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ORIGINAL ARTICLE

The distribution of CD44+/CD24- cancer stem cells in breast cancer and its relationship with prognostic factors

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Summary

Purpose: *The purpose of this study was to investigate the correlation between the percentages of $CD44^+/CD24^-$ cancer stem cells (CSCs) and the clinicopathological and prognostic factors in breast cancer patients.*

Methods*: Twenty three women who underwent surgery for breast cancer were enrolled in this study. The mean age of the patients was 46.65 years and 52% had early-stage disease. Tumor tissues obtained during surgery were digested enzymatically. $CD44^+/CD24^-$ cell phenotype was identified by using surface marker antibodies and percentages were determined by surface marker expression of the cells.*

Results: *Sixty five percent of the tumors were positive for estrogen (ER)/ progesterone receptors (PR) and 38% of the tumors were positive for HER-2. All of the patients with hormone receptor positive tumors had ER positive tumors, while only 11 patients had PR positive breast cancer. $CD44^+/CD24^-$ cells were present in all tumor tissues. The mean proportion of the $CD44^+/CD24^-$ cells was 1.43±1.6. The mean percentages of $CD18^+$ cells and $MUC1^+$ were 27.9±26.5% and 6.07±11.34%, respectively. The percentage of $CD18^+$ cells was significantly higher in PR positive tumors (p=0.042). There was no significant correlation between the percentage of $CD44^+/CD24^-$ cells and clinicopathological features.*

Conclusion: *This study showed that $CD44^+/CD24^-$ cells were present in all tumor tissues. The percentage of $CD44^+/CD24^-$ cells was higher in early-stage disease, yet without statistical significance. No correlation was found between prognostic factors and the percentage of the $CD44^+/CD24^-$ cells.*

Key words: *breast cancer, cancer stem cell, $CD44^+/CD24^-/Lin^-$, $CD18^+$, MUC1*

Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer deaths among women. It constitutes approximately 30% of malignant diseases and 16% of cancer-related deaths in women [1]. The incidence of breast cancer is gradually increasing; however, mortality has remained stable or decreased in many industrialized countries within the past 15 years. Increased detection of early-stage disease with screening and improvements in systemic therapies caused a decrease in breast cancer mortality. But metastatic breast cancer is still unlikely to be cured by any means, despite improved systemic treatments [2].

The clinicopathological manifestations of breast cancer are becoming further complicated due to different morphological characteristics, molecular profile and clinical behavior of the disease. World Health Organization identified 18 different histological types of breast cancer that are characterized with different clinical behavior. Moreover, in addition to their morphological

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structures, breast cancers are divided into different groups based on their biological characteristics such as proliferative potential, hormone receptor status and overexpression of HER-2. Beside the differences in histological types, the cells located in the tumor show a phenotypical and genotypical heterogeneity [3].

Understanding the underlying molecular causes of such a heterogeneous disease will provide insights into the mechanisms of breast cancer genesis. This will also provide effective treatment of disease and the prevention of metastases. For this purpose, several hypotheses have been proposed. One of them is the CSC hypothesis which supports that breast cancer is derived from a small cell group with a tumor-forming capacity. This small cell group is called CSC due to its normal stem cell-like characteristics. Experimental evidence supports that CSCs are responsible for cancer formation, tumor invasion, metastasis and the development of resistance to several kinds of treatments [4]. Various methods are used to identify these CSCs [5,6]. One of them is the identification of CSCs according to their surface markers. The phenotype of breast CSCs is identified by $CD44^+/CD24^-/Lin^-$ surface markers. It was shown that the cells with a $CD44^+/CD24^-/Lin^-$ phenotype could form tumor in immune-deficit mice while those lacking this phenotype could not. In another study, 9 tumor samples were used, one of them was taken only from primary tumor tissue and the others were obtained from metastatic tissue [7].

In the present study, we aimed to investigate the distribution of cells with a $CD44^+/CD24^-/Lin^-$ phenotype which are related with cancer development and the relationship between these distribution rates and prognostic factors.

Methods

Patients

Twenty three women for whom surgery was planned due to breast cancer were included in this study between May 2010 and January 2011. Stage I, IIA and IIB tumors were defined as early-stage breast cancer; stage IIIA, IIIB, IIIC and IV tumors were defined as advanced-stage breast cancer.

Collection of biopsy samples

The tumor tissues were obtained from the patients who underwent surgery for breast cancer in Kocaeli University Hospital, Department of General Medicine; 0.5x0.5x0.5cm biopsy samples were taken from the tumor which was macroscopically assessed by a pathologist.

These samples were sent to the human cell culture laboratory in Kocaeli University Center for Stem Cell and Gene Therapies Research and Practice in a solution containing HBSS (Hanks' Balanced Salt Solution 1x; GIBCO Invitrogen Corporation) with 100 units/ml penicillin and 100 mg/ml streptomycin (P-S; GIBCO Invitrogen Corporation) without $CaCl_2$ and $MgCl_2$ in a conical test tube at 2-8 °C within 10 min and were stored until the beginning of digestion procedures.

Mechanical and enzymatic digestion of biopsy samples

Tumor biopsy samples were washed 5-6 times in 5 or 6 100x20 mm sterile cell culture dishes (BD Falcon Biosciences) each containing 15 ml HBSS supplemented with 100 units/ml penicillin and 100 g/ml streptomycin in order to remove blood and microorganisms. Then, they were transferred to a dry dish without HBSS. Unwanted surrounding connective tissue was removed by dissecting under a stereomicroscope. The biopsy sample was minced, the biggest tissue piece being $1x1x1mm^3$ by incising the biopsy sample by curve tipped scissors. Tumor dissociation enzyme solution (Tumor dissociation enzyme reagent; DCS Innovative Diagnostik-Systeme) was prepared by dissolving the enzyme in 10 ml complete assay medium (Complete assay medium CAM; Innovative Diagnostik-Systeme) and sterilizing it by passing through 0.22 µm cell strainer. Five ml of this solution were used for the assay and the remaining was stored at -20 °C until the next assay. After that, an additional 5 ml CAM were added into 50 ml conical tube containing 5 ml tumor dissociation enzyme solution and minced tumor tissue pieces were transferred into it. The tube was left to 2-hr enzyme incubation in 37°C shaking water bath. During the incubation, tissue was controlled every 30 min and subjected to vortex. At the end of incubation, 20 ml CAM were added into the tube and centrifuged at 400 g for 10 min. The supernatant was removed and the pellet was resuspended by adding 10 ml fresh CAM and centrifuged at 400 g for 5 min. After this procedure was repeated twice, the last pellet was resuspended in 1 ml CAM and passed through 70 micrometer and 40 micrometer cell filters (BD Falcon Biosciences), respectively. It was centrifuged again, the supernatant was removed, the pellet was resuspended in 1.5 ml CAM and sent immediately to the flow cytometry laboratory.

Examination of breast cancer stem cells by flow cytometry

To confirm that breast CSCs maintained their phenotypic characteristics after isolation of the cancer tissue, they were subjected to flow cytometry analysis. Cancer cells were harvested and resuspended in 1.5 ml CAM (their own culture medium) at a concentration of $1x10^6$ cells/ml. Flow cytometry was performed on FACSCalibur (BD Biosciences, San Diego, CA). The data were analysed with Cell Quest software (BD Biosciences, San

Diego, CA), and the forward and side scatter profiles allowed to gate out debris and dead cells. Immunophenotyping of breast CSCs was performed with antibodies against the following human antigens which are directly conjugated to various fluorochromes, depending on the experiment: anti-CD44 (Hyaluronate/lymphocyte homing-associated cell adhesion molecule-HCAM; PE), anti-CD24 (Signal transducer, heat stable antigen; PE), CD227 (cell surface mucin; MUC1). Lineage marker antibodies were anti-CD2 (cell adhesion molecule; FITC), -CD3 (T cell receptor; PerCP), -CD10 (N-cadherin/common leukocyte lymphocytic leukaemia antigen-CALLA; PE), -CD16 (Fc receptors FcγRIIIa (CD16a) and FcγRIIIb (CD16b); FITC), -CD18 (Integrin beta-2; FITC), -CD19 (B-lymphocyte antigen; PerCP-Cy5.5), CD31 (Platelet endothelial cell adhesion molecule (PECAM-1); FITC), CD140b (Beta-type platelet-derived growth factor receptor b; PE). Ten microliters of antibodies of cell surface markers and isotype controls were added and incubated for 30 min. At the end of this period, 500 microliters stain buffer were added, and then immunophenotyping was performed using FACSCalibur instrument (BD Bioscience, San Diego, USA). The data were analysed with Cell Quest software (BD Bioscience) and the forward and side scatter profile gated out debris and dead cells. In addition, after using Fixation/permeabilization kit (BD Bioscience) for cytoplasmic markers, 20 µl PE Cytokeratin 14, 15, 16 and 19 set (BD Bioscience) were added to the cells. After 30-min incubation, 500 µl stain buffer was added. Flow cytometry analysis of the lineage$^-$ cell lines CD44$^+$ / CD24$^-$ and MUC1 $^+$ cells distribution was evaluated. On lineage $^+$ cells, CD18 $^+$ cells distributions were evaluated. All of the antibodies were purchased from Becton Dickinson.

Sphere formation assay

To further investigate whether breast CSCs would also have the potential to generate mammospheres, we applied a previously described method [6] for the culturing of mammary gland stem/progenitor cells to pathologic specimens obtained from breast lesions. Breast cancer cells were trypsinised from the stem monolayer culture and cell suspensions were then seeded in 6-well, 1% agarose-coated culture plates, with approximately 1x10^6 cells per well. Breast cancer cells' spheroids were grown in DMEM-F12 supplemented with 10 ng/mL EGF, 20 ng/mL Bfgf, 10% FBS, 5 mg/mL insulin , 1 mg/mL hydrocortisone, 5% penicillin and were kept at 37°C with 5% CO_2. After 3-5 days in culture, spheroids were examined under an inverted microscope equipped with a digital camera. Spheroids were removed after 3-5 days of culture [8].

Statistics

SPSS for Windows version 13.0 was used for statistical analysis. Mann Whitney-U test was used for comparisons and Spearman's rho correlation test was used for correlations. A p value <0.05 was accepted as statistically significant.

Results

Patient demographic characteristics

Twenty three women who underwent surgery for breast cancer were included in the study. The mean patient age was 46.65±10.98 years (min-max: 24-79). Fifty two percent (N:12) of the patients were postmenopausal and had early-stage

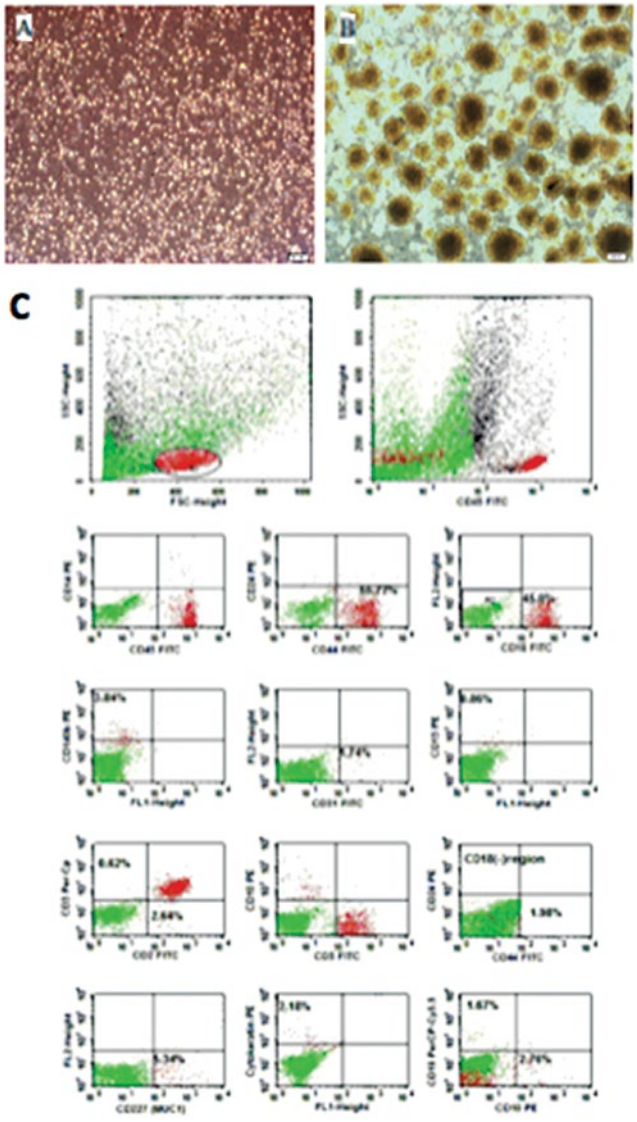

Figure 1. A : Culture of isolated breast cancer cells growing as nonadherent mammospheres (Scale bar: 200 µm). **B**: Tumor spheres formed by breast cancer cells after 5-day culture. The solid, circular formations represent tumor spheres (Scale bar: 200 µm). **C**: FACS analysis for double staining of CD44 and CD24, CD2, CD3, CD19, CD10, CD18, CD16, MUC1 (CD227), Cytokeratin, and CD16 expression in breast cancer cells (CD44 55-77%, CD24-, CD18 45%, MUC1 5.3%, CD2-, CD3-, CD19-, CD10-, CD16-, cytokeratin-). Breast cancer stem cells identified in the samples were CD44+/CD24-/Lin-.

disease. Fifteen patients (65%) had ER positive tumors. Fifty seven percent (N:13) of the patients had grade III and 30% (N:7) had grade II tumors.

Sphere formation assay

Tumor-derived single cells showed ability to proliferate and form clonally derived mammospheres when cultured in 6-well, 1% agarose-coated culture plates. (Figures 1A and B).

Identification of tumorigenicity markers

Breast cancer cells were heterogeneous with respect to expression of a variety of cell surface markers (CD44, CD24, CD16, CD18, CD31, CD2, CD3, CD5, CD19, Cytokeratine, MUC1, CD45, CD14) (Figure 1C). The distribution of surface markers is shown in Table 1.

$CD44^+/CD24^-Lin^-$ cells' distribution was higher in postmenopausal women but without statistical significance (p=0.17). The distribution of $CD44^+/CD24^-Lin^-$ cells was higher in early-stage disease, yet without statistical significance (p=0.090). As the number of involved lymph nodes increased, $CD44^+/CD24^-Lin^-$ cells distribution decreased, but the difference was not significant (p=0.535). The distribution of $CD44^+/CD24^-Lin^-$ cells increased with tumor grade, but without statistical significance (p=0.84). There was no statistically significant difference between the distribution of $CD44^+/CD24^-Lin^-$ cells and ER/PR status (p=0.49). In HER-2 negative tumors, $CD44^+/CD24^-Lin^-$ cells' distribution was increased, however, without statistical significance (p=0.19) (Table 2).

There was no statistical significance between the distribution of $CD18^+$ cells and menopausal status, stage, ER and HER-2 status. But, there was a statistically significant relationship between PR status and the distribution of $CD18^+$ cells (p=0.042). In PR positive tumors, distribution of $CD18^+$ cells was higher. $CD18^+$ cells' distribution decreased as the number of involved lymph nodes increased, but this relationship was not significant (p=0.193) (Table 3).

$MUC1^+$ cell distribution was lower in postmenopausal women, however without statistical significance (p=0.424). When patients were analyzed according to the stages of breast cancer, the distribution of $MUC1^+$ cells was found to be lower in the early stages of disease, but again without statistical significance (p=0.25). It was observed that the distribution of $MUC1^+$ cells increased, as the number of involved lymph nodes increased, but this increase was not statistically significant (p=0.264). The distribution of $MUC1^+$ cells de-

Table 1. Distribution of tumor surface markers

Markers	*Mean ± SD*	*Min-max*
$CD44^+/CD24^-$	1.43±1.16	0.12-4.8
$CD18^+$	27.9±26,5	0,53-81
$MUC1^+$	6.07±11.34	0.19-44.5

Table 2. Distribution of $CD44^+/CD24^-$ cells according to prognostic factors

Prognostic factors	*$CD44^+/CD24^-$ Mean ± SD*	*p value*
Menopausal status		0.17
Premenopausal	1.03 ± 0.70	
Postmenopausal	1.88 ± 1.43	
Stage		0.90
Early	1.61 ± 1.48	
Advanced	1.24 ± 0.71	
Number of involved lymph nodes		0.535
0-3	1.67 ± 1.44	
4 and more	1.12 ± 0.61	
Grade		0.84
I	1.25 ± 0.80	
II	1.43 ± 1.22	
III	1.87 ± 1.91	
ER status		0.69
Positive	1.48 ± 1.18	
Negative	1.34 ± 1.20	
PR status		0.49
Positive	1.17 ± 0.76	
Negative	1.61 ± 1.44	
HER-2 status		0.19
Positive	1.04 ± 0.65	
Negative	1.64 ± 1.34	

Table 3. Distribution of $CD18^+$ cells according to prognostic factors

Prognostic factors	*CD18 Mean ± SD*	*p value*
Menopausal status		0.970
Premenopausal	28.16 ± 24.64	
Postmenopausal	27.75 ± 29.61	
Stage		0.350
Early	33.21 ± 28.14	
Advanced	22.24 ± 24.58	
Number of lymph nodes		0.193
0-3	34.12 ± 27.14	
4 and more	19.96 ± 24.66	
Grade		0.520
I	21.04 ± 26.03	
II	33.93 ± 25.26	
III	18.26 ± 18.93	
ER status		0.061
Positive	37.08 ± 28.08	
Negative	10.87 ± 10.86	
PR status		0.042
Positive	39.5 ± 22.8	
Negative	17.38 ± 26	
HER2 status		0.605
Positive	22.05 ± 23.79	
Negative	31.12 ± 28.1	

Table 4. Distribution of MUC1+ cells according to the prognostic factors

Prognostic factors	*MUC1 Mean ± SD*	*p value*
Menopausal status		0.424
Premenopausal	9.09 ± 14.98	
Postmenopausal	2.77 ± 3.65	
Stage		0.250
Early	4.27 ± 9.75	
Advanced	8.03 ± 13.05	
Number of lymph nodes		0.264
0-3	4.09 ± 9.36	
4 and more	8.64 ± 13.59	
Grade		0.200
I	10.08 ± 12.33	
II	4.86 ± 12.02	
III	1.97 ± 0.55	
ER status		0.272
Positive	5.32 ± 11.44	
Negative	7.47 ± 11.79	
PR status		0.350
Positive	5.54 ± 12.1	
Negative	6.55 ± 10.6	
HER2 status		0.561
Positive	7.16 ± 11.97	
Negative	5.49 ± 11.38	

creased as the tumor grade increased; however this decrease was also not statistically significant (p=0.20). There was no relationship between the distribution of MUC1+ cells and ER/PR status. In addition, no relationship was found between the distribution of MUC1+ cells and HER-2 expression (p=0.561) (Table 4).

Discussion

In contrast to the scholastic hypothesis, a small group of cells within the tumor is the cause of cancer development according to CSC hypothesis. This cell group leads to tumorigenesis, while non tumorigenic cells form the majority of the tumor with their offsprings [9]. Many studies have shown that cells which have a tumor-initiating characteristic were identified in breast cancer and in other solid tumors in animal models and cell lineage studies. These cells were identified due to their $CD44^+/CD24^-$ surface marker expression in these studies [6,7,10]. In our study, breast CSCs were identified by using $CD44^+/CD24^-$ phenotype as in other studies. In lineage-negative cells, $CD44^+/CD24^-$ phenotype was used. The lineage markers defined by Al Hajj et al. [7] were used in the present study and were antigens associated with the presence of normal cell (CD2, CD3, CD10, CD16, CD18, CD140B). In the same study lineage markers were not expressed by CSCs. In our study, in addition to these surface markers, cells were also assessed for cytokeratin which is another epithelial cell marker of normal breast tissue and defined in the study of Dontu et al. [11]. In addition to identification of CSCs with surface markers, the assessment could also be done by determining the DNA content of these cells by flow cytometry or by determining the tumor-forming capacity of these CSCs in cell lines. But in our study only surface markers were studied due to limited facility.

In the study by Al Hajj et al., it was reported that $CD44^+/CD24^-$ cells were present in all breast cancer tissue samples [7]. In the study by Honeth et al., $CD44^+/CD24^-$ cells were observed in 31% of all tumor samples [12]. In another study similar to the study of Honeth et al., $CD44^+/CD24^-$ cells were detected in 56% of tumor samples [13]. The reason of the different results between these two recent studies and the study of Al Hajj et al. was attributed to the techniques used and the tissue difference. While metastatic tissues and cell suspension were generally used in the study of Al Hajj et al., immunohistochemical staining and primary breast cancer tissue were used in the other studies. We used primary tumor tissues in our study and $CD44^+/CD24^-$ cells were present in all tumor samples. These results were consistent with the results of Al Hajj et al. The reason why our results were different from the other two studies may be the use of cell suspension in our study.

Stem cell distribution, in other words their percentage, in solid tumors such as breast cancer is considered as 1-2% of the total tumor cells [7]. Similarly, the distribution of $CD44^+/CD24^-$ cells was 1.43±1.16% in our study. If the tumor-initiating group within the tumor has a stem cell-like characteristic, the distribution may be thought like stem cell-like, and thus the distribution results in our study may be regarded as significant. In addition to this, in the study by Abraham et al., primary breast tumor tissues were immunohistochemically stained for CD44 and CD24, and the percentage of $CD44^+/CD24^-$ cells was between 0 and 80% in the tissues. The percentage of $CD44^+/CD24^-$ cells was below 10% in approximately 3/4 of them (78%) [14]. In another study conducted by immuhistochemical staining, the percentage of $CD44^+/CD24^-$ cells was 0-70% and the distribution of $CD44^+/CD24^-$ cells was below 10% in 97.6% of the tissues [13]. Similar results were obtained in the study by Honeth et al. [12]. In another study conducted by immunohistochemical staining, the mean percentage of $CD44^+/CD24^-$ cells was reported as 4.4% [15]. The percentage of stem cells in

normal breast tissue was reported as 0.2, 1 and 5% [16]. The difference between these studies with a higher distribution ratio and our study may be attributed to the technique used. In our study, $CD44^+/CD24^-$ mononuclear cells could be distinguished with lineage markers. In other studies, the percentages were higher since these mononuclear cells could not be distinguished.

Abraham et al. have reported that there was no inverse correlation between distribution of $CD44^+/CD24^-$ cells and life expectancy [14]. As the stage of the disease increases the life expectancy of patients decreases. In relation with this information, Mylona et al. reported that they found an inverse correlation between the distribution of $CD44^+/CD24^-$ cells and stage (p=0.068). In the discussion part, it was also stated that these results were contradicted by studies supporting that the cells with $CD44^+/CD24^-$ phenotype have invasive genes and they affect disease-free survival in a negative manner [13,17,18]. When the distribution of surface markers was evaluated by the stage of disease, the distribution of cells with a $CD44^+/CD24^-/Lin^-$ phenotype were higher in the early stage but without statistical significance (p=0.90). As indicated by Waterworth et al., factors determining the distribution of $CD44^+/CD24^-$ cells may be the signalling pathways interacting with CSCs which have a role in tumor formation [19].

In the study by Abraham et al., it was reported that there was no relationship between the number of involved lymph nodes and the percentage of $CD44^+/CD24^-$ cells [14]. Similarly, in the study by Honeth et al. on basal-like breast cancers, no correlation was found between the number of lymph nodes and the percentage of $CD44^+/CD24^-$ cells [14]. In a meta-analysis evaluating 12 studies, similar results were obtained in terms of number of lymph nodes and the percentage of $CD44^+/CD24^-$ cells [20]. Moreover, Mylona et al. showed a significant inverse relationship between lymph nodes and percentage of $CD44^+/CD24^-$ cells in their study (p=0.019) [13]. In our study, no relationship was found between the number of lymph nodes and the percentage of $CD44^+/CD24^-$ cells.

Aulmann et al. investigated a possible relationship between CSC distribution and tumor grade, and reported no relationship [15]. In a meta-analysis published in 2010, a relationship was reported between CSC distribution and the histological high grade characterized by biologically agressive phenotype [20]. When the tumor grade and the percentage of $CD44^+/CD24^-$ cells were compared in our study, the percentage of $CD44^+/CD24^-$ cells increased as the tumor grade increased; however, this relationship was not statistically significant. This result may be explained by the limited number of patients included in our study.

In two different studies reported before, no relationship was detected between the percentage of $CD44^+/CD24^-$ cells and ER/PR status [13,14]. However, in a meta-analysis, it was stated that there was a statistically significant relationship between ER and PR negativity and CSC distribution [20]. Similarly to the other two studies, no relationship was detected between the percentage of $CD44^+/CD24^-$ cells and ER status. However, it was observed that the percentage of $CD44^+/CD24^-$ cells was higher in PR negative tumors compared to PR positive ones although without statistical significance, probably due to the limited number of patients which might lead to the statistical insignificance.

A correlation between HER-2 negativity and $CD44^+/CD24^-$ phenotype has been shown in the study of Honeth et al. [12]. In the study by Aulmann et al., the number of $CD44^+/CD24^-$ cells was higher in tumors with HER-2 overexpression [15]. In addition, no relationship between HER-2 status and CSC distribution was reported in a meta-analysis [20]. In the present study, although the percentage of $CD44^+/CD24^-$ cells was higher in HER-2 negative tumors, this result lacked statistical significance. Since the distribution of HER-2 positive (8 patients) and negative (15 patients) tumors was not equal among our patients, statistical significance could not be determined.

Although there was no relationship between stem cell distribution and the age of patients in the studies reported [12,13,15], there was a positive correlation between the percentage of $CD44^+/CD24^-$ cells and the age of the patients in our study.

CD18 is a mononuclear cell surface marker. In a study conducted in China in 2000, it was reported that $CD18^+$ expression was higher in lymph node negative tumors compared to lymph node positive ones. This result supports the relationship between cancer formation and immunity [21]. In our study, the distribution of $CD18^+$ cells was higher in some tumors when the tissues were examined in terms of lineage antigens. Therefore, we analyzed whether there is a relationship between the percentage of $CD18^+$ cells and tumor stage and lymph node involvement. The distribution of $CD18^+$ cells was higher in the early disease stage, but without statistical significance. The percent-

age of $CD18^+$ cells was also higher in lymph node negative tumors, again without statistical significance. When the distribution of $CD18^+$ cells was evaluated according to ER/PR status, it was shown that $CD18^+$ distribution was significantly higher in PR positive tumors (p=0.042). No information on this topic was reported in the literature.

MUC1, expressed in the apical part of glandular epithelium, is a big glycosylated transmembrane mucin. It has many physiological roles such as adhesion, development and differentiation. Increased expression of MUC1, especially in breast cancer and colon, kidney, prostate and gastrointestinal cancer and its delocalization in the membrane were found to be associated with poor prognosis and short life expectancy. This molecule is also being studied as a new target in antitumor vaccines [22]. Therefore, we evaluated whether there is any relationship between MUC1 and stage and grade and observed that the distribution of $MUC1^+$ cells was higher in advanced-stage breast cancers. However, this result lacked statistical significance, maybe due to the limited number of patients. There was also an inverse relationship between the distribution of $MUC1^+$ cells and the grade of the tumor that could not reach statistical significance.

In conclusion, $CD44^+/CD24^-$ cells were present in all tumor tissues in our study and the distribution of these cells was 1.43±1.16%, similar to the literature. This cell distribution was higher in the early-stage disease, although without statistical significance. No correlation between prognostic factors and the distribution of $CD44^+/CD24^-$ cells was established. The distribution of $CD18^+$ cells was significantly higher in PR positive tumors and no information on this topic is to be found in the literature.

Although there are some positive improvements, absence of any relationship between $CD44^+/CD24^-$ cell phenotype and clinicopathological features reveals that more research is needed to understand cancer genesis.

Conflict of interests

The authors declare no confict of interests.

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