CHARACTERISTICS OF THE METASTASIS-ASSOCIATED CIRCULATING CELLS: FEATURES OF SIDE SCATTER PARAMETERS

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It is difficult to detect the circulating tumor cells (CTCs) being through the epithelial–mesenchymal transition (EMT) terminal phase, since these do not express epithelial markers or show weak expression of those. This hampers assessment of the CTC prognostic potential. It has been shown that the circulating cells (CCs) with the $CD45^{-}EpCAM^{-}CK7/8^{-}CD24^{+}N\text{-}cadherin^{-}$ phenotype are associated with the risk of metastasis in breast cancer (BC). The study aimed to test CCs based on the side scatter parameters considering the expression of epithelial cell markers and CD11b. CC phenotypes were assessed by flow cytometry within the regions with low (SSC^{low}) and high (SSC^{high}) side scatter in 11 donors and 20 female patients with BC. All the $CD45^{-}EpCAM^{-}CK7/8^{-}CD24^{+}N\text{-}cadherin^{-}$ CCs were represented by the $CD11b^{-}$ and $CD11b^{+}$ phenotypes found in both SSC^{low} and SSC^{high} regions. Among eight $CD45^{-}mEpCAM^{-}CK7/8^{-}CD24^{+}N\text{-}cadherin^{-}$ CC phenotypes with different variants of co-expression of epithelial markers (E-cadherin, panCK, and icEpCAM) and CD11b found in patients, six showed signs of epithelial nature based on one of the markers, while another two showed no epithelial traits and predominated over other phenotypes (only these two phenotypes were found in donors). The differences in light scattering parameters of the CCs with the same phenotype is one more characteristic, the prognostic value of which remains to be uncovered. The E-cadherin and panCK expression in the absence of mEpCAM and presence of icEpCAM suggest that some CCs are tumor cells in the state of pronounced EMT. CCs showing co-expression of CD11b and epithelial markers can emerge due to hybridization with myeloid cells.

Keywords: breast cancer, circulating cells, flow cytometry, side scatter

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Compliance with ethical standards: the study was approved by the Ethics Committee of the Cancer Research Institute, Tomsk National Research Medical Center of the Russian Academy of Sciences (protocol No. 8 dated 17 June 2016) and conducted in accordance with Federal Laws of the Russian Federation (No. 152, 323, etc.), the Declaration of Helsinki (1964) and all later amendments and additions that regulate scientific research involving human biomaterial. All subjects submitted the informed consent to participation in the study.

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ХАРАКТЕРИСТИКА МЕТАСТАЗ-АССОЦИИРОВАННЫХ ЦИРКУЛИРУЮЩИХ КЛЕТОК ПРИ РАКЕ МОЛОЧНОЙ ЖЕЛЕЗЫ: ОСОБЕННОСТИ ПАРАМЕТРОВ БОКОВОГО СВЕТОРАССЕЯНИЯ

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Детекция циркулирующих опухолевых клеток (ЦОК), находящихся в терминальной стадии эпителиально-мезенхимального перехода (ЭМП), затруднена, поскольку они не экспрессируют или имеют слабую экспрессию эпителиальных маркеров. Это осложняет изучение их прогностического потенциала. Показано, что циркулирующие клетки (ЦК) с фенотипом $CD45^{-}EpCAM^{-}CK7/8^{-}CD24^{+}N\text{-}cadherin^{-}$ ассоциированы с риском метастазирования при раке молочной железы (РМЖ). Целью исследования было изучить ЦК в зависимости от параметров бокового светорассеяния, с учетом экспрессии маркеров эпителиальности и CD11b. У 11 доноров и 20 пациенток с РМЖ методом проточной цитометрии проводили оценку фенотипов ЦК в областях с низким (SSC^{low}) и высоким (SSC^{high}) боковым светорассеянием. Все $CD45^{-}EpCAM^{-}CK7/8^{-}CD24^{+}N\text{-}cadherin^{-}$ ЦК были представлены фенотипами $CD11b^{-}$ и $CD11b^{+}$, которые встречались как в SSC^{low}, так и в SSC^{high} областях. Из восьми обнаруженных у пациенток фенотипов $CD45^{-}mEpCAM^{-}CK7/8^{-}CD24^{+}N\text{-}cadherin^{-}$ ЦК с разными вариантами коэкспрессии эпителиальных маркеров (E-cadherin, panCK, и icEpCAM) и CD11b, шесть имели признаки эпителиальности по одному из маркеров, еще два не имели признаков эпителиальности и преобладали над прочими (у доноров встречались только такие два фенотипа). Различие параметров светорассеяния ЦК с одинаковыми фенотипами является дополнительной характеристикой, прогностическое значение которой предстоит выяснить. Экспрессия E-cadherin и panCK при отсутствии mEpCAM и наличие icEpCAM позволяют полагать, что часть ЦК являются опухолевыми в состоянии выраженного ЭМП. ЦК, коэкспрессирующие CD11b и эпителиальные маркеры, могут возникать вследствие гибридизации с миелоидными клетками.

Ключевые слова: рак молочной железы, циркулирующие клетки, проточная цитометрия, боковое светорассеяние

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Distant metastasis is the main cause of malignant neoplasm adverse outcomes. Cells of primary tumors capable of intravasation and generating the circulating tumor cells (CTCs) are the source of hematogenous metastasis. CELLSEARCH, the conventional method to determine CTCs, is based on isolation of CD45-negative, EpCAM-positive, and cytokeratin 8-, 18- and/or 19-positive CTCs from peripheral blood [1]. To date, various CTC detection and isolation techniques have been developed: separation by density gradient centrifugation; dielectrophoresis (method to isolate CTCs based on the cells' dielectric properties); microfluidic chip-based cell separation method; CTC enrichment involving the use of the magnetic-activated cell sorting (MACS) system (involves CTC labeling with the MACS superparamagnetic microspheres covered with the antibodies specific for the CTC surface antigens); use of magnetic beads covered with a thin layer of the hydrogel containing antibodies against EpCAM; reverse transcription polymerase chain reaction (RT-PCR); flow-cytometry-based detection methods [2]. In patients with late-stage breast cancer (BC), CTCs are found in 60% of cases, while in patients with early-stage disease these are found in 20–30% of cases [3]. The use of flow cytometry allows one to identify a broad range of functional markers in each CTC. With this technology, heterogeneity of stemness sign manifestations and the epithelial–mesenchymal transition manifestations in BC has been shown [4, 5]. In BC, progression-free survival is associated with CTCs. With the CTC counts ≥ 6, relapses and metastasis are more frequent, and survival rate is lower [6]. We have previously reported the cells in peripheral blood with the $CD45^{-}mEpCAM^{-}CK7/8^{-}CD24^{+}N\text{-}cadherin^{-}$ phenotype, which are associated with high risk of metastasis, along with the classic CTCs that express membrane EpCAM (mEpCAM) [7]. In contrast to CTCs, these cells have been classified as circulating cells (CCs). The nature and origin of CCs are poorly understood.

The important characteristics of any cell present in the bloodstream, including tumor cells, involve biophysical parameters that can partially reflect their morphofunctional state. Measurement of the cells' biophysical properties (such as electrical impedance, radio-frequency conductivity, light scattering from cells at various angles) has provided the basis for the automated hematology analysis methods. Flow cytometry techniques enable gathering information about the cells' size and structure through the detection of forward and side scatter parameters. As cells passes through the light stream emitted by a laser, both cell fluorescence and light scattering in various directions are recorded. Forward scatter (FSC) considers intensity of the light scattered at small angles of up to 10° (with detectors located along the laser beam) and provides data about cell size. Side scatter (SSC) considers intensity of the light scattered at the angles of up to 90° (with detectors positioned perpendicularly to the laser beam direction), depends on cell density, and characterizes the complexity of intracellular structures [8] and the extent of cytoplasmic granularity [9]. Furthermore, studies suggest that various cell structures have a subtle impact on side scatter parameters. For instance, light scattering at an angle of 5–30° is primarily caused by the cell nucleus, while scattering at angles of 50–130° is attributed by small organelles, such as mitochondria, peroxisomes, lysosomes, and granules [10].

In 2023, advancements in this method led to the development of an approach where cell granularity, as estimated based on SSC, can be used to differentiate functional lymphocyte subpopulations. Specifically, naïve undifferentiated lymphocytes were within the SSClow region, whereas cytotoxic lymphocytes characterized by high granule contents were detected in the SSC^{high} region [9].

The study aimed to clarify phenotypic characteristics of the $CD45^{-}mEpCAM^{-}CK7/8^{-}CD24^{+}N\text{-}cadherin^{-}$ cells in blood of female patients with breast cancer. The investigation focused on the analysis of these cells based on side scatter (SSC) properties and evaluation of epithelial cell markers such as E-cadherin, cytokeratins AE1/AE3 (pan Cytokeratin), as well as intracellular expression of the EpCAM adhesion molecules (icEpCAM). We assessed the indicated parameters in both female patients with invasive ductal carcinoma of no special type (IDC NST) and healthy donors.

METHODS

A prospective study involved 11 donors and 20 patients with IDC NST, who underwent treatment at the Cancer Research Institute, Tomsk National Research Medical Center RAS. Inclusion criteria for the study group: morphologically verified diagnosis of invasive ductal carcinoma of no special type; primary tumor extent T_1-$4N_0$-$3M_0$; luminal B $Her2^{-}$ (9 patients), luminal B $Her2^{+}$ (4 patients), triple negative (5 patients) and $HER2^{+}$ (2 patients) molecular biological subtypes. Exclusion criteria for the study group: other breast cancer histologic types; multiple primary malignant tumors; exacerbation of chronic inflammatory disorder. The group of donors was matched to patients based on age, and the primary inclusion criterion was the absence of any exacerbation of chronic inflammatory disorders. Venous blood samples were collected into EDTA-treated vacuum test tubes before surgery and neoadjuvant chemotherapy. Further sample preparation was performed in accordance with the previously reported protocol [11]. Monoclonal antibodies were used to stain surface markers: BV570-anti-CD45 (clone HI30, mouse IgG1; Sony Biotechnology, USA), PE-Cy7-anti-N-cadherin (clone 8C11, mouse IgG1; Sony Biotechnology, USA), BB700-anti-CD24 (clone ML5, mouse IgG2a; BD Horizont, USA), R718-anti-EpCAM (CD326) (clone EBA-1, mouse IgG1; BD Biosciences, USA), BV-421-anti-CD11b (clone ICRF44, mouse IgG1; BioLegend, UK), PE-Dazzle594-anti-E- cadherin (CD324) (clone 67A4, rat IgG1; Sony Biotechnology, USA). Intracellular staining was performed using the following: PE-anti-CK7/8 (clone CAM 5.2, mouse IgG2a; BD Biosciences, USA), eFlour660-anti-panCK (clone AE1/3, mouse IgG1; Invitrogen, USA), BV605-anti-EpCAM (CD326) (clone 9C4, IgG2b; Sony Biotechnology, USA). The MCF-7 breast cancer cells were used as positive controls when estimating fluorescence of antibodies against epithelial markers, whereas U937 promonocytic cells served as negative controls. The events detected within the high signal intensity region (7^{th} decade and above) were considered to be positive based on epithelial markers.

Immunofluorescence was conducted using the Novocyte 3000 flow cytofluorometer (ACEA Biosciences, USA) with the acompanying the NovoExpress 1.3.0 software package (ACEA Biosciences, USA). The granularity of the cells was evaluated based on the side scatter (SSC) parameter individually for each case. In the FSC/SSC two-dimensional plot, the SSC^{low}-circulating cells were localized within the region corresponding to populations of agranulocytes, which include lymphocytes and monocytes. SSC^{high}-cells were positioned within the region corresponding to the granulocyte population on the FSC/SSC two-dimensional plot. The median levels of the boundary between the SSC^{low} and SSC^{high} regions for this cytometer was 0.40 (0.36–0.45) (Fig. 1).

Statistical data processing was performed using the GraphPadPrism 9 software package (GraphPad Software, San

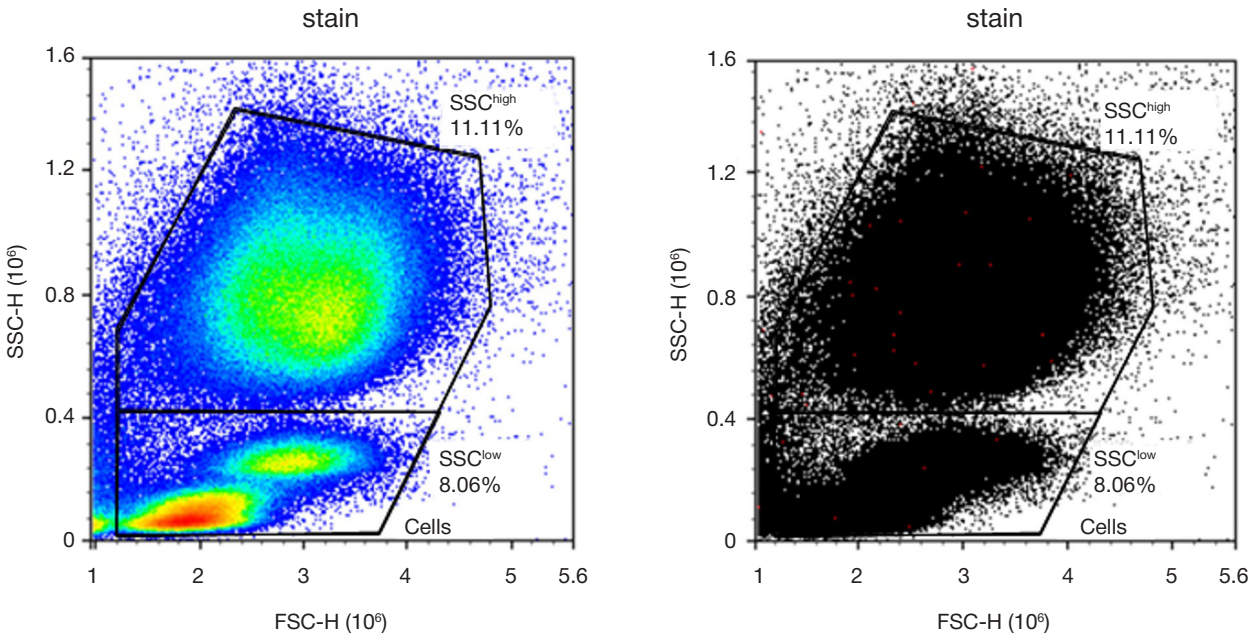

Fig. 1. Distribution of CCs ($CD45^-mEpCAM^-CK7/8^-CD24^+Ncadh^-$) within the SSC^{low} and SSC^{high} regions (cells of interest are *highlighted in red*)

Diego, CA, USA). Fischer's exact test was used to compare the abundance of various cell phenotypes. The cell phenotype counts were compared with each other using the nonparametric Wilcoxon test, and the nonparametric Mann–Whitney test was used to compare cell phenotype counts between donors and patients; the data were presented as Me (Q_1; Q_3). The results were considered significant at $p < 0.05$.

RESULTS

CC abundance and counts within the SSC^{low} and SSC^{high} regions in donors and patients with IDC NST

To clarify the CC nature we evaluated expression of the CD11b marker of myeloid origin, as well as of epithelial cell markers: E-cadherin, pan-Cytokeratin, and intracellular EpCAM (icEpCAM). Among 16 possible CC phenotypes, eight were found in the peripheral blood of patients with IDC NST. Furthermore, each cell expressed only one of the above epithelial markers. We compared the abundance (Table 1) and counts (Table 2) of these cell populations based on the side scatter degree: SSC^{low} or SSC^{high}.

Table 1 represents only those cell phenotypes that were detected in blood of patients with IDC NST. There were no differences in the abundance of cells with the studied CC phenotypes between the SSC^{low} and SSC^{high} regions. Regardless of the SSC parameter and CD11b expression, in the majority of cases (85–100%) CCs with phenotypes 1 and 5 showing no expression of epithelial markers were found. In 20–35% of cases, there were E-cadherin$^+$ cells among CCs. In six CC phenotypes, expression of only one studied epithelial marker was observed.

The total number of cells showing expression of epithelial markers (regardless of the CD11b expression and SSC parameter) was 1.9 cells per 1 mL of whole blood. CCs with phenotypes 1 and 5 were not only more often found in blood, but were the most numerous. The median of other six phenotypes was close to zero.

The number of CCs with phenotype 5 within the SSC^{high} region was 24 times higher, than that within the SSC^{low} region ($p < 0.0001$) (Table 2). In contrast to breast cancer patients, only two phenotypes of the studied cells were found in donors. It should be noted that these were the same most abundant phenotypes 1 and 5. There were no differences in abundance of the specified cell phenotypes depending on their location within the SSC^{low} and SSC^{high} regions, and the counts were not the same. The number of cells with phenotype 5 $CD11b^+Ecadherin^-panCK^-icEpCAM^-$ was 12.5 times higher within the SSC^{high} region compared to the SSC^{low} region ($p = 0.0020$), while the number of cells with phenotype 1 $CD11b^-E\text{-}cadherin^-panCK^-icEpCAM^-$, on the contrary, was 7.5 times higher within the SSClow region

Table 1. Comparison of CC abundance within the SSC^{low} and SSC^{high} regions in patients with IDC NST

CC phenotype ($CD45^-mEpCAM^-CK7/8^-CD24^+Ncadh^-$)	Abundance, % (abs.)	
	$n = 20$	
	SSC^{low}	SSC^{high}
(№1) $CD11b^-E^-cadh^-panCK^-icEpCAM^-$	85 (17/20)	100 (20/20)
(№2) $CD11b^-E^-cadh^-panCK^-icEpCAM^+$	0 (0/20)	10 (2/20)
(№3) $CD11b^-E^-cadh^-panCK^+icEpCAM^-$	5 (1/20)	10 (2/20)
(№4) $CD11b^-E^-cadh^+panCK^-icEpCAM^-$	35 (7/20)	20 (4/20)
(№5) $CD11b^+E^-cadh^-panCK^-icEpCAM^-$	95 (19/20)	100 (20/20)
(№6) $CD11b^+E^-cadh^-panCK^-icEpCAM^+$	10 (2/20)	10 (2/20)
(№7) $CD11b^+E^-cadh^-panCK^+icEpCAM^-$	10 (2/20)	25 (5/20)
(№8) $CD11b^+E^-cadh^+panCK^-icEpCAM^-$	15 (3/20)	25 (5/20)

Table 2. Comparison of CC counts within the SSC^{low} and SSC^{high} regions in patients with IDC NST

CC phenotype (CD45$^-$mEpCAM$^-$CK7/8$^-$CD24$^+$Ncadh$^-$)	Number of cells Me (Q_1–Q_3) / mL		p
	n = 20		
	SSC^{low}	SSC^{high}	
	a	b	
(№1) CD11b$^-$E$^-$cadh$^-$panCK$^-$icEpCAM$^-$	47.25 (10.38–97.25)	19.50 (3.13–48.50)	
(№5) CD11b$^+$E$^-$cadh$^-$panCK$^-$icEpCAM$^-$	11.75 (5.00–36.50)	287.00 (73.00–1132.00)	$p_{a-b} < 0.0001$

Table 3. Comparison of CC abundance and counts within the SSC^{low} and SSC^{high} regions in donors

CC phenotype (CD45$^-$mEpCAM$^-$CK7/8$^-$CD24$^+$Ncadh$^-$)			SSC^{low}	SSC^{high}	p
			a	b	
(№1) CD11b$^-$Ecadh$^-$panCK$^-$icEpCAM$^-$	abundance, % (abs.)	1	91 (10/11)	82 (9/11)	
	number of cells Me (Q_1–Q_3)	2	7.50 (1.50–12.50)	1.00 (0.50–7.50)	$p_{a-b} = 0.0156$
(№5) CD11b$^+$Ecadh$^-$panCK$^-$icEpCAM$^-$	abundance, % (abs.)	3	100 (11/11)	100 (11/11)	
	number of cells Me (Q_1–Q_3)	4	2.00 (1.00–7.50)	25.00 (3.50–61.00)	$p_{a-b} = 0.0020$; $p_{2-4} = 0.0020$

compared to the SSC^{high} region (p = 0.0156). Furthermore, in the SSC^{high} region, the number of cells with phenotype 5 CD11b$^+$Ecadherin$^-$panCK$^-$icEpCAM$^-$ was 25 times higher, than the number of cells with phenotype 1 CD11b$^-$Ecadherin$^-$panCK$^-$icEpCAM$^-$ (p = 0.0020) (Table 3). It is noteworthy that donors had no CCs showing expression of any epithelial cell marker used.

Comparison of the abundance and counts of various CC phenotypes in donors and patients with IDC NST

Comparison of the abundance and counts of various CC phenotypes in blood of donors and patients with IDC NST is of special interest due to the possibility that there is an association between CC identification in blood and the presence of cancer. We compared the abundance of CCs with the CD45$^-$mEpCAM$^-$CK7/8$^-$CD24$^+$Ncadherin$^-$ phenotypes and different variants of CD11b, E-cadherin, pan-Cytokeratin, and icEpCAM co-expression within the SSClow and SSChigh regions (Fig. 2). Two CC phenotypes were most often found within the SSC^{low} and SSC^{high} regions in both donors and patients with IDC NST: phenotype 1 CD11b$^-$Ecadherin$^-$panCK$^-$icEpCAM$^-$ and phenotype 5 CD11b$^+$Ecadherin$^-$panCK$^-$icEpCAM$^-$, i.e. cells the do not express the E-cadherin, pan-Cytokeratin, and icEpCAM epithelial cell markers (Fig. 2A, B). The same CC phenotypes (1 and 5) turned out to be both most abundant and most numerous in both regions (Fig. 2C, D). Cells with phenotype 4 CD45$^-$mEpCAM$^-$CK7/8$^-$CD24$^+$Ncadherin–CD11b$^-$Ecadherin$^+$panCK$^-$icEpCAM$^-$ were significantly more often found within the SSC^{low} region (p = 0.0331) (Fig. 2A), and the counts were higher at the division level (p = 0.0522) (Fig. 2C) in patients with IDC NST compared to donors. It is worthwhile to emphasize once again that no cells showing expression of epithelial cell markers were found in donors. In patients with IDC NST, CCs showing expression of epithelial cell markers were found, but the abundance was low.

The abundance of the cells showing expression of any epithelial marker within the SSC^{low} region was higher in patients with IDC NST, than in donors (no such cells were found in donors), regardless of the CD11b expression (p = 0.0331 and p = 0.0331, respectively), while within the SSC^{high} region this was reported for the CD11b$^+$ cells only (p = 0.0116) (Fig. 3A). The counts of such cells were higher in patients with IDC NST compared to donors only for the CD11b$^+$ cells within the SSChigh region (p = 0.0129) (Fig. 3B).

DISCUSSION

The assessment of the side scatter parameter (SSC) divided the studied cells into two distinct populations, which are located within the SSC^{low} and SSC^{high} regions. This division indicates differing level of cellular organization complexity, as suggested by the physical nature of the SSC parameter. Specifically, these differences encompass variations in the number of organoids and the extent of cytoplasmic granularity [8].

This criterion is likely to actually show cytoplasmic granularity, since the number of cells within the SSC^{low} and SSC^{high} regions depended on the CD11b myeloid marker expression in the cells. The number of the CCs with phenotype 5, which expressed CD11b, in both donors and cancer patients was higher within the SSC^{high} region, than within the SSC^{low} region. At the same time, there were no differences in the number of the CCs having the same phenotype, but showing no CD11b expression (phenotype 1) between the SSC^{low} and SSC^{high} regions. Assesment of the abundance has shown that each of the eight CC phenotypes was equally likely to be found within the the SSC^{low} and SSC^{high} regions. This suggests that the cells showing expression of the same cytokeratins and/or CD11b show different intracellular organization complexity. Based on the results of the study of lymphocytes with different functional activity [9], it can be noted that the cells showing high activity are located within the SSC^{high} region. This observation can likely to be applied for the studied CCs. Thus, CCs with identical phenotypes but differing in biophysical properties — and consequently in functional characteristics — may vary in their association with metastasis mechanisms. This nuanced understanding underscores the complexity of cellular behavior in cancer metastasis and underscores the potential significance of SSC parameters in evaluating cellular function and malignancy potential.

When discussing the CC epithelial traits, some methodological features of the study should be clarified. The study involved the utilization of the anti-EpCAM monoclonal antibodies labeled with two different fluorescent markers. This dual-labeling technique permitted the distinct detection and differentiation of EpCAM expression at the membrane and within the cell.

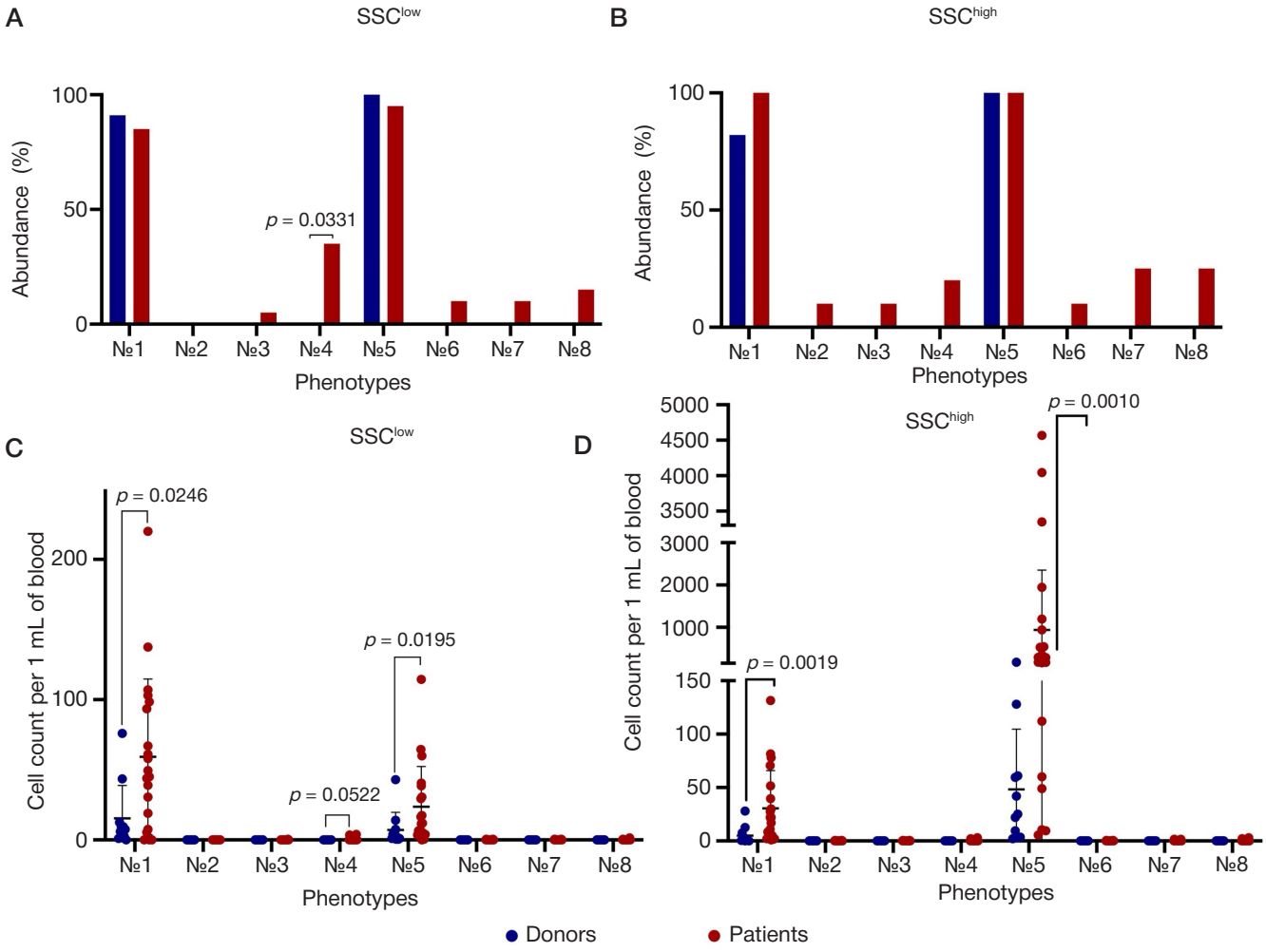

Fig. 2. Comparison of the abundance and counts in blood of donors and patients with IDC NST (main phenotype: CD45⁻mEpCAM⁻CK7/8⁻CD24⁺Ncadh⁻ — with co-expression of CD11b, E-cadherin, pan-Cytokeratin и icEpCAM) within the SSC^{low} and SSC^{high} regions. **A.** Comparison of CC abundance in donors and patients within the SSC^{low} region. **B.** Comparison of CC abundance in donors and patients within the SSChigh region. **C.** Comparison of CC counts in donors and patients within the SSC^{low} region. **D.** Comparison of CC counts in donors and patients within the SSC^{high} region

Specifically, antibodies with the first label were used for surface staining, while those with the second label were added post-permeabilization. The anti-EpCAM antibodies used in our study were produced by the EBA-1 and 9C4 cell clones. These antibodies are designed to detect the EpCAM marker both on the membrane and inside the cell, contingent upon whether the permeabilization phase is included in the procedure. [12]. It is noteworthy that staining following permeabilization may not exclusively reveal intracellular EpCAM expression due to the potential presence of accessible antigenic epitopes on the surface, even after the use of excess antibodies in the initial surface staining phase. However, in our study, not a single case exhibited cells with simultaneous EpCAM expression on both the membrane and intracellularly (within the cytoplasm or nucleus). This observation strongly suggests a genuine intracellular localization of EpCAM expression in the cells we analyzed.

The loss of membrane EpCAM expression during the EMT may occur due to molecule translocation [13, 14] or result from regulated intramembrane proteolysis (RIP) and endocytosis of mEpCAM, leading to its eventual degradation in proteosomes [15, 16]. In cases where there is an absence of mEpCAM and presence of icEpCAM alongside the expression of the other epithelial markers (E-cadherin and pan-Cytokeratin), it is plausable that some of the studied CCs are tumor cells undergoing pronounced EMT. These cells could, therefore, be considered CTCs. However, contemporary research suggests that these CCs may also be of non-tumor origins; for example, bone marrow-derived epithelial progenitor cells have been identified [17, 18]. There are data that the cells originating from the bone marrow can express proteins of epithelial cells and become epithelial cells in many organs. These cells do not express CD45 leukocyte marker, however, they do exhibit cytokeratins expression and are detected only following prior epithelial damage. This is considered to be an argument in favor of the fact that the bone marrow-derived epithelial progenitor cells are intended for regeneration [19–21]. Given these findings, it's possible that the studied CCs expressing epithelial markers—yet showing no CD45 expression—might be normal cells originating from the bone marrow. The absence of such cells in healthy donors does not contradict this hypothesis, as their prevalence might only rise to detectable levels under specific conditions, such as in loci undergoing regeneration or

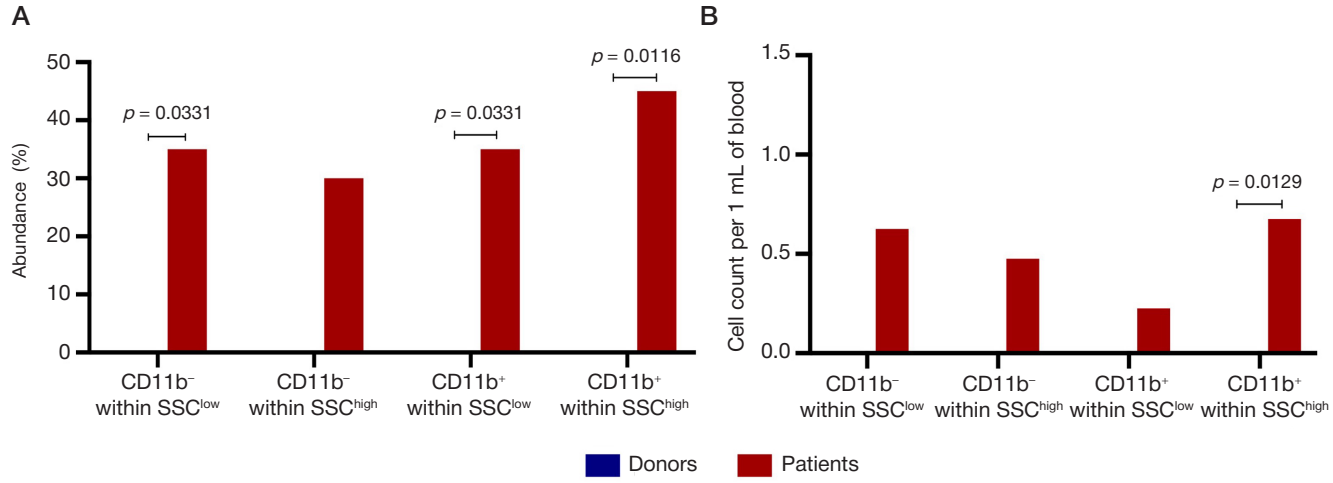

Fig. 3. Total abundance and counts of the CD11b⁻ and CD11b⁺ cell that express any epithelial cell marker (main phenotype: CD45⁻mEpCAM⁻CK7/8⁻CD24⁺Ncadh⁻, epithelial cell markers: E-cadh, panCK, icEpCAM) within the SSC^{low} and SSC^{high} regions. **A.** Comparison of the total abundance of the CC epithelial markers in donors and patients with IDC NST within the SSC^{low} and SSC^{high} regions. **B.** Comparison of total counts of the CC epithelial markers in donors and patients with IDC NST within the SSC^{low} and SSC^{high} regions

within the carcinoma microenvironment, which is commonly described as a "non-healing wound" [22, 23].

Two CC phenotypes, 1 and 5, are deprived of epithelial cell markers. CCs with phenotype 1 expressed CD24 only, while that with phenotype 5 expressed both CD24 and CD11b⁺. CCs with phenotypes 1 and 5 were not only more often found compared to other phenotypes detected in blood, but were also most numerous. As for origin of such cells, it can be assumed that these are epithelial cells, in which epithelial traits have been lost after achieving the EMT terminal phase, or these CCs are of non-epithelial origin and belong to another unknown population.

The CC phenotypes 1–4 were similar to phenotypes 5–8 based on eight studied markers out of nine. The only difference between these groups of phenotypes was CD11b⁺ expression in CCs with phenotypes 5–8. As is well known, integrin CD11b is expressed mainly on monocytes/macrophages and neutrophils, as well as some subpopulations of dendritic cells. CD11b represents an integrin alpha-M subunit (αM CD11bCD18), which is part of the αMβ2 heterodimer. This integrin serves as a receptor of fibrinogen and the ICAM-1 endothelial adhesion molecule [24]. CD11b mediates cell adhesion, chemotaxis, migration, phagocytic activity and inhibits inflammatory responses initiated via Toll-like receptors [25]. Immunosuppression is one of the most important functions of the myeloid cells that express CD11b [26]. What could be the origin of the CD11b⁺ CCs that we found? The CD11b expression is observed at the rather late promonocytic differentiation stage, while CD45 has to be expressed much earlier: at the monoblast stage [27]. In this regard, it is doubtful that the CD45⁻D11b⁺ CCs considered can belong to myeloid elements (especially cells that express epithelial markers). It is appropriate to consider the CCs showing co-expression of CD11b⁺ and epithelial markers as the hybrid cells emerging due to hybridization with myeloid cells. In this case, expression of leukocyte markers is expected. Not excluded the rarely studied and discussed EMT mechanism, in which not fibroblastic, but leukocyte traits manifest itself. Such EMT variant is substantiated in one of the reports [28]. Finally, if we accept the hypothesis of bone marrow origin of the CCs with epithelial traits, the presence of a myeloid marker can result from the nonlinear bone marrow stem cell differentiation process with the presence of cells with atypical phenotypes in the differentiation continuum.

It is important to note several limitations of the study:

The prospective character of the study has made it impossible to find out whether phenotypic features of the combination of SSC parameters with the CD11b⁺ expression and the presence of epithelial cell traits are associated with hematogenous metastasis and metastasis-free survival. A longer monitoring period will be required to adequately address these questions.

A small sample of donors (n = 11) could limit detection of rare CC phenotypes in the control group. The control group expansion will make it possible to clarify the presence of CCs showing expression of epithelial markers in donors.

Heterogeneity of the studied group of patients with IDC NST also represents a limitation of the study. Perhaps, assessment of the studied parameters in the groups of patients more homogenous based on molecular biological subtype will make it possible to reveal the associations of the CCs having epithelial traits with the less favorable BC subtypes.

CONCLUSIONS

The studied CCs represent a heterogenous population. All cell phenotypes were found within both SSC^{low} and SSC^{high} regions. There was a larger number of the CCs showing CD11b expression within the SSC^{high} region (p = 0.0020 for donors; p < 0.0001 for patients with IDC NST). In BC patients, among eight detected CC CD45⁻mEpCAM⁻CK7/8⁻CD24⁺N-cadherin⁻ phenotypes with different variants of co-expression of the epithelial markers (E-cadherin, pan-Cytokeratin, and icEpCAM) and CD11b, six phenotypes had epithelial cell traits based on one marker only. All the CC phenotypes were represented by two variants depending on the CD11b expression. Cells with two phenotypes, CD45⁻mEpCAM⁻CK7/8⁻CD24⁺N-Ecadherin⁻panCK⁻icEpCAM⁻CD11b⁻ and CD45⁻mEpCAM⁻CK7/8⁻CD24⁺N-Ecadherin⁻panCK⁻icEpCAM⁻CD11b⁺, were most frequently detected and numerous. Only these two CC phenotypes without epithelial traits were found in donors. The side scatter values of the CCs with the same phenotype represent an additional characteristic. Future studies will need to clarify the role of this trait in the association of the studied CCs with distant metastasis.

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