ASSESSMENT OF METASTATIC TRAITS OF THE CELLS WITH HYBRID PHENOTYPE IN BREAST CANCER

Mukhamedzhanov RK[1,4], Grigoryeva ES[2], Tashireva LA[2], Perelmuter VM[2], Zavyalova MV[1,2], Savelieva OE[2,3] ✉

[1] Siberian State Medical University, Tomsk, Russia
[2] Cancer Research Institute, Tomsk National Research Medical Center, Russian Academy of Sciences, Tomsk, Russia
[3] Saint-Petersburg State Pediatric Medical University, Saint-Petersburg, Russia
[4] Regional Children's Clinical Hospital, Vladimir, Russia

Nowadays, great attention is paid to the study of circulating tumor cells (CTCs) due to their key role in distant metastasis. At the same time there is little data on the properties of circulating cells showing simultaneous expression of the leukocyte and epithelial markers and their possible role in tumor progression and chemotherapy resistance. The study was aimed to assess subpopulations of cells with hybrid epithelial/leukocyte phenotype and estimate the features of stemness, epithelial–mesenchymal transition (EMT), and integrin interface, which determine the cells' possible metastatic properties in breast cancer (BC). The survey data from 128 patients with invasive breast carcinoma of no special type were included. Multicolor flow cytometry was used to assess the population structure and metastatic potential of the cells circulating in blood and primary tumor cells with hybrid phenotype. The primary tumor cell suspension was prepared by mechanical disaggregation. The high degree of heterogeneity was noted in the population of cells with hybrid phenotype, including the combination of the stemness and EMT features, and diverse integrin interface. Cells with hybrid phenotype are involved in the mechanisms underlying lymph node and distant metastasis. In lymph node metastasis, metastatic potential of these cells is associated with the stemness features (p = 0.0422) and co-expression of β3-, β4-, and αVβ5-integrins (p = 0.0338). In distant metastasis, metastatic potential of hybrid cells is associated with the stemness features (p = 0.015) and is not associated with the EMT features and integrin expression.

Keywords: hybrid cells, circulating tumor cells, metastasis, stemness, epithelial-mesenchymal-transition, integrins

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Compliance with the ethical standards: the study was approved by the Ethics Committee of the Cancer Research Institute, Tomsk National Research Medical Center (protocol № 10 of 24 April 2015) and conducted in accordance with the Federal Laws of the Russian Federation (№ 152, 323, etc.), the Declaration of Helsinki (1964) and all subsequent amendments and supplements regulating the research involving human biomaterials. The informed consent to study participation was submitted by all subjects.

✉ **Correspondence should be addressed:** Olga E. Savelieva
Litovskaya, 2, Saint-Petersburg, 194100; olga_chechina@mail.ru

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ОЦЕНКА МЕТАСТАТИЧЕСКИХ ХАРАКТЕРИСТИК КЛЕТОК С ГИБРИДНЫМ ФЕНОТИПОМ ПРИ РАКЕ МОЛОЧНОЙ ЖЕЛЕЗЫ

Р. Х. Мухамеджанов[1,4], Е. С. Григорьева[2], Л. А. Таширева[2], В. М. Перельмутер[2], М. В. Завьялова[1,2], О. Е. Савельева[2,3] ✉

[1] Сибирский государственный медицинский университет, Томск, Россия
[2] Научно-исследовательский институт онкологии, Томский национальный исследовательский медицинский центр Российской академии наук, Томск, Россия
[3] Санкт-Петербургский государственный педиатрический медицинский университет, Санкт-Петербург, Россия
[4] Областная детская клиническая больница, Владимир, Россия

Изучению циркулирующих опухолевых клеток (ЦОК) в последнее время уделяют большое внимание, благодаря их ведущей роли в формировании отдаленных метастазов. В то же время мало данных о свойствах циркулирующих клеток с одновременной экспрессией лейкоцитарных и эпителиальных маркеров и их возможной роли в опухолевой прогрессии и резистентности к химиотерапии. Целью исследования было изучить субпопуляции клеток с гибридным эпителиально-лейкоцитарным фенотипом, а также оценить признаки стволовости, эпителиально-мезенхимальный переход (ЭМП) и интегриновый интерфейс, обусловливающие их возможные метастатические свойства при раке молочной железы (РМЖ). В работу включены данные исследования 128 больных инвазивной карциномой неспецифического типа молочной железы. Для оценки популяционного состава и метастатического потенциала циркулирующих в крови клеток и клеток первичной опухоли с гибридным фенотипом использовали метод многоцветной проточной цитометрии. Суспензию клеток первичной опухоли готовили методом механической дезагрегации. В популяции клеток с гибридным фенотипом отмечена высокая степень гетерогенности, включая комбинацию признаков стволовости, ЭМП и разнообразный интегриновый интерфейс. Клетки с гибридным фенотипом принимают участие в механизмах лимфогенного и гематогенного метастазирования. При лимфогенном метастазировании метастатический потенциал этих клеток ассоциирован с признаками стволовости (p = 0,0422) и коэкспрессией β3-, β4- и αVβ5-интегринов (p = 0,0338). При гематогенном метастазировании метастатический потенциал гибридных клеток ассоциирован с признаками стволовости (p = 0,015) и не связан с признаками ЭМП и экспрессией интегринов.

Ключевые слова: гибридные клетки, циркулирующие опухолевые клетки, метастазы, стволовость, эпителиально-мезенхимальный переход, интегрины

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✉ **Для корреспонденции:** Ольга Евгеньевна Савельева
ул. Литовская, д. 2, г. Санкт-Петербург, 194100; olga_chechina@mail.ru

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Nowadays, great attention is paid to the study of circulating tumor cells (CTCs). This is due to their key role in distant metastasis and, therefore, in adverse outcomes of cancer. Today, the data are available on their subpopulation composition [1], stem-like properties [2], epithelial–mesenchymal transition (EMT) [3], chemotherapy resistance [4]; the genomic profiling data of CTCs have been also published [5]. Furthermore, the importance of integrin expression in CTCs for metastasis in breast cancer (BC) has been shown; the CTC integrin interface can be associated with the location of distant metastases [6–7]. The data on the correlation of peripheral blood levels of these cells with the survival rate and the risk of distant metastasis in BC [8], lung cancer [9], ovarian cancer [10], colorectal cancer [11], etc. are provided. Thus, in 2019 it was shown that the presence of CTCs showing stemness and partial EMT features was associated with adverse disease outcomes and reduced overall survival rate. Cells with the same phenotype turned out to be resistant to chemotherapy [12].

When studying various CTC populations, we have found unusual cells showing simultaneous expression of the CD45 leukocyte and CD326 (EpCAM) epithelial markers. It turned out that other researchers obtained similar results. Thus, cells with the $CD45^{+}CK^{+}EpCAM^{+}$ phenotype were found in blood of patients with BC, and in 90% of cases expression of the CD68 macrophage marker was also noted [13]. Cells with the $CD45^{+}EpCAM^{+}$ phenotype were found in primary tumors and pleural effusion of all surveyed patients with non-small cell lung cancer. Moreover, the higher percentage of such cells was associated with adverse outcome [14].

Hybridization (cell fusion) between tumor cells and macrophages or leukocytes is the most probable mechanism underlying generation of cells showing simultaneous expression of the leukocyte and epithelial markers. It has been noted that formation of hybrid cells is associated with many body's physiological processes, such as muscle and bone tissue formation, wound healing [15]. The $CD45^{+}EpCAM^{+}$ cells' physiological role is confirmed by detection of these cells in blood of healthy donors. However, biological value of these cells together with their role in physiological and disease processes is poorly understood.

Due to the lack of knowledge about the cells showing simultaneous expression of the leukocyte and epithelial markers and the data on their possible role in tumor progression and chemotherapy resistance [16], our study was aimed to assess subpopulations of cells with hybrid epithelial/leukocyte phenotype and estimate the features of stemness, EMT, and integrin interface, which determine the cells' possible metastatic properties in breast cancer (BC).

METHODS

Patients

Survey results of 128 patients treated in the clinics of the Cancer Research Institute, Tomsk National Research Medical Center, in 2015–2020 were included in the study. Inclusion criteria: invasive breast carcinoma of no special type; age 29–76 years (average age: 52.56 ± 11.57; $T1^{-}4N0^{-}3M0^{-}1$). Exclusion criteria: breast cancer of other histological type; multiple primary malignant tumors; exacerbation of chronic inflammatory disorder. To assess the population structure and metastatic traits of the circulating cells with hybrid phenotype, venous blood was collected from 108 patients before neoadjuvant chemotherapy. The association between cells with hybrid phenotype and lymph node or distant metastasis was estimated in the group of patients without neoadjuvant chemotherapy (n = 79). To assess properties of the cell populations with the primary tumor hybrid phenotype, surgical material obtained from 35 patients during surgical treatment (radical mastectomy or sectoral resection) was studied. These patients were not prescribed neoadjuvant therapy. The basic clinical and morphological parameters are provided in Table. 1.

Sample preparation for flow cytometry

The patients' venous blood was collected before the course of neoadjuvant chemotherapy and surgical treatment in the morning in the fasting state: 12 mL in the EDTA vacuum tubes. The entire volume of blood was used to prepare cell concentrate by sedimentation at 37 °C for 90 min at an angle of 45° with subsequent collection of buffy coat with cells on the boundary between the erythrocyte sediment and the separated blood plasma, as well as the entire supernatant, in accordance with the method by R.A. Pospelova [17].

Fresh cancer tissue samples were mechanically disaggregated using the BD Medimachine System (BD; USA) for cell suspensions. The total cell count of the resulting suspensions was determined using the Luna-II Cell Counter system (Logos Biosystems; Korea).

Flow cytometry of samples and data processing

After Fc blocking with the Human TruStain FcX™ Fc Receptor Blocking Solution (Biolegend; USA), 5 μL of the BV570 anti-human CD45 (clone HI30; Sony Biotechnology, USA), BV650 anti-human CD326 (EpCAM) (clone 9C4; Sony Biotechnology, USA), BV510 anti-human CD44 (clone G44-26; BD Horizon, USA), PerCP/Cy5.5 anti-human CD24 (clone ML5; Sony Biotechnology, USA), PE/Cy7 anti-human N-Cadherin (clone 8C11; Sony Biotechnology, USA) monoclonal antibodies and 7-AAD Viability Staining Solution (Sony Biotechnology; USA) were added to the primary tumor cell concentrate and/or cell suspension and incubated in the dark at room temperature for 20 min. Cell concentrate was also supplemented with 5 μL of the BV 421-anti-β3 integrin (clone VI-PL2; BD Biosciences, USA), Alexa Fluor 488-anti-β4 integrin (clone 422325; R&D Systems, USA), BV Alexa Fluor 750-anti-αVβ5 integrin (clone P5H9; R&D Systems, USA) monoclonal antibodies. The unstained control was processed in parallel. After incubation, 500 μL of the OptiLyse C buffer (Beckman Coulter; France) were added to the samples for erythrocyte lysis, then the samples were washed in 2 mL of the CellWASH solution (BD Biosciences; USA) for 10 min at 300 g with subsequent removal of supernatant. During the intracellular staining phase each stained sample was supplemented with 250 μL of the BD Cytofix/Cytoperm solution (BD Biosciences; USA), incubated in the dark for 30 min at 4 °C, and then twice washed in 1 mL of the BD Perm/Wash buffer (BD Biosciences; USA) when centrifuged at 300 g for 6 min. A total of 50 μL of the BD Perm/Wash buffer (BD Biosciences; USA) were added to the samples, the stained sample was supplemented with 5 μL of the AF647-anti-human CK7/8 antibody (clone CAM5.2; BD Pharmingen, USA) and incubated at 4 °C for 20 min. After that each sample was washed in 1 mL of the CellWASH buffer (BD Biosciences; USA) by centrifugation at 300 g for 6 min. In the final phase 500 μL of the Cell Staining Buffer (Sony Biotechnology; USA) were added to precipitate, and the sample was resuspended.

Nonspecific staining was controlled using appropriate isotype antibodies. The BC MCF-7 cell line was used as a positive control for antibodies against epithelial markers EpCAM

Table 1. Characteristics of patients with invasive breast carcinoma of no special type

Parameter	Parameter value	Frequency, % (abs.)
Age	52.56 ± 11.57	
Molecular subtype	Luminal A	32.8% (42/128)
	Luminal B	47.7% (61/128)
	Triple negative	15.6% (20/128)
	HER2neu+	3.9% (5/128)
Stage	I	25.0% (32/128)
	IIA	48.4% (62/128)
	IIB	17.2% (22/128)
	IIIA	1.6% (2/128)
	IIIB	4.7% (6/128)
	IIIC	3.1% (4/128)
Lymph node metastasis	Yes	34.4% (44/128)
	No	65.6% (84/128)
Distant metastasis	Yes	9.4% (12/128)
	No	90.6% (116/128)
Estrogen receptors	Positive	80.5% (103/128)
	Negative	19.5% (25/128)
Progesterone receptors	Positive	71.9% (92/128)
	Negative	28.1% (36/128)
HER2 receptors	Positive	51.6% (66/128)
	Negative	48.4% (62/128)
Ki67 expression	< 20%	35.9% (46/128)
	>20%	64.1% (82/128)
Tumor size	< 2 cm	28.1% (36/128)
	2–5 cm	67.2% (86/128)
	> 5 cm	4.7% (6/128)
Menstrual status	Postmenopausal	41.4% (53/128)
	Premenopausal	54.7% (70/128)
	Perimenopausal	3.9% (5/128)
Neoadjuvant chemotherapy	Yes	38.3% (49/128)
	No	61.7% (79/128)
Tumor grade	1	17.2% (22/128)
	2	63.3% (81/128)
	3	19.5% (25/128)
Recurrence	Yes	5.5% (7/128)
	No	94.5% (121/128)

and CK7/8 and a negative control for CD45. The histiocytic lymphoma U937 cell line was used as a negative control for antibodies against the above epithelial markers and a positive control for CD45.

The samples were analyzed in the Novocyte 3000 flow cytometer (ACEA Biosciences; USA) using NovoExpress 1.3.0 (ACEA Biosciences; USA). The concentration of circulating cells per 1 mL of blood and the concentration of primary tumor cells per 1000 tumor cells were calculated. When gating cells with hybrid phenotype, the cells were first analyzed in the FSC vs. SSC mode, then singlets were isolated in the FSC-A vs. FSC-H mode. After that viable cells were isolated based on the 7-AAD negative stain, and then cell fluorescence parameters were analyzed in appropriate channels. The gating strategy for cells with hybrid phenotype is provided in Fig. 1.

Statistical analysis

Statistical processing of the results was performed using the IBM SPSS Statistics 22 (Armonk; USA) and GraphPad Prism 8.3.1 (GraphPad Software; USA) software packages. All the studied parameters were tested for normality using the Shapiro–Wilk test. Parameters were described using median (Me) and interquartile range (Q_1–Q_3). The differences in parameters were assessed using the Mann–Whitney U test and the Wilcoxon signed-rank test. Fisher's exact test was used to estimate the differences in the traits' frequency. Spearman's rank correlation coefficient was calculated to determine the relationships between the traits. The differences were considered significant at $p < 0.05$ (5%).

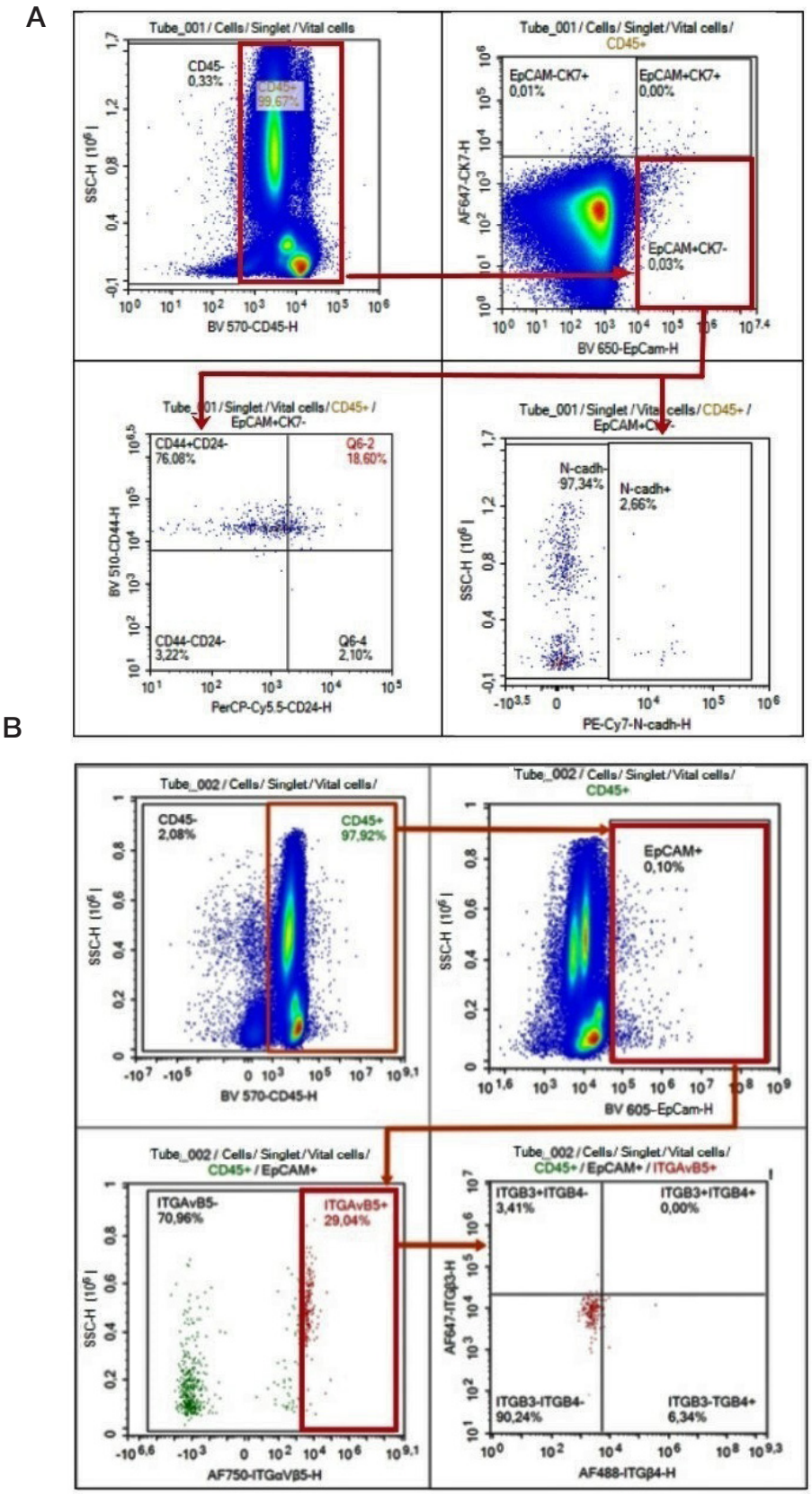

Fig. 1. Gating strategy for the populations of cells with hybrid phenotype and expression of the leukocyte, epithelial, stem and EMT markers (**A**), and integrin receptors (**B**) exemplified by the cells circulating in blood

Logistic regression was used as a multivariate method to assess the relationships between the traits and build the prognostic models. When building mathematical models, the threshold values were determined by ROC analysis. The probability of an event was calculated using the following formula: $P = e^Y/(1+e^Y)$, where P was the probability of a trait; Y was a regression equation value; e was a mathematical constant equal to 2.72. When the probability $P \geq 50\%$, the risk of the event was considered to be high; when the probability $P < 50\%$, the risk was considered to be low. The differences were considered significant at $p < 0.05$ (5%).

Table 2. Frequency of cells with hybrid phenotype in patients with breast cancer

№	Phenotype	Frequency, %	Significance level
Blood			
1	$CD45^+EpCAM^+CK7/8^-$	93.5% (101/108)	$p_{1-2} = 0.1137$; $p_{1-3} = 0.0045$
2	$CD45^+EpCAM^+CK7/8^+$	86.1% (93/108)	$p_{2-3} = 0.2785$
3	$CD45^+EpCAM^-CK7/8^+$	79.6% (86/108)	
Primary tumor			
4	$CD45^+EpCAM^+CK7/8^-$	94.6% (35/37)	$p_{4-1} = 1.0000$; $p_{4-5} = 0.0123$; $p_{4-6} = 0.0001$
5	$CD45^+EpCAM^+CK7/8^+$	70.3% (26/37)	$p_{5-2} = 0.0452$; $p_{5-6} = 0.2305$
6	$CD45^+EpCAM^-CK7/8^+$	54.1% (20/37)	$p_{6-3} = 0.0046$

RESULTS

Subpopulation composition of cells with hybrid phenotype

Flow cytometry was used to estimate the expression of the CD45 leukocyte marker and the EpCAM and CK7/8 epithelial markers in the circulating cells and primary tumor cells. The $CD45^+$ cell populations showing co-expression of two epithelial markers ($CD45^+EpCAM^+CK7/8^+$) and mono-expression of one epithelial marker ($CD45^+EpCAM^+CK7/8^-$, $CD45^+EpCAM^-CK7/8^+$) were found in blood and primary tumors of the majority of patients (Table 2).

The largest population most often found in both blood and primary tumor was represented by cells with the $CD45^+EpCAM^+CK7/8^-$ phenotype (Table 2; Fig. 2A).

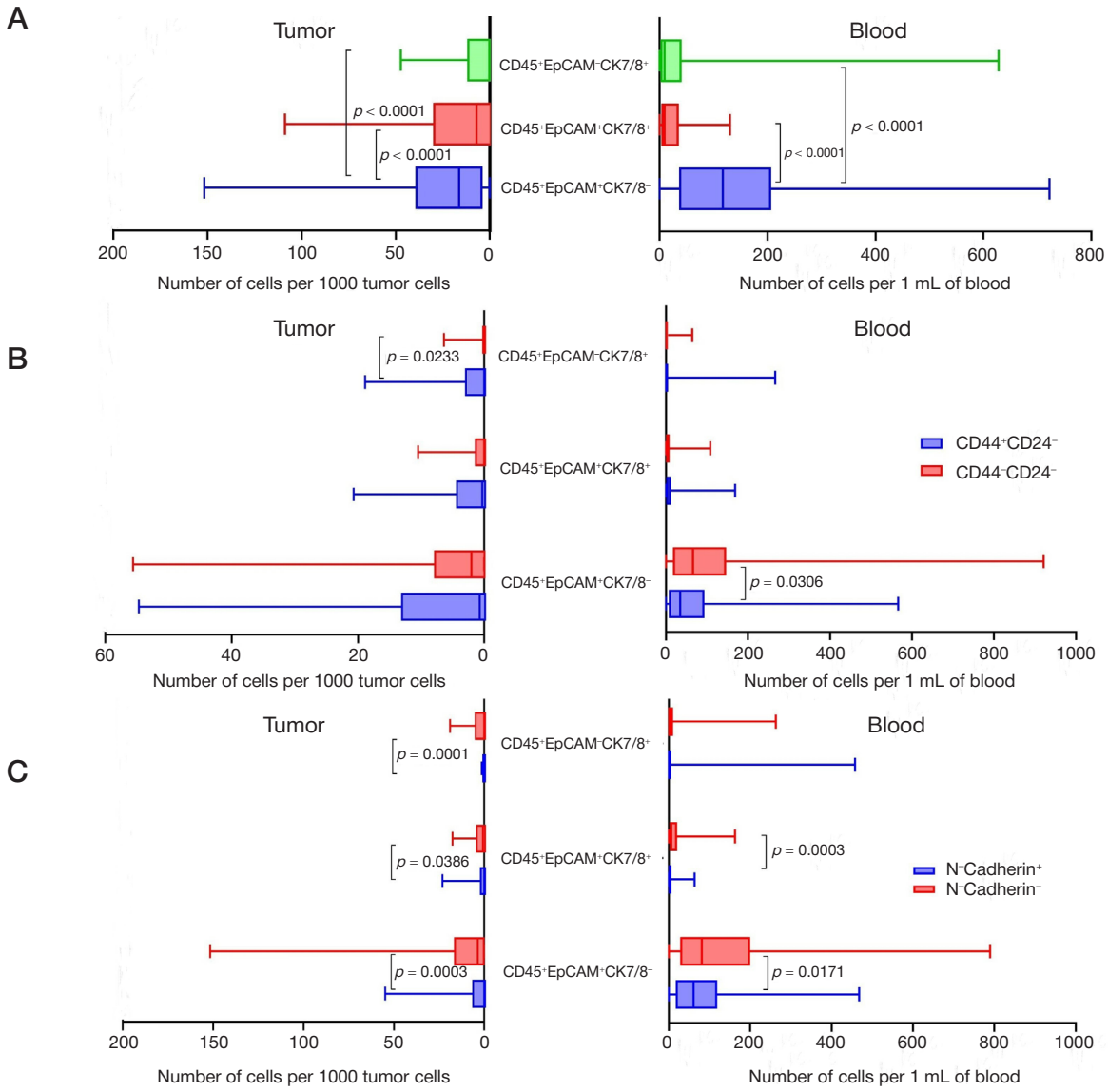

Fig. 2. Number of cells with hybrid phenotype in blood and primary tumors of patients with breast cancer. **A**. Number of cells with various combinations of the CD45 leukocyte and EpCAM and CK7/8 epithelial markers expression. **B**. Number of cells showing stem features. **C**. Number of cells showing features of epithelial–mesenchymal transition

Table 3. Frequency of cells with hybrid phenotype showing stem features in patients with breast cancer

	№	Phenotype	Frequency, %	Significance level
Blood	CD45$^+$EpCAM$^+$CK7/8$^-$			
	1	CD44$^+$CD24$^-$	85.7% (54/63)	$p_{1-2} = 0.0540$
	2	CD44$^-$CD24$^-$	96.8% (61/63)	
	CD45$^+$EpCAM$^+$CK7/8$^+$			
	3	CD44$^+$CD24$^-$	77.8% (49/63)	$p_{3-4} = 0.4179$
	4	CD44$^-$CD24$^-$	69.8% (44/63)	
	CD45$^+$EpCAM$^-$CK7/8$^+$			
	5	CD44$^+$CD24$^-$	47.6% (30/63)	$p_{5-6} = 0.4760$
	6	CD44$^-$CD24$^-$	55.6% (35/63)	
Primary tumor	CD45$^+$EpCAM$^+$CK7/8$^-$			
	7	CD44$^+$CD24$^-$	62.2% (23/37)	$p_{7-8} = 0.2030$; $p_{7-1} = 0.0126$
	8	CD44$^-$CD24$^-$	78.4% (29/37)	$p_{8-2} = 0.0048$
	CD45$^+$EpCAM$^+$CK7/8$^+$			
	9	CD44$^+$CD24$^-$	51.4% (19/37)	$p_{9-10} = 0.3497$; $p_{9-3} = 0.0081$
	10	CD44$^-$CD24$^-$	37.8% (14/37)	$p_{10-4} = 0.0030$
	CD45$^+$EpCAM$^-$CK7/8$^+$			
	11	CD44$^+$CD24$^-$	43.2% (16/37)	$p_{11-12} = 0.0811$; $p_{11-5} = 0.6842$
	12	CD44$^-$CD24$^-$	21.6% (8/37)	$p_{12-6} = 0.0015$

No significant correlations were revealed for the number of cells with hybrid phenotype in blood and primary tumors of BC patients.

Assessment of the metastatic traits in cells with hybrid phenotype

Acquisition of the stemness and EMT features by tumor cells, including CTCs, is associated with their capability of self-renewal, anticancer therapy resistance, and metastatic potential increase [3, 12, 18]. Furthermore, the integrin expression in CTCs plays an important role in metastasis and is likely to promote targeting distant organs by these cells, thereby determining the location of prospective metastases [6–7]. The same properties can be possessed by CTCs with hybrid phenotype. In this regard we have analyzed metastatic potential of the cells with hybrid phenotype circulating in blood and primary tumor cells by assessing the features of stemness, EMT, and expression of integrin receptors.

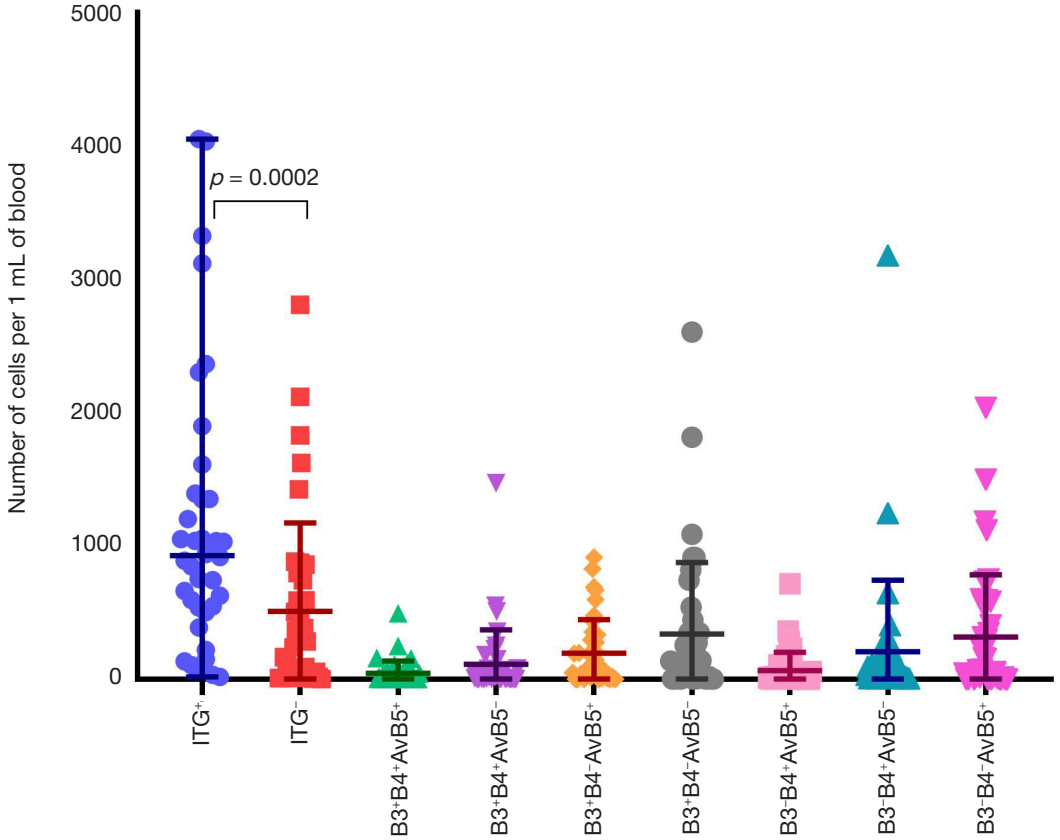

Fig. 3. Number of cells with hybrid phenotype showing expression of β3, β4, and αVβ5 integrin molecules in blood of patients with breast cancer

Table 4. Frequency of cells with hybrid phenotype showing EMT features in patients with breast cancer

	№	Phenotype	Frequency, %	Significance level
Blood	CD45⁺EpCAM⁺CK7/8⁻			
	1	N-cadherin⁺	92.1% (58/63)	$p_{1-2} = 0.7175$
	2	N-cadherin⁻	95.2% (60/63)	
	CD45⁺EpCAM⁺CK7/8⁺			
	3	N-cadherin⁺	65.1% (41/63)	$p_{3-4} = 0.0235$
	4	N-cadherin⁻	84.1% (53/63)	
	CD45⁺EpCAM⁻CK7/8⁺			
	5	N-cadherin⁺	52.4% (33/63)	$p_{5-6} = 0.5909$
	6	N-cadherin⁻	58.7% (37/63)	
Primary tumor	CD45⁺EpCAM⁺CK7/8⁻			
	7	N-cadherin⁺	45.9% (17/37)	$p_{7-8} = 0.0004$; $p_{7-1} = 0.0000$
	8	N-cadherin⁻	86.5% (32/37)	$p_{8-2} = 0.1419$
	CD45⁺EpCAM⁺CK7/8⁺			
	9	N-cadherin⁺	29.7% (11/37)	$p_{9-10} = 0.0340$; $p_{9-3} = 0.0009$
	10	N-cadherin⁻	56.8 (21/37)	$p_{10-4} = 0.0042$
	CD45⁺EpCAM⁻CK7/8⁺			
	11	N-cadherin⁺	5.4% (2/37)	$p_{11-12} = 0.0001$; $p_{11-5} = 0.0000$
	12	N-cadherin+⁻	45.9% (17/37)	$p_{12-6} = 0.2988$

Detection of the stemness features

The results of the analysis of the stemness features in the populations of cells with hybrid phenotype in blood and primary tumor by flow cytometry are provided in Table 3 and Fig. 2B. It was determined that the stemness features were found in all populations of cells with hybrid phenotype, in both blood and primary tumor. However, no significant differences in the frequency of cells possessing and not possessing stem-like properties were revealed. Cells with the CD45⁺EpCAM⁺CK7/8⁻CD44⁺CD24⁻ and CD45⁺EpCAM⁺CK7/8⁺CD44⁺CD24⁻ phenotypes were less common in primary tumor than in blood ($p = 0.0126$ and $p = 0.0081$, respectively) (Table 3).

Quantification of the cell populations with hybrid phenotype demonstrated that the CD45⁺EpCAM⁺CK7/8⁻CD44⁻CD24⁻cells showing mono-expression of the EpCAM epithelial marker and no stemness features prevailed in blood ($p = 0.0306$); there were significantly more hybrids possessing stem-like properties among the CD45⁺EpCAM⁻CK7/8⁺ cells of the tumor ($p = 0.0233$) (Fig. 2B).

Detection of the EMT features

The EMT features in the populations of cells with hybrid phenotype were estimated via detection of N-cadherin in the cells by flow cytometry (Table 4; Fig. 2C).

Expression of N-cadherin was found in all populations of cells with hybrid phenotype, in both blood and primary tumor. However, N-cadherin-positive cells were significantly less common than N-cadherin-negative cells (Table 4).

Quantification of cells showing EMT features demonstrated that the number of cells with hybrid phenotype showing N-cadherin expression in both blood and primary tumor was significantly lower than the number of cells with no N-cadherin expression (Fig. 2C).

Integrin interface assessment

The results of analysis of the β3, β4, and αVβ5 integrin expression in circulating cells with hybrid phenotype by flow cytometry are provided in Table 5 and Fig. 3. Estimation of the studied cells' frequency showed that cells with the β3⁺β4⁺αVβ5⁺ phenotype were the least common ($p = 0.0003$) (Table 5).

Table 5. Frequency of cells with hybrid phenotype showing integrin expression in patients with breast cancer

№	Phenotype	Frequency, %	Significance level
1	β3⁺β4⁺αVβ5⁺	64.1% (25/39)	$p_{1-8} = 0.0003$
2	β3⁺β4⁺αVβ5⁻	82.1% (32/39)	$p_{2-8} = 0.0564$
3	β3⁺β4⁻αVβ5⁺	92.3% (36/39)	$p_{3-8} = 0.6151$
4	β3⁺β4⁻αVβ5⁻	87.2% (34/39)	$p_{4-8} = 0.2002$
5	β3⁻β4⁺αVβ5⁺	82.1% (32/39)	$p_{5-8} = 0.0564$
6	β3⁻β4⁺αVβ5⁻	97.4% (38/39)	$p_{6-8} = 1.0000$
7	β3⁻β4⁻αVβ5⁺	97.4% (38/39)	$p_{7-8} = 1.0000$
8	β3⁻β4⁻αVβ5⁻	97.4% (38/39)	

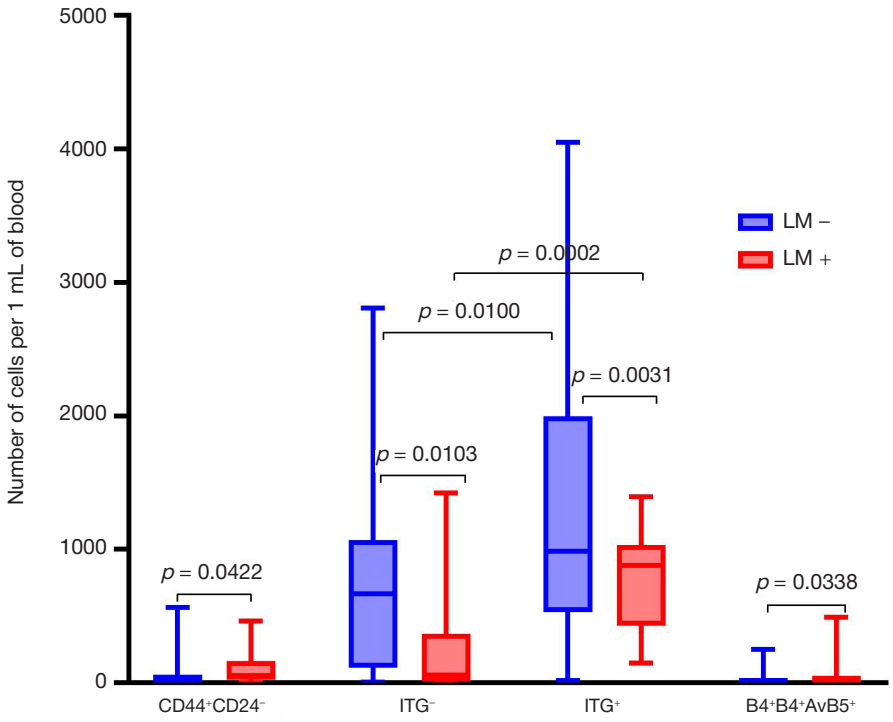

Fig. 4. Stem features and integrin expression in circulating cells with hybrid phenotype in breast cancer patients with lymph node metastasis

The number of circulating cells with hybrid phenotype showing expression of the β3- and/or β4- and/or αVβ5 integrin receptors (ITG+) was significantly higher ($p = 0.0002$) than the number of cells showing no expression of these molecules (ITG–) (Fig. 3).

Association between metastatic traits of cells with hybrid phenotype and lymph node metastasis

Comparative analysis of the metastatic traits in cells with hybrid phenotype revealed the association of the cells showing the stemness features and the expression of integrin molecules with lymph node metastasis (LM).

Thus, the level of "stem" $CD45^+EpCAM^+CK7/8^-CD44^+CD24^-$ cells in blood turned out to be significantly higher ($p = 0.0422$) in patients with LM than in patients with no LM (Fig. 4).

The levels of ITG^- and ITG^+ cells with hybrid phenotype were significantly higher in patients with no LM than in patients with LM ($p = 0.0103$ and $p = 0.0031$, respectively) (Fig. 4). At the same time, patients with LM has a significantly larger number of ITG^+ cells ($p = 0.0002$) relative the number of ITG^- cells. The increase in the number of $\beta3^+\beta4^+\alpha V\beta5^+$ cells ($p = 0.0338$) compared to patients with no metastasis was found in blood of patients with LM (Fig. 4).

The logistic regression models confirmed the role in lymph node metastasis played by cells with hybrid phenotype.

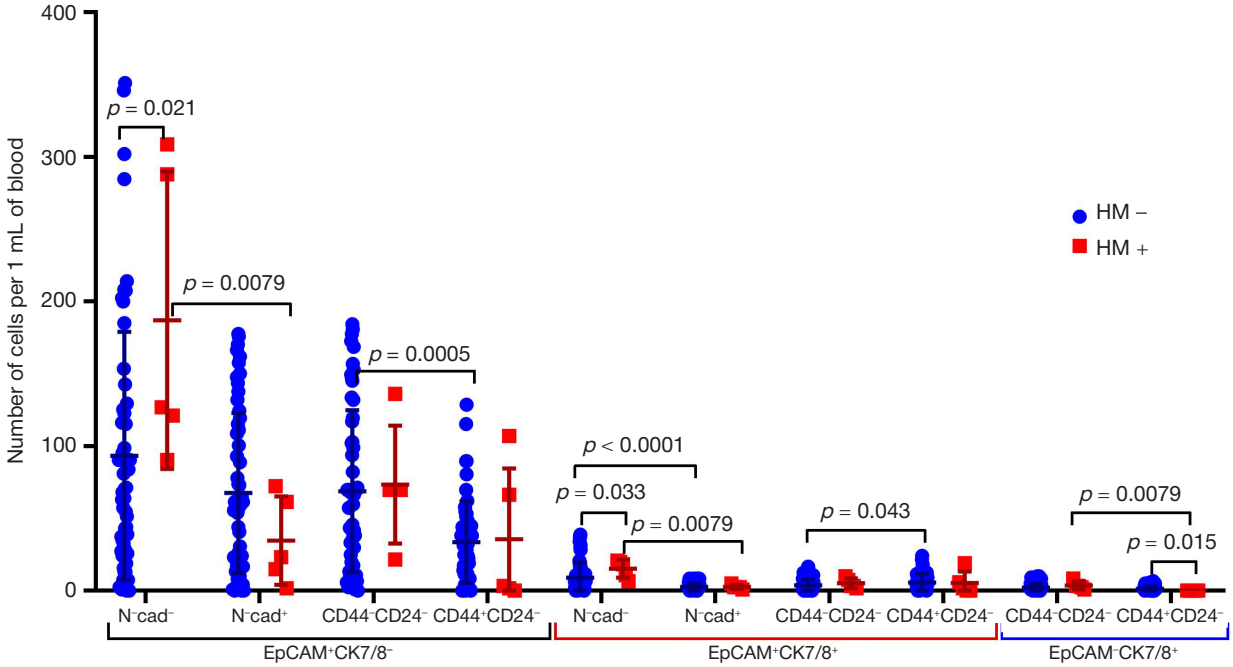

Fig. 5. Stem and EMT features in circulating cells with hybrid phenotype in breast cancer patients with distant metastasis

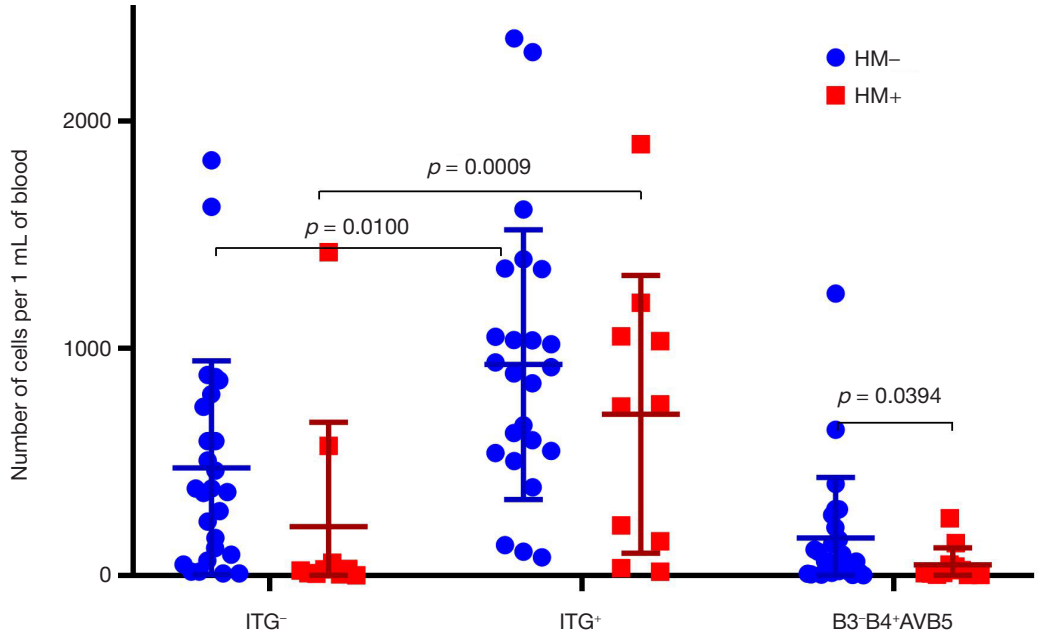

Fig. 6. Integrin expression by cells with hybrid phenotype in breast cancer patients with distant metastasis

Thus, the risk of LM in BC patients turned out to be associated with the presence of cells with the $CD45^+EpCAM^+CK7/8^+$ and $CD45^+EpCAM^-CK7/8^+$ phenotypes showing stemness features in blood. The mathematical model is as follows:

$$Y = -2.4 + 2.7X_1 - 1.0X_2,$$

where Y is the regression equation value; –2.4 is the regression coefficient of the constant term; X_1 is the level of $CD45^+EpCAM^+CK7/8^+$ hybrid cells in blood ($X_1 = 1$ when the frequency is less than 14.94 cells per 1 mL of blood, $X_1 = 2$ when the frequency exceeds 14.94 cells per 1 mL of blood); 2.7 is the regression coefficient of this trait; X_2 is the level of $CD45^+EpCAM^-CK7/8^+CD44^+CD24^-$ cells in blood ($X_2 = 1$ when the frequency exceeds 2.49 cells per 1 mL of blood, $X_2 = 2$ when the frequency is less than 2.49 cells per 1 mL of blood); –1.0 is the regression coefficient of this trait.

The model's sensitivity is 79% and specificity is 85% ($\chi^2 = 18.49$; $p = 0.0001$).

Thus, the study has shown that cells with hybrid phenotype have such properties, as stemness and co-expression of β3-, β4-, and αVβ5-integrins, that are likely to contribute to the mechanisms underlying lymph node metastasis in BC.

Association between metastatic traits of cells with hybrid phenotype and distant metastasis

Comparative analysis of the metastatic traits of cells with hybrid phenotype showed that cells with no features of EMT or stemness were associated with distant metastasis (DM).

Thus, patients with DM showed a significant increase in the frequency of $CD45^+EpCAM^+CK7/8^-N\text{-}cadh^-$ and $CD45^+EpCAM^+CK7/8^+N\text{-}cadh^-$ cells ($p = 0.021$ and $p = 0.033$, respectively) compared to patients with no DM, and the decrease in the frequency of $CD45^+EpCAM^+CK7/8^-N\text{-}cadh^+$ and $CD45^+EpCAM^+CK7/8^+N\text{-}cadh^+$ cells ($p = 0.0079$ and $p = 0.0079$, respectively) relative to N-cadherin-negative cells (Fig. 5).

The number of $CD45^+EpCAM^-CK7/8^+CD44^+CD24^-$ cells in individuals with DM was significantly lower than the number of $CD45^+EpCAM^-CK7/8^+CD44^-CD24^-$ cells ($p = 0.015$) (Fig. 5).

No significant differences in the frequency of cells with hybrid phenotype showing and not showing features of stemness and EMT were found in primary tumors of individuals with DM.

Comparative analysis of the properties possessed by cells with hybrid phenotype in individuals with DM revealed an increase in the frequency of cells showing integrin expression (Fig. 6).

The number of ITG^- cells was significantly lower than the number of ITG^+ cells in patients with no DM or patients with DM ($p = 0.0100$ and $p = 0.0009$, respectively) (Fig. 6). The decrease in the number of β3–β4+αVβ5– circulating cells ($p = 0.0394$) associated with DM relative to no DM was also found (Fig. 6).

The risk of DM in BC patients turned out to be associated with the presence of $CD45^+EpCAM^+CK7/8^+$ cells in blood, regardless of the presence of the features of stemness or EMT, and $CD45^+EpCAM^-CK7/8^+$ cells showing stemness features. The mathematical model is as follows:

$$Y = 63.5 - 31.8X_1 - 30.0X_2,$$

where Y is the regression equation value; 63.5 is the regression coefficient of the constant term; X_1 is the frequency of $CD45^+EpCAM^+CK7/8^+$ hybrid cells in blood ($X_1 = 1$ when the frequency is less than 14.94 cells per 1 mL of blood, $X_1 = 2$ when the frequency exceeds 14.53 cells per 1 mL of blood); –31.8 is the regression coefficient of this trait; X_2 is the frequency of $CD45^+EpCAM^-CK7/8^+CD44^+CD24^-$ cells in blood ($X_2 = 1$ when the frequency exceeds 2.49 cells per 1 mL of blood, $X_2 = 2$ when the frequency is less than 2.49 cells per 1 mL of blood); –30.0 is the regression coefficient of this trait.

The model's sensitivity is 100.0% and specificity is 98.3% ($\chi^2 = 29.52$; $p = 0.0000004$).

DISCUSSION

A biological phenomenon of hybridization in cancer is still a source of debate. Despite numerous in vitro and in vivo studies conducted in the recent decades, there is still no evidence that hybrid tumor cells can cause tumor progression.

Fusion of normal cells and cancer cells is considered to be the most probable mechanism underlying hybrid cell

generation. Thus, *in vitro* studies revealed spontaneous fusion of normal breast epithethelial cells and cancer cells, cancer cells only, epithelial tumor cells and endothelial cells, epithelial tumor cells and stromal cells [19]. It was also noted that the processes of cell fusion and hybrid cell generation were enhanced after using radiotherapy and chemotherapy due to local inflammation in the tumor microenvironment and tissue regeneration processes [20–21]. Discovering the biological nature of cells with hybrid phenotype is still a pressing issue.

Subpopulation analysis of cells with hybrid phenotype has shown that these cells can express one epithelial marker (EpCAM or CK7/8) or both markers. According to modern concepts, the EpCAM and CK7/8 markers are expressed mainly by epithelial cells [22]. However, there is evidence of the EpCAM expression in the bone marrow-derived precursor cells, such as early erythroid precursors [23]. Under physiologic conditions, precursor cells are recruited from the bone marrow during reparative regeneration if needed [24]. In tumor process, these cells are involved in generation and maintenance of the tumor and premetastatic niches and, therefore, contribute to the emergence of metastatic foci in distant organs [25].

Thus, the cells showing expression of CK7/8 and CD45 ($CD45^+EpCAM^+CK7/8^+$ and $CD45^+EpCAM^-CK7/8^+$) are likely to by hybrids of leukocytes/macrophages and epithelial tumor cells, while the $CD45^+EpCAM^+CK7/8^-$ cell population can be represented by both leukocyte-epithelial hybrids and bone marrow-derived hematopoietic progenitor cells.

Assessment of the metastatic traits of cells with hybrid phenotype has shown that these cells are involved in the mechanisms underlying LM and DM. Thus, the logistic regression data suggest that both metastasis types are associated with the same patterns: the increase in the number of circulating $CD45^+EpCAM^+CK7/8^+$ cells and the decrease in the number of $CD45^+EpCAM^-CK7/8^+$ cells showing stemness features. Furthermore, in LM, the increase in blood levels of $CD45^+EpCAM^+CK7/8^-$ cells showing stemness features and $CD45^+EpCAM^+$ cells showing co-expression of β3-, β4-, and αVβ5-integrins is observed. In DM, there is an increase in the number of $CD45^+EpCAM^+CK7/8^-$ and $CD45^+EpCAM^+CK7/8^+$ cells showing no EMT features along with the decrease in the number of the same cells showing EMT features and $CD45^+EpCAM^+$ cells showing mono-expression of β4-integrin.

Acquisition of stem-like properties by hybrid cells can (by analogy with CTCs) contribute to their anticancer therapy resistance and metastatic potential enhancement. The increase in the number of β3-, β4-, and αVβ5-expressing cells with hybrid phenotype observed in LM also suggests enhancement of their metastatic potential. Integrin β3, expressed mainly by platelets, hematopoietic cells, and angiogenic endothelial cells, is responsible for adhesion in hemostasis, wound healing, and angiogenesis. The association of integrin β3 with tumor growth, lymph node and bone marrow metastasis, as well as reduced patient survival, has been shown [26]. Integrin β4 is expressed mainly by epithelial cells. In BC, integrin β4 promotes tumor invasion, increases tumor cell viability, and contributes to angiogenesis [27]. Integrin αVβ5 is a positive regulator of the tumor cells' stemness, it contributes to their growth and invasion [28]. As we have earlier reported, CTCs express the CXCR4 pro-migratory marker [29]. Elevated expression of CXCR4 and integrin molecules in tumor cells can ensure their high migration potential and promote dissemination to various organs. As a result, the hybrid cells can acquire properties that are necessary for metastasis [30].

Today, it is unknown whether the hybrids of leukocytes and epithelial tumor cells can divide indefinitely to form tumors in distant organs, i.e. play the role of tumor seeds. It is not known whether hybrid circulating cells can have a function of niche formation. However, it is already clear that hybrid cells of the tumor and peripheral blood are associated with both LM and DM.

CONCLUSIONS

Thus, the population of cells with hybrid phenotype, just like CTCs, is characterized by high degree of heterogeneity, including the combination of the features of stemness, EMT, and diverse integrin interface. Cells with hybrid phenotype are involved in lymph node and distant metastasis. LM is associated with such metastatic traits of the circulating cells with hybrid phenotype, as the stemness features and co-expression of β3-, β4-, and αVβ5-integrins. In DM, metastatic traits of cells with hybrid phenotype are associated with the stemness features, but not with the EMT features and integrin expression. Understanding of the involvement of cells with hybrid phenotype in metastasis can be useful in terms of improving anti-metastatic therapy.

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