of those isoforms, especially v6, v7, v8, and v10, unfavorably correlates with certain clinical determinants of plasma cell tumors, including duration of patients' survival time [54, 55].

An important finding of this analysis is the demonstration of differential expression of CD56 in PCL patients. In most patients with primary PCL, the bone marrow and peripheral blood plasma cells did not show CD56 expression, although in approximately 45% of patients, expression of this antigen was found. The CD56 antigen, identical with a 140 kD isoform of adhesion molecule N-CAM, shows an ability both to homotypic binding in intercellular interactions and to heterotypic binding with heparane sulfate — an extracellular matrix proteoglican [52]. The N-CAM is an example of a 'foreign' antigen for plasma cells with respect to lymphoid cell ontogenesis. Its acquisition, according to the first reports on this antigen expression in MM, imprints a 'mark of malignancy' on plasma cells [13, 56, 57]. However, some authors have questioned whether CD56 expression is exclusively limited to myeloma plasma cells [58].

On the other hand, a lack of CD56 expression is considered to be an immunophenotypic marker of leukemic cells in PCL. In addition, the appearance of a decreased CD56 expression on bone marrow plasma cells is considered an unfavorable factor and a hallmark of leukemic transformation of MM. Generally, the absence of N-CAM expression is attributed to a less mature plasma cell immunophenotype [43, 58–60].

As mentioned above, in our study, in 55% of primary PCL cases malignant plasma cells did not express CD56, while the incidence of CD56 positivity in secondary PCL did not differ from that observed in MM cases. Lack of CD56 expression on malignant plasma cells in 81% of patients with primary PCL or secondary PCL has been reported [22]. The effect of CD56 absence on malignant plasma cells on PCL development may result both from weaker myeloma cell-to-cell and myeloma cell-to-bone cell interactions in $CD56^{-/weak}$ MM and increased secretion of MMP-9 by $CD56^-$ cells leading to degradation of basal membrane and extramedullary spreading of malignant cells [22, 34].

The demonstration of CD56 expression heterogeneity in PCL patients in our study contrasts with opinions previously published. However, in one study performed on a larger group of PCL patients, the authors paid attention to a differential CD56 expression on leukemic cells [61]. Moreover, our previous study found that CD56 expression carries no distinct adverse prognosis and that lack of CD56 expression does not define a unique clinicopathological or prognostic entity in multiple myeloma [4].

In all analyzed PCL cases, a strong expression of IL-6 receptor (CD126) was found. IL-6 is widely accepted as the most relevant growth factor for myeloma cells *in vitro* and *in vivo* [15, 62, 63]. IL-6 affects the survival and proliferation of myeloma cells via autocrine and/or paracrine mechanisms. Our observation may explain the aggression of plasma cell proliferation in PCL and suggests that the application of IL-6 antibody in combination with IL-6 R antibody in PCL therapy could be more effective than IL-6 antibody alone [8, 12].

More than a quarter of our PCL patients' plasma cells displayed CD117 expression. C-kit (CD117), a product of c-kit protooncogene, is a 145 kD transmembrane protein with activity of class III receptor tyrosine kinase. Recent studies have found c-kit antigen to be a 'tumor associated marker'. Together with CD38 and CD138, it may be of value in identifying the malignant clone in minimal residual disease [3]. It may also prompt a consideration of the usefulness of therapy with tyrosine kinase inhibitors in the management of c-kit positive plasma cell proliferations [2, 5]. Mateo et al. suggested that the expression of CD117 is associated with more favorable outcomes [34, 64]. However, we did not observe this in our present or previous studies [3].

In a quarter of our PCL patients, plasma cells showed CD71 expression. Proliferating neoplastic cells require an adequate supply of iron. Thus the expression of transferrin receptor 1 (CD71), one of the 'classical' markers upregulated upon B-cell activation, is important. Ng et al. [65] have reported that an anti-human tranferrin receptor IgG3–avidin fusion protein inhibits the proliferation of human malignant B cells and plasma cells (by inducing lethal iron deprivation) and suggested that the molecule may be useful in the treatment of B-cell malignancies such as MM and PCL.

Lack of B-cell marker- $CD19^-$ expression was observed in all our PCL patients. We must emphasize that in one case of PCL with IgEκ monoclonal protein in a patient's bone marrow, apart from a population of large granular $CD38^{++}$, $CD138^+$, $CD56^+$, $CD45^-$, $CD19^-$ cells corresponding immunophenotypically to plasma cells, there was also present a less numerous $CD19^{+dim}$, $CD38^{++}$, $CD138^+$cell subset with lymphoplasmocyte features.

In the study by Bataille et al. [34], CD19 was found in 2.5% (nine out of 362) myeloma cases. Lack of CD19 constitutes a marker of plasma cell malignancy. The role of the absence of CD19 expression, if any, has yet to be elucidated.

The expression of a second lymphocyte marker, CD20, was assessed in seven cases of PCL only; in three cases, its presence was found. In the study by Robillard et al. [66], CD20 was found in 14% (29 out of 209) of myeloma cases at diagnosis.

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