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ARE CD44$^+$/CD24$^-$ CELLS THE ASSUMED CANCER STEM CELLS IN BREAST CANCER?

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Identification and characterization of the population of cancer stem cells (CSC) depends on several cellular markers, which combination is specific for the phenotype of CSC in the corresponding tumor. Several markers of CSC have already been identified in breast cancer (BC), but there are no universal indicators that could specifically identify the CSC in BC. ***Aims*****: To determine the validation of the CSC model for cell surface markers such as CD44 and CD24 and their clinical significance.** ***Materials and Methods*****: Primary tumor samples of 45 patients with invasive BC without chemotherapy prior to surgery exposure were examined in paraffin blocks. CD44 and CD24 antigens expression was evaluated by the percentage of positive cells using different chromogens and the MultiVision detection system by immunohistochemical method. In this research the evaluation was determined by the following criteria: (-), negative — expression in < 10% of tumor cells; (+), positive — expression in ⩾10% of cells. The same scoring system was applied for the expression of CD44$^+$/CD24$^-$.** ***Results*****: 62.2% of investigated patients are patients older than 50 years and most of them with stage II of disease (71.0%) and luminal tumor subtypes (68.9%). We analysed the expression of CD44, CD24 and CD44$^+$/CD24$^-$ for different patients with dividing them into two groups. The group A consists of patients with unfavorable prognosis (relapses and metastases have occurred in the first three years after diagnosis), and the group B — with a favourable prognosis (the development of metastases after three years). Median disease-free survival in the group A is 19 months, in the group B — 46 months. The difference between the overall survival (OS) curves in the groups A and B is statistically significant ($p < 0.001$), the risk of death was higher in the group A (hazard ratio (HR) 5.9; confidence interval (CI) 2.3–15.2). The content of CD44 cells did not differ statistically between groups A and B ($p = 0.18$), but there was a tendency for increasing in OS with the existence of CD44$^+$ cells ($p = 0.056$). The distribution of the expression of CD24 marker did not differ between the groups ($p = 0.36$) as well as the OS curves ($p = 0.59$). Analysis of the expression of CD44$^+$/CD24$^-$ which were considered as possible CSC, revealed a paradoxical increase ($p = 0.03$) of the frequency in patients of the group B (40.9%) compared to the group A (8.7%). Nevertheless, the comparison of the clinical outcomes did not reveal a statistically significant difference in the survival curves in the groups with existence and absence of CD44$^+$/CD24$^-$ expression ($p = 0.08$). The analysis showed the increasing of the risk of worse clinical outcomes in the cases of expression absence of CD44$^+$/CD24$^-$ (HR 2.8; CI 1.1–6.8).** ***Conclusions*****: As a result of our research, the analysis of the quantity of assumed stem cells of the BC, which were identified by immunohistochemistry as CD44 and CD24 cells, failed to detect a statistically significant relation between groups of patients with different prognosis, and the identification of their expression is not enough for the characteristics of CSC. The obtained data demonstrating the worst clinical outcome in the cases of absence of CD44$^+$/CD24$^-$ expression apparently require further investigations and the validation of the immunohistochemical method with the determination of the cut-off line in defining of CD44 and CD24 status.**

Key Words**: breast cancer, cancer stem cells, CD44, CD24, CD44$^+$/CD24$^-$ cells.**

According to modern concepts tumors originate from normal cells because of accumulated genetic and epigenetic changes, but to identify these tumor-forming cells is rather difficult. Stem cells were suggested as attractive models of "tumor-initiating" or "tumorigenic" cells [1, 2] as they possess a lot of characteristics similar to tumor cells, including the self-renewal capacity and differentiation into various clones and an ability to induce cellular heterogeneity, to migrate and penetrate into surrounding tissues. Cancer stem cells (CSC) have been identified as a subgroup of tumor cells with stem properties. They are responsible for the growth, progression and relapse of the tumor [1, 3].

The hypothesis of the key role of CSC in prediction the biological aggressiveness of cancer was proposed many years ago and has been revived with new experimental approaches recently [4]. However, the high heterogeneity of CSC within a tumor as well as the absence of certain criteria for "stem" (belonging to CSC) require further investigations in this direction.

The identification and the description of the population of CSC depend mainly on several cellular markers, the combination of which is specific for the phenotype of CSC in the corresponding tumor. Several supposing CSC markers have already been characterized in breast cancer (BC) but so far there is no universal combination of markers which could specifically identify CSC of BC.

Thus, expression of the cell surface marker CD44 was identified in experiments on cell lines associated with proliferation and invasion of BC cells [5]. The dominant tumorogenic effect of CD44 with

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Abbreviations used: AJCC – American Joint Committee on Cancer; ASCO/CAP – American Society of Clinical Oncology/College of American Pathologists; BC – breast cancer; CD – cluster of differentiation; CI – confidence interval; CSC – cancer stem cells; ER – estrogen receptor; HER2 – human epidermal growth factor receptor 2; HR – hazard ratio; IHC – immunohistochemistry; LN – lymph node; PFS – progression-free survival; OS – overall survival; PR – progesterone receptor.

evidence of worse clinical behavior and a shorter progression-free survival (PFS) [6, 7] has provided the rationale using of the CD44 marker alone or in combination with other surface markers in order to determine cells with "stem" properties.

The other marker, the membrane protein CD24, also plays the certain role in tumorigenesis and its expression may be sufficient in promotion tumor metastasis and increasing proliferation of tumor cells [8]. At the same time, the effect of CD24 expression on tumorigenicity and invasiveness is inconsistent and varies from positive to negative [9].

The combination of these markers in BC was investigated by Al-Hajj et al. [10]. They demonstrated for the first time that $CD44^+/CD24^{-/low}$ cells from the tumor pleural effusion of patients with BC were more tumorigenic in mice and received xenografts have reproduced the heterogeneity of the original tumor. This specific immunophenotype $CD44^+/CD24^{-/low}$ has been presented as CSC of BC and as a powerful predictor of short PFS, overall survival (OS) and as a risk of distant metastases [10, 11].

But, unfortunately, the further researches did not provide the convincing evidence that $CD44^+$ tumor cells are stem cells in reality [12]. This is confirmed by the research [13] in which the analysis of the number of supposed stem cells in BC (identified immunohistochemically as $CD44^+/CD24^-$ cells) was not related with clinical outcomes and survival, although the tumors which have developed distant metastases (mainly in the bone) had a higher share of $CD44^+/CD24^-$ cells.

The other study [14] points out that the signature $CD44^+/CD24^-$ has identified a more basal cell phenotype than tumorigenicity and $CD44^-/CD24^+$ cells showed signs of luminal epithelial cells.

Hence, these investigations have demonstrated the ability to form breast tumors by tumor cells with $CD44^+/CD24^{-/low}$ characteristics and simultaneously the amount of $CD44^+/CD24^-$ in BC cell lines did not predict a tumorigenic potential. Our task was to determine the validation of the CSC model by the surface markers CD44 and CD24, $CD44^+/CD24^-$ and indicate their clinical significance and connection with patient outcomes.

MATERIALS AND METHODS

In the present research, primary tumor samples of 45 patients with invasive BC were studied in paraffin blocks. The patients were operated at the National Cancer Institute (Kyiv) for the period from 2008 to 2012 and they did not receive chemotherapy before surgery. All the patients gave their written informed consent. The study was approved by the Local Medical Ethics Committee.

The evaluation variables were age, axillary lymph node (LN) metastasis status, tumor stage, estrogen receptor (ER) status, progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) status.

We also analyzed PFS and censored OS. The correlations between marker expression and patient survival time from the date of surgery were studied as well. The average follow-up time was 53.9 ± 20.4 months (M ± SD).

The disease stage was collected from the patient's medical history and was coded according to the American Joint Committee on Cancer (AJCC), the tumor phenotype was classified according to the St. Gallen consensus [15]. ER and PR status were determined by immunohistochemistry (IHC) using American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines [16]. HER2 status was also determined by IHC. HER2-positive tumors were defined as 3+ and HER2-negative tumors were defined as 0 or 1+ using ASCO/CAP guidelines [17]. The baseline characteristics of the patients are summarized in Table 1.

Table 1. Patient characteristics

Patient characteristics	Number of patients, n (%)
All cases	45
Age, years	
< 50	17 (37.8)
⩾ 50	28 (62.2)
LN metastasis	
N^-	20 (44.4)
N^+	25 (55.6)
Stage (AJCC)	
I	4 (9.0)
IIA	16 (35.5)
IIB	16 (35.5)
IIIA	9 (20.0)
Intrinsic subtypes[a]	
Luminal A	14 (31.1)
Luminal B (HER2-negative)	17 (37.8)
Triple negative BC	11 (24.4)
HER2-positive	3 (6.7)

Note: [a]St. Gallen's intrinsic subtype's classification.

Sections of tumor tissue 3 μm thick were made on SuperFrost Plus adhesive glasses (Menzel, Germany) from formalin fixed paraffin blocks, which then were deparaffinized in xylene and rehydrated in a 96% ethanol solution. For antigen retrieval, paraffin tissue sections were immersed into citrate buffer (pH 6.0 for Ki-67 and HER2) and into TRIS-EDTA buffer (pH 9.0 for the rest) heated to 98 °C for the period of 40 min. Then the sections were rinsed out with TRIS-HL buffer.

The cells were incubated with antibodies at room temperature within 30 min. CD44 and CD24 were detected by the MV Detection System (MultiVision anti-rabbit/HRP + anti-mouse/AP polymers — Thermo Scientific, USA) with anti-mouse alkaline phosphatase + anti-rabbit peroxidase polymers using various chromogenes — blue chromogen CD24 and red chromogen CD44 (Table 2). Then the sections were stained with Light Green for a short period of time, dehydrated and mounted. Staining was performed with EnVision™ FLEX+ System (Dako, Denmark) to evaluate the expression of ER, PR, HER2, Ki-67.

The expression of cell surface markers CD44 and CD24 was graded in terms of the percentage of positive cells with membrane staining in each block as well as the intensity of staining. Pathologist who was blinded to clinical diagnosis scored all cases. Staining for each of CD44, CD24 and $CD44^+/CD24^-$ was evaluated separately using our scoring system.

There is no common cut-off value for evaluating of the expression of CD44 and CD24 nowadays and in the research the evaluation was determined according to the following criteria: (–), negative — expression

in < 10% of tumor cells; (+), positive — expression of ⩾ 10% of tumor cells. The same scoring system described for the antigens CD44 and CD24 was applied for the expression of the proportion of $CD44^+/CD24^-$ [18].

Table 2. Antibodies and immunohistochemical techniques

Antibody	Clone	Source	Dilution	Incubation temperature/time
ER	EP1 Rabbit monoclonal	DAKO, Denmark	RTU	4 °C/overnight
PR	PgR 636 Mouse monoclonal	DAKO, Denmark	RTU	4 °C/overnight
HER2	Rabbit polyclonal	DAKO, Denmark	1:750	Room temperature, 30 min
Ki-67	MIB1 Mouse monoclonal	DAKO, Denmark	RTU	Room temperature, 30 min
CD24	SN3 Mouse monoclonal	Thermo Scientific, USA	1:200	Room temperature, 30 min
CD44	Rabbit polyclonal	Thermo Scientific, USA	1:100	Room temperature, 30 min

Note: RTU – ready–to–use.

Statistical analysis. The data were analysed using MedCalc v.17.6 (MedCalc Software Inc, Broekstraat, Belgium). In order to analyze and report the data descriptive statistics were used. The χ^2 test was used for comparison of qualitative data. For survival time estimation the Kaplan — Meier survival analysis was used. The survival proportions (with standard error) were calculated. The median survival times are reported with their 95% confidence interval (CI). For the comparison between the survival curves logrank test was used, hazard ratio (HR) with CI was calculated. The significance threshold was set at $p < 0.05$.

RESULTS AND DISCUSSION

We have analyzed the patients' clinicopathological data (Table 1). 62.2% of patients were the patients over 50 years old and the majority of them (71.0%) had the stage II of disease and luminal tumor subtypes (68.9%).

The patients were divided into two groups: group A — with an unfavorable prognosis (the occurrence of relapses and metastases in the first three years after staging the diagnosis), and group B — with a favorable prognosis (development of metastases after three years of the disease). Median PFS in group A was 19 months (CI 17–22 months), in group B — 46 months (CI 43–52 months). In addition, the 1-year old PFS in group A was 82.6 ± 7.9% *vs* 100% in group B.

The difference between OS curves in groups A and B is statistically significant ($p < 0.001$) (Fig. 1). Median OS in group A was 53.3 months (CI 39–58 months), in group B — 80 months (CI 75–95 months). The 5-year OS of patients in group A was 23.4 ± 10.2% and in group B — 92.3 ± 7.4%, respectively, the risk of death was higher in group A (HR 5.9; CI 2.3–15.2).

The analysis of clinicopathological data has not correlated with the existence of metastases in regional LN ($p = 0.10$) and the tumor phenotype ($p = 0.12$) of the patients in group A with an unfavorable clinical outcome given above (Table 3). Although there were more patients with non-luminal subtypes (43.5%) in group A associated with a worse prognosis than group B (18.2%), the distribution between the groups did not differ statistically significant ($p = 0.12$). The statistically significant difference was in the distribution by stages ($p = 0.009$); there were more patients with stages I and IIA in group B.

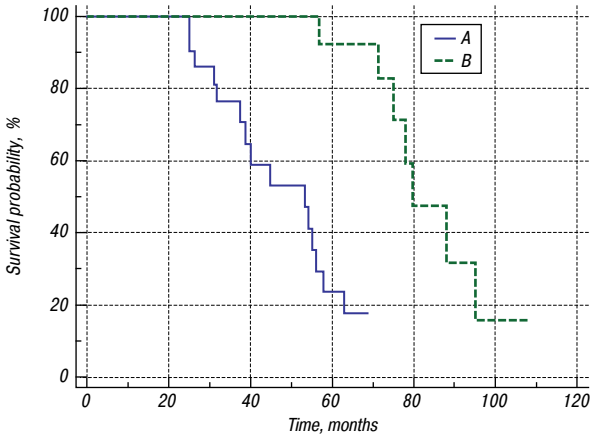

Fig. 1. Survival analysis according group A (patients with an unfavorable prognosis) and B (patients with a favorable prognosis)

Using the alleged data an unfavorable clinical outcome may be associated with the existence of CSC, we performed an analysis of the expression of cell surface markers of identified CD44 and CD24, their combinations of $CD44^+/CD24^-$ in different groups of patients.

Table 3. Clinicopathological characteristics of patients' groups with different outcomes and expression of CD44, CD24, $CD44^+/CD24^-$

Clinicopathological characteristics	Group A, n (%)	Group B, n (%)	*p*-value
All cases	23 (51.1)	22 (48.9)	
Age, years			
< 50	6 (26.1)	11 (50)	0.18
⩾ 50	17 (73.9)	11 (50)	
LN metastasis			
N^-	7 (30.4)	13 (59.1)	0.10
N^+	16 (69.6)	9 (40.9)	
Stage (AJCC)			
I	0 (0.0)	4 (18.2)	0.009
IIA	7 (30.4)	9 (40.9)	
IIB	13 (56.5)	3 (13.6)	
IIIA	3 (13.1)	6 (27.3)	
Intrinsic subtypes			
Luminal A	5 (21.7)	9 (40.9)	0.12
Luminal B (HER2-negative)	8 (34.8)	9 (40.9)	
Triple negative BC	7 (30.4)	4 (18.2)	
HER2-positive	3 (13.1)	0 (0.0)	
CD44			
Positive (%)	6 (26.1)	11 (50.0)	0.18
Negative (%)	17 (73.9)	11 (50.0)	
CD24			
Positive (%)	8 (34.8)	4 (18.2)	0.36
Negative (%)	15 (65.2)	18 (81.8)	
$CD44^+/CD24^-$			
Positive (%)	2 (8.7)	9 (40.9)	0.03
Negative (%)	21 (91.3)	13 (59.1)	

We had been observing a different expression of CD44, CD24, and a different distribution in groups A and B (Fig. 2). Some tumors contained only $CD44^+$ (red) or only $CD24^+$ (blue) cells, while the others had a combination of two cell types.

Expression of cell surface markers CD44 and CD24 and their combinations $CD44^+/CD24^-$ were analyzed.

Expression of CD44 cell did not differ statistically between groups A and B ($p = 0.18$). Nevertheless, the lack of expression was observed in 73.9% of cases in group A. The expression of the $CD44^+$ phenotype was observed

in half of the group B. Some studies showed that the tumors consisting mainly of $CD44^+$ cells may have worse clinical behavior [7]. Probably a certain role in the expression of $CD44^+$ is represented by the TGF-β signaling pathway which is known to play a dual role in the progression of tumors. On the one hand, it is one of the most powerful inhibitors of cell proliferation; on the other, it promotes the invasion, angiogenesis, epithelial-mesenchymal transition and metastasis [6, 9, 19].

Our results show that cells with different phenotypes, even within the same type of tumor and tissue, respond differently to the activation of TGF-β. Median OS in the absence of $CD44^+$ expression was 63 months (CI 54–78 months) and with high expression — 88 months (CI 56–95 months) ($p = 0.056$). There is a tendency for an increasing in OS with the existence of $CD44^+$ cells.

The distribution of the expression of another surface marker of CD24 did not differ either between the groups ($p = 0.36$) or the OS curves ($p = 0.59$). Median OS with low expression or absence of $CD24^+$ was 71 months (CI 57–88 months) and in the presence of expression — 56 months (CI 39–78 months). In this case, low expression or lack of expression were noted in 81.8% of cases in group B, whereas in group A there were more cells with positive expression of the phenotype $CD24^+$. This observation contradicts the hypothesis that $CD24^+$ cells are more differentiated and less tumor-bearing cells. But it conforms the concept of linking the expression of CD24 with tumor progression and metastatic behavior [8].

Since the detection of $CD24^+$ and $CD44^+$ cells involves several steps and it cannot be ruled out the possibility that the procedure has changed the expression of some antigens. We analyzed the simultaneous detection of $CD44^+/CD24^-$ as presumed CSC. The state of expression of cells with immunophenotype $CD44^+/CD24^-$ revealed a paradoxical increase ($p = 0.03$) of its frequency in patients of group B (40.9%) compared with group A (8.7%). The existing data conflicts with earlier reports indicating that phenotype $CD44^+/CD24^-$ correlated with an unfavorable prognosis [18, 20].

Nevertheless, the comparison of clinical outcomes did not reveal a statistically significant difference in the survival curves in the groups with the presence and absence of $CD44^+/CD24^-$ expression ($p = 0.08$). Median OS in the presence of $CD44^+/CD24^-$ expression was 88 months (CI 88–95 months), whereas the absence of it was 63 months (CI 54–78 months) (Fig. 3). The same trend of higher survival in the presence of $CD44^+/CD24^-$ expression was observed for 3 years of survival (100% *vs* 84.4 ± 6.2%, respectively) and 5-year of survival (85.7 ± 13.2% *vs* 50.2 ± 9.7%, respectively). This analysis allows to make a conclusion that the risk of unfavourable clinical outcomes increases in the absence of $CD44^+/CD24^-$ expression (HR 2.8; CI 1.1–6.8).

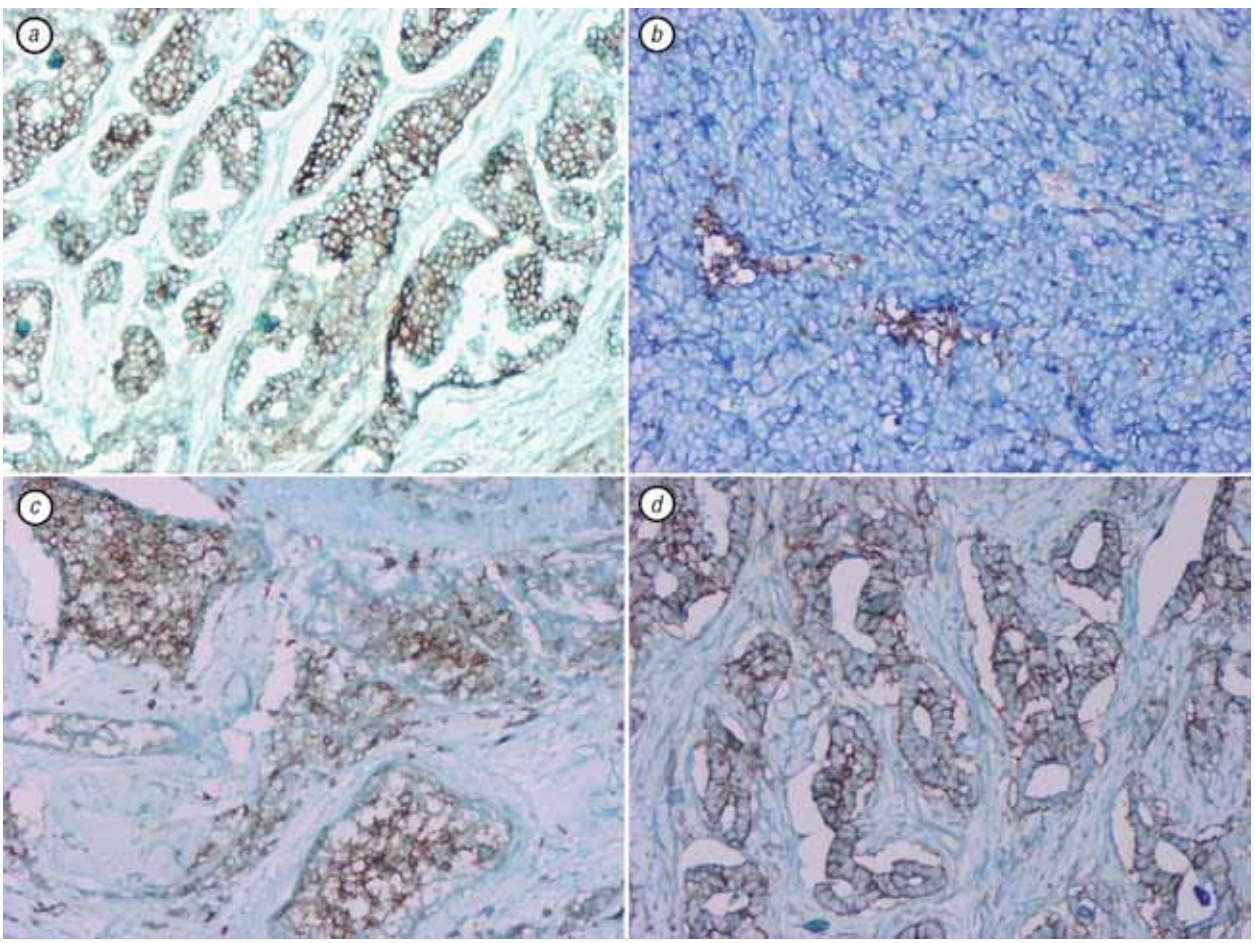

Fig. 2. Immunohistochemical profiles of expression CD44 (red) and CD24 (blue): *a* — $CD44^+$ cells; *b* — $CD24^+$ cells; *c* — $CD44^+/CD24^-$ cells (group A); *d* — $CD44^+/CD24^-$ cells (group B). Immunohistochemical staining using various chromogenes patterns of CD44, CD24 on paraffin-embedded tissue sections of breast carcinoma, × 100

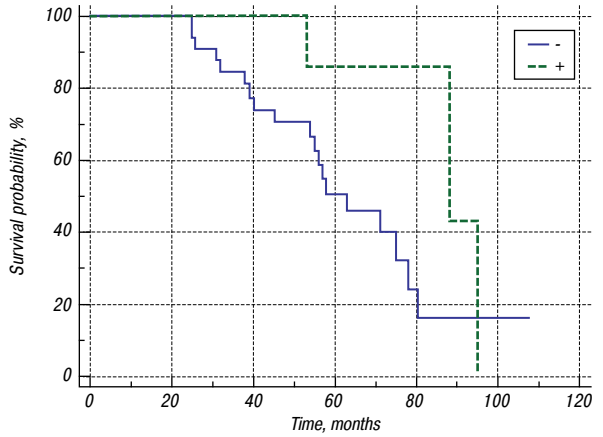

Fig. 3. Survival analysis according to the presence (+) and absence (–) of expression $CD44^+/CD24^-$

According to the results of our investigation, the analysis of the number of supposed stem cells of BC identified by IHC as $CD44^+$ and $CD24^-$ cells did not reveal a statistically significant relation between groups of patients having different prognosis. Immunohistochemical analysis of tumors among the samples of the BC showed high heterogeneity in the expression of the selected immunophenotypes. The expression of a single cell surface marker may not be sufficient to identify it without an alternative and to determine its tumorigenic potential and, hence, the clinical outcome.

The data obtained which demonstrate the worst clinical outcome in the absence of $CD44^+/CD24^-$ expression are discordant to the data of other researchers [9, 18]. This result is also confirmed by the observed trend of higher survival in the presence of $CD44^+/CD24^-$ and $CD44^+$ phenotypes. But, the obtained data also indicate that studies in experimental systems may not reflect the behavior of cells in the tumor in patients. In addition, the usage of different immunohistochemical scoring method in defining CD44 and CD24 status and the classification of the status of CD44 and CD24 expression may be the cause of the discrepancy between our investigation and previous studies.

We acknowledge the limitations of this study. Firstly, this is strictly a population-based cancer registry investigation. Secondly, it includes an insufficiently adequate number of BC cases. Thirdly, there is no definite technique for evaluating stem cells and the search for markers identifying CSC still continues.

To sum it up, based on our data, the markers CD44 and CD24 do not reflect the features of CSC and unfavorable prognosis and do not clarify the role and clinical significance of the immunophenotype $CD44^+/CD24^-$. These markers require further studies, especially clinical trials and validation of the immunohistochemical method.

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