



The Expressions of Cancer Stem Cell Markers and Non-classical HLA antigens in Breast Tumors

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Abstract

Objective: There is a strong relationship between the cancer stem cells (CSCs) and poor prognosis, metastasis and recurrence. In addition to this CSCs are resistant to chemotherapy and radiotherapy. Therefore, current treatment approaches may be ineffective to elimination of CSCs. The tumor cells have various adaptations for escaping from immune cells. The human leukocyte antigen (HLA) -G, which expressions restricted with fetal tissues and HLA-E are kind of tumor immune evasive adaptations. In this study, we aimed to investigate the relationship between the CSCs and immune evasive adaptations of breast tumors.

Methods: We immunohistochemically evaluated that the expressions of cluster of differentiation (CD) 44, CD133, Homeobox protein Nanog, octamer-binding transcription factor (Oct) 3/4, HLA-G and HLA-E in the advanced stage breast cancer tissues (n=10) and the non-malignant breast biopsies (n=10).

Results: We detected that the significantly increased expressions of especially Nanog (p<0.001) and also CD44 (p<0.001), CD133 (p<0.001) and Oct3/4 (p<0.001) in the advanced stage breast tumor group compared with non-malignant breast biopsies group, but the HLA-G (p<0.001) and HLA-E (p<0.001) decreased.

Conclusion: These findings suggested that, malignant breast tumors may have CSC-like cells, and these cells may play role for occurring malignant behavior. To proliferation and tumor formation, the immune evasion is essential for both of malignant and benign tumors. Higher expressions of HLA-G and HLA-E may be an indication that the non-malign tumors more immune evasive than the malign tumors. However, further prospective studies are needed to confirm our findings.

Keywords: Breast tumor, cancer stem cell, human leukocyte antigen G, human leukocyte antigen E, tumor immune evasion

Introduction

In *in vitro* culture conditions, cancer stem cells (CSCs) stand out as non-adherent spheroid colony-forming cells and are differentiated from the other tumor cells by specific markers such as cluster of differentiation (CD)133, CD44, Aldehyde dehydrogenase (ALDH), Homeobox protein Nanog, sex determining region Y-box (Sox) 2, and octamer-binding transcription factor (Oct)4 (1-3). These cells are strongly associated with poor prognosis, metastasis, and recurrence, and thus they are potential therapy targets (4). However, it has been shown that the CSCs are chemotherapy and radiotherapy-resistant cells, and conventional anti-cancer therapies may be inadequate (5). It is quite important to specifically target the CSCs, but unfortunately the specific markers of CSCs are also expressed in many adult stem or progenitor cells (6, 7). The identification of biomarkers specific to CSCs is thus essential for therapeutic approaches to eliminate CSCs.

Breast cancer (BC) is a multifactorial tumor that includes anomalies in estrogen, progesterone, and receptor tyrosine-protein kinase erbB-2 (HER2) receptors. The tumor progression and survival rates of BC patients improve by using combinations of chemotherapy and anti-HER2 monoclonal antibody (mAb) therapy. However, it has been reported that triple-negative BC patients have worst prognosis, greater recurrence, and shorter survival (8, 9). According to current literature, the most accepted phenotype of spheroid colony-forming BC-CSCs is CD44^{high}CD24^{low}, and some researchers have reported that CD44^{high}CD24^{low} BC-CSCs also have decreased HER2 expression and that these cells are resistant to anti-HER2 mAb therapy (10, 11).

Tumor cells can escape from the immune system by various adaptations, one of which is alteration of major histocompatibility (MHC) class I antigens. Intracellular tumor-related antigens are presented to cytotoxic T lymphocytes (CTLs) via the MHC class Ia antigens, which activates CTLs to selectively kill the tumor cells, and therefore MHC class Ia antigens are crucial to the generation of an effective anti-cancer immune response (12, 13). However, most tumor cells can downregulate the expressions of MHC class Ia antigens to escape from CTL-dependent cytotoxicity (14, 15). The natural killer (NK) cells are another type of anti-tumor cytotoxic cells, but the NK-mediated

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anti-tumor response is not tumor specific, and the tumor tissue distributions of NK cells are low compared to peripheral blood and they have short half-lives (16-18). Due to the lack of MHC class Ia antigens, the tumor cells become targets of NK cells (19). To evade NK-dependent cytotoxicity, some tumor cells can alter their expression pattern of MHC class Ib antigens such as HLA-G, HLA-E, and HLA-F. The expression of HLA-G is restricted to fetal tissues, and it plays an important role in the development of maternal immune tolerance to the semi-allogeneic fetus (20). The interaction of HLA-G with the immunoglobulin-like transcript (ILT) receptor 2, ILT-4, and killer cell immunoglobulin-like receptor leads to suppression of NK cells, CTLs, B lymphocytes, and dendritic cells (21). HLA-E is not restricted to fetal tissues, but it can inhibit NK cells by interacting with the CD94/NKG2A receptors, and HLA-E expression can be stimulated in the presence of HLA-G (22).

Conventional therapy approaches might be ineffective in eliminating CSCs, and immunotherapy is a new and promising approach to treating cancer. The effect of immunotherapy consists of activating patient immune cells against tumor cells or blocking the inhibitory signals released by tumor cells (23, 24). Anti-HER2 mAb therapy targets HER2-expressing BC tumor cells and blocks the HER2 receptors. Additionally, the Fc domains of anti-HER2 antibodies stimulate the NK cells by interacting with CD16 (Fc γ R), and this leads to cytolysis of tumor cells. However, it has been shown that some tumor cells can evade the antigen-dependent cytotoxic effects of NK cells by stopping or reducing the expression of HER2 (10, 11).

In the current literature, several studies have investigated BC-CSCs and MHC class Ib antigens, but they have not been evaluated together (8-11). To show the immune-evasive alterations of BC-CSCs, we aimed to investigate the relationship between the CSCs and non-classical MHC Ib antigens in histopathologically confirmed advanced-stage BC and non-cancer breast biopsy samples by using immunohistochemical staining of CD44, CD133, Nanog, Oct3/4, HLA-G, and HLA-E.

Material and Methods

This is a retrospective study and was approved by the Ethics Committee of Celal Bayar University Medical School (19.11.2014/20478486-383). From 2009 to 2011, 10 histopathologically confirmed advanced-stage BC and 10 non-malignant breast biopsy samples were obtained from the pathology laboratory of Merkezefendi State Hospital and enrolled for immunohistochemical evaluation. All samples came from breast tissues that had been collected for diagnostic purposes. The 4 μ m sections were obtained from formalin-fixed, paraffin-embedded tissues and stained with the primary antibodies CD133 (clone 3F10 mouse monoclonal IgG1, Biorbyt, USA), CD44 (DF1485 mouse monoclonal IgG1, Santa Cruz Biotechnology, USA), Nanog (mouse monoclonal IgG1, Biorbyt, USA), Oct3/4 (clone C-10 mouse monoclonal IgG2b, Santa Cruz Biotechnology, USA), HLA-G (clone 4H84 mouse monoclonal IgG1, Santa Cruz Biotechnology, USA), and HLA-E (clone 3H2679 mouse monoclonal IgG1, Santa Cruz Biotechnology, USA) all diluted at 1/100. In brief, the deparaffinization procedure was accomplished in xylene for 1 hour. Rehydration was done in sequential descending alcohol series for 2 minutes each. After leaving in distilled water for 5 minutes, the tissues were delineated on the object slide, washed in phosphate buffered saline (PBS) for 10 minutes, and then left in trypsin for 15 minutes. The primary antibody was then

applied in an incubator at 37°C and washed with PBS. Afterwards the biotinylated secondary antibody was applied and washed with PBS before incubating with the enzyme conjugate and 3,3'-diaminobenzidine tetrahydrochloride (DAB). Then sections were counterstained with Mayer's hematoxylin (Zymed Laboratories) and mounted with entellan.

Immunostaining was evaluated semi-quantitatively by H-SCORE analysis. All slides were examined and photographed with an Olympus DP72 digital camera integrated with an Olympus BX51 light microscope. Immunostaining intensity was categorized into the following scores: 0 (no staining), 1 (weak but detectable staining), 2 (moderate staining), and 3 (intense staining). An H-SCORE value was derived for each specimen by calculating the sum of the percentage of cells for the nuclear and cytoplasmic immunoreaction of the sections that were stained at each intensity category multiplied by its respective score according to the formula $H\text{-score} = \sum P_i (i+1)$, where i =intensity of staining with a value of 1, 2, or 3 (weak, moderate, or strong, respectively) and P_i is the percentage of stained cells for each intensity, varying from 0% to 100%. For each slide, 10 different fields were evaluated microscopically at 200 \times magnification. H-SCORE evaluations were performed independently by at least two investigators blinded to the source of the samples as well as to each other's results. The average score of both was utilized.

Statistical Analysis

The statistical analysis were performed using Statistical Package for Social Sciences version 20.0 (IBM Corp.; Armonk, NY, USA) software. Means and standard deviations were used to present the data. Statistical comparisons between the groups were performed using the Mann-Whitney U-test, and p-values <0.05 were accepted as significant.

Results

The median age of the BC patients was 59 years, and the median age of the non-cancer patients was 41 years. Patient data, including age, gender, histological differentiation, lymph node metastasis, and tumor size were collected and summarized in Table 1. The CD44 ($p < 0.001$), Nanog ($p < 0.001$), and Oct3/4 ($p < 0.001$) expression levels were significantly increased in the BC tumor tissues compared with the non-malignant tissues, but the HLA-G ($p < 0.001$) and HLA-E ($p < 0.001$) expression levels were significantly decreased. CD marker expressions of BC tumor tissues and non-malignant tissues are shown in Figure 1, pluripotency markers are Figure 2 and nonclassical MHC antigens are Figure 3. The graphical comparisons of H-SCORE values of all markers are presented in Figure 2.

Discussion

In this study, we showed significantly increased expression of CD133, CD44, Nanog, and Oct3/4 in the advanced-stage BC tumor sections compared with the non-malignant biopsy sections, while the expression of HLA-G ($p < 0.001$) and HLA-E ($p < 0.001$) was significantly decreased.

Due to their strong correlation with poor prognosis, metastasis, and recurrence, CSCs are very attractive cells as therapeutic targets in cancer therapy. Much research focus has been on ways to accurately identify CSCs. However, these cells are heterogenic subsets

Table 1. The demographic and histopathological data of advanced-stage breast tumors and non-malignant breast biopsies

	Advanced-stage breast tumors	Non-malignant breast biopsies
Age	59±9	41±14
H-score values		
CD44	253±11	203±40
CD133	258±17	204±38
NANOG	219±4	119±7
OCT3/4	125±6	113±7
HLA-G	180±27	233±20
HLA-E	169±13	247±14
Stage of tumors		
IIIA	n: 7	NA
IIIB	n: 3	
Histopathological findings		
- Lobular carcinoma	n: 1	
- Invasive ductal carcinoma	n: 9	
- Fibrocystic breast disease		n: 5
- Fibroadenoma		n: 5
- Apocrine metaplasia		n: 2
- Ductal ectasies		n: 2
Lymph node metastasis		
N2	n: 6	NA
N3	n: 4	
Tumor size		
T1 (UP TO 2 CM)	n: 1	NA
T2 (2-5 CM)	n: 9	
NA: not available; N2: cancer has spread to 4 to 9 lymph nodes under the arm, or cancer has enlarged the internal mammary lymph nodes; N3: cancer has spread to 10 or more axillary lymph nodes, with at least one area of cancer spread greater than 2mm; T1: tumor is 2 cm or less across; T2: tumor is more than 2 cm but not more than 5 cm across; Stage IIIA: the tumor is not more than 5 cm across (or cannot be found) (T0 to T2). It has spread to 4 to 9 axillary lymph nodes, or it has enlarged the internal mammary lymph nodes (N2). The cancer hasn't spread to distant sites (M0). Stage IIIB: the tumor has grown into the chest wall or skin (T4), and one of the following applies: It has not spread to the lymph nodes (N0), It has spread to 1 to 3 axillary lymph nodes and/or tiny amounts of cancer are found in internal mammary lymph nodes on sentinel lymph node biopsy (N1), It has spread to 4 to 9 axillary lymph nodes, or it has enlarged the internal mammary lymph nodes (N2), The cancer hasn't spread to distant sites (M0)		

of tumor cells and can have different phenotypes, thus the exact detection of CSCs is extremely difficult. In vitro studies have shown that most CSCs share expression patterns of specific molecules such as CD133, CD44, ALDH, Sox2, Nanog, and Oct4 (1-3, 25).

In this study, we evaluated the expression of CD44 and CD133 as cell surface markers of CSCs (26). Basal membrane adhesion is the first step in the metastasis of cancer cells, and the highly glycosylated surface molecule CD44 plays a crucial role in this process (27-29). Several studies have demonstrated that increased CD44 expression is strongly correlated with malignant BC, but not with clinical outcome (29-31). CD133, also called Prominin-1, is a well-defined cell surface marker of CSCs, and its expression is strongly

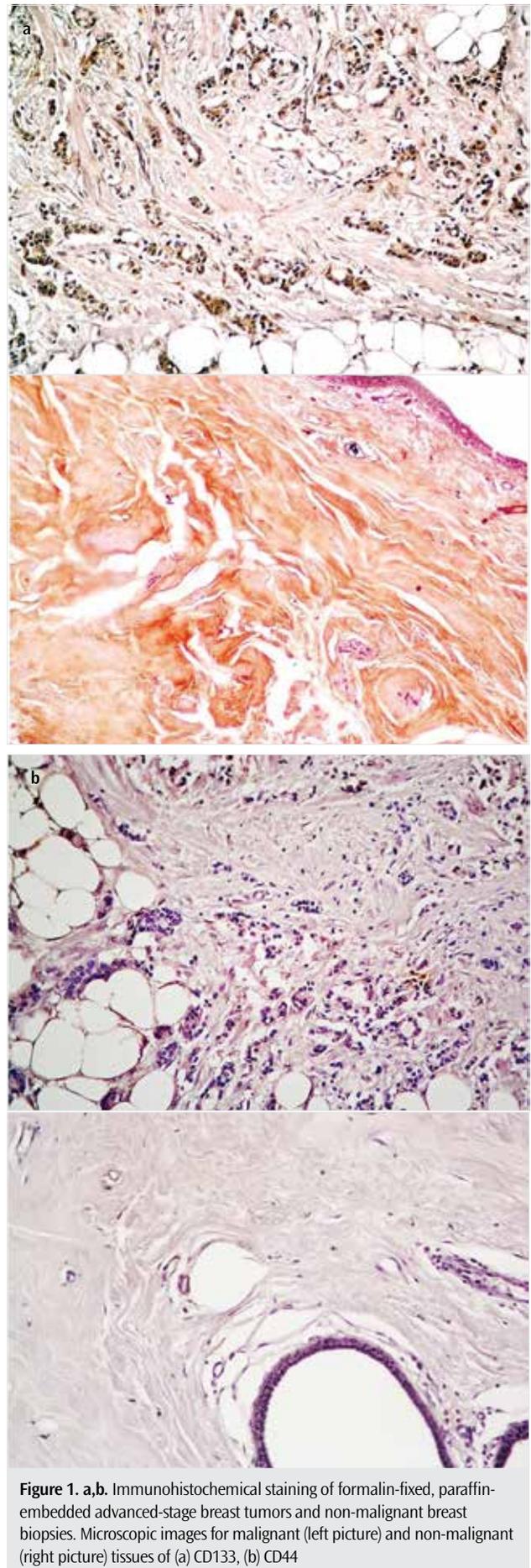


Figure 1. a,b. Immunohistochemical staining of formalin-fixed, paraffin-embedded advanced-stage breast tumors and non-malignant breast biopsies. Microscopic images for malignant (left picture) and non-malignant (right picture) tissues of (a) CD133, (b) CD44

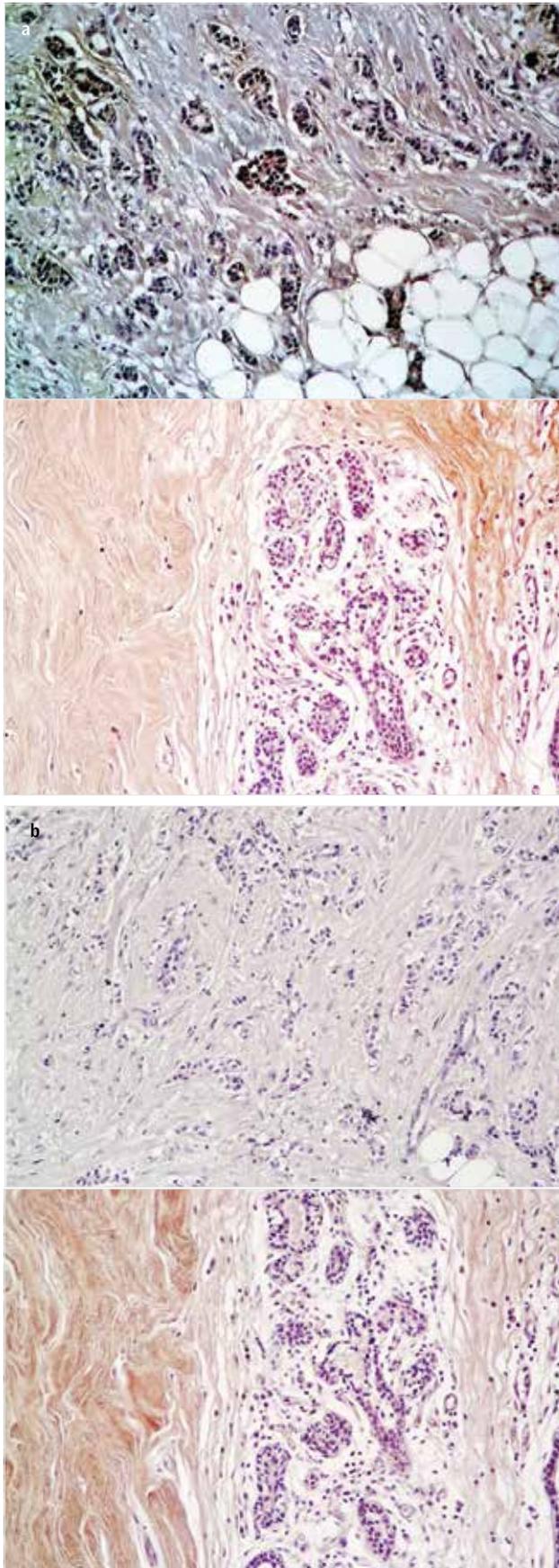


Figure 2. a,b. Immunohistochemical staining of formalin-fixed, paraffin-embedded advanced-stage breast tumors and non-malignant breast biopsies. Microscopic images for malignant (left picture) and non-malignant (right picture) tissues of (a) Nanog, (b) Oct3/4

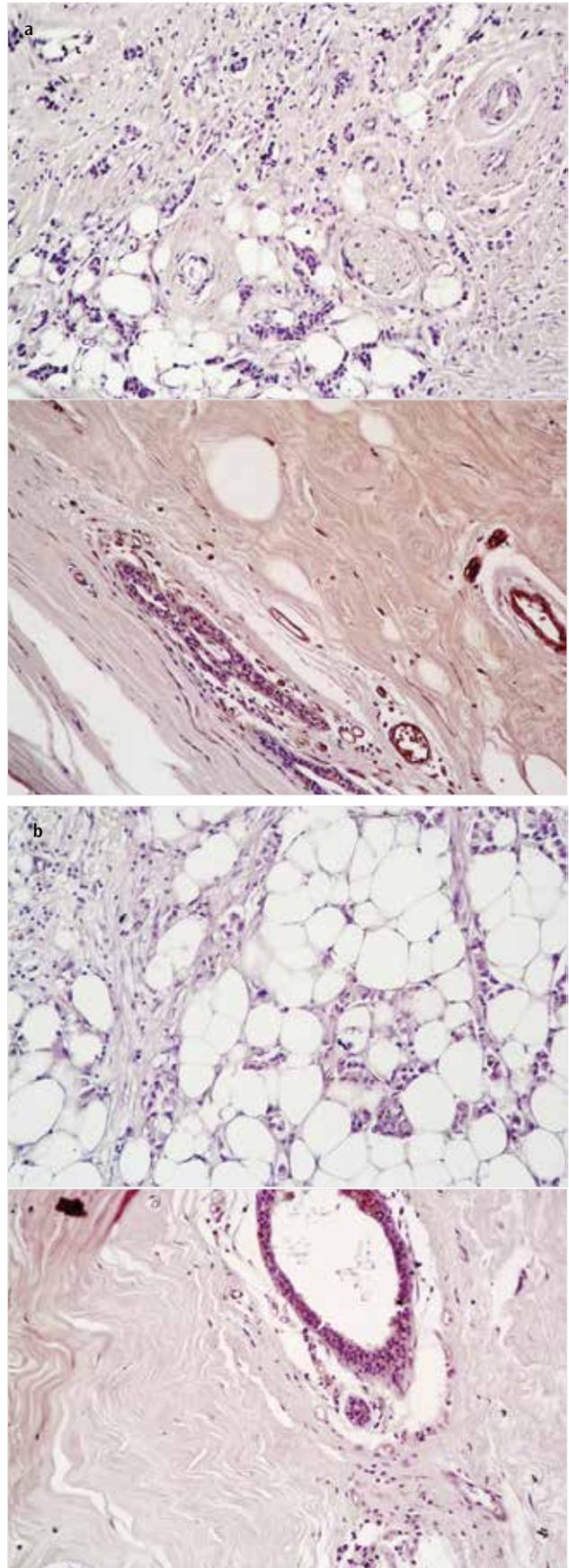


Figure 3. a,b. Immunohistochemical staining of formalin-fixed, paraffin-embedded advanced-stage breast tumors and non-malignant breast biopsies. Microscopic images for malignant (left picture) and non-malignant (right picture) tissues of (a) HLA-G, (b) HLA-E

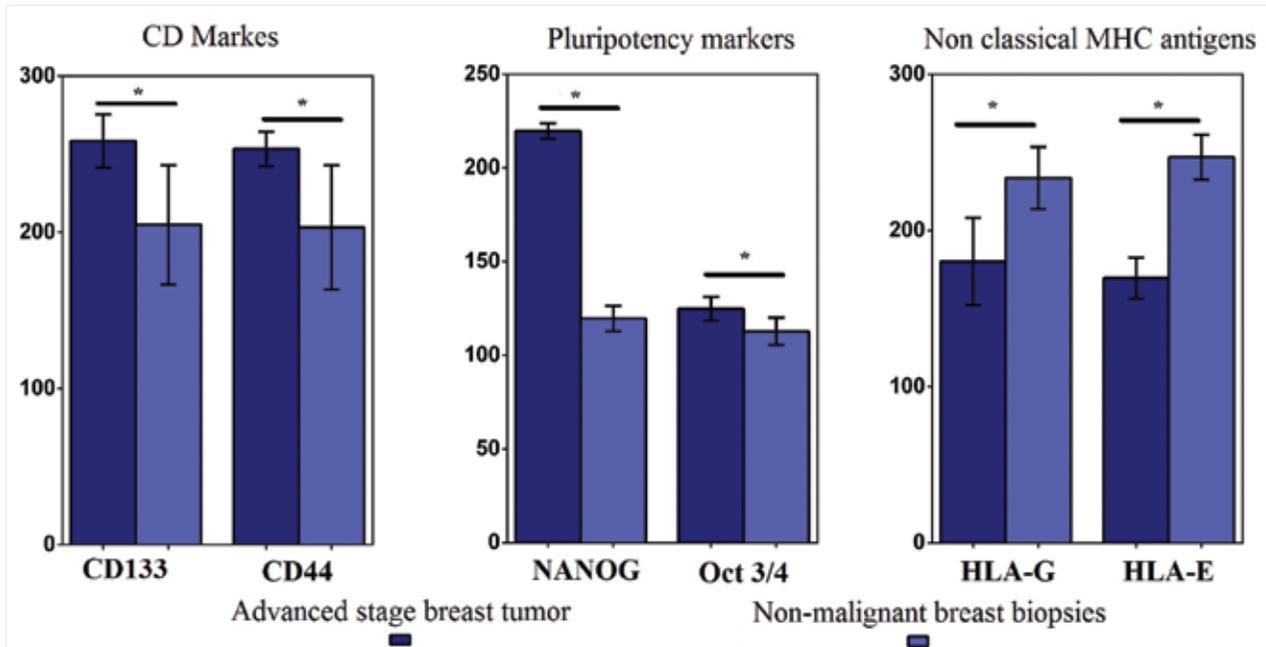


Figure 4. The comparison chart of H-scores of all markers. Data are presented as mean±SD (standard deviation). (*statistically significant, $p < 0.05$)

associated with poor prognosis, metastasis, and recurrence (32). CD133 can also be detected in healthy cells like hematopoietic stem cells and neuronal and glial stem cells (6), so the main function of CD133 in tumor progression remains unknown. Contrary to CD44, it has been shown that CD133 is associated with tumor angiogenesis, metastasis, and poor prognosis in malignant BC patients (33-35). We found significantly increased expression of both CD44 and CD133 in the advanced-stage BC tissue compared with non-cancer breast biopsy tissues. These findings may be indicative of the presence of CSC-like cells in the advanced-stage BC, but not in the non-malignant breast biopsies.

To further support the presence of CSCs, we evaluated the pluripotency-related transcription factors Nanog and Oct3/4. Similar to embryonic stem cells (ESCs), CSCs have self-renewal, undifferentiated, and pluripotency properties (7, 36, 37). Human Nanog is one of the crucial transcription factors that is strongly related with self-renewal and maintaining the undifferentiated state of ESCs (38). It has been reported that Nanog expression up-regulates most cancer types such as carcinoma in situ, embryonal carcinomas, and cervical and ovarian cancers (39). Mass spectrophotometric analysis of frozen BC specimens showed that Nanog is one of the most highly increased proteins (40). Oct4, similar to Nanog, is an essential transcription factor for maintaining the stemness of both ESCs and CSCs (41-43). The over-expression of Nanog and Oct4 is correlated with poor prognosis, metastasis, and reduced survival (42, 44, 45). Similar to cell surface markers of CSCs, we found that the expression of Nanog and Oct3/4 was significantly increased in the advanced-stage BC tissue compared with non-cancer breast biopsy tissues. These findings also support the presence of CSC-like cells in advanced-stage BC, but not in non-malignant breast biopsies.

As immune-evasive adaptation markers, we evaluated the expression of HLA-G and HLA-E in the tumor tissue and biopsy sections. Increased expression of HLA-G in BC has been shown to play an important role in the inhibition of immune cells (46, 47). It has

been reported that there is correlation between recurrence and poor prognosis and the expression of both HLA-G and HLA-E (48). In this study, we could not evaluate breast tissue samples from healthy subjects. However, we found reduced expression of HLA-G and HLA-E in the advanced-stage BC tissues compared with non-malignant breast biopsies. Although they are not malignant tumors, fibrocystic breast disease, fibro adenoma, apocrine metaplasia, and ductal ectasia may be considered benign but still tumors (49); therefore, they might have immune-evasive adaptations. The higher expression of HLA-G and HLA-E in non-malignant breast biopsies suggests to us that benign tumors of the breast might be more adapted to evading the immune system.

This study has some limitations, and one of the most important was the low sample number with regard to the incidence of BC. In addition to this, we could not evaluate the breast tissue samples from healthy subjects. Therefore, our results require validation via studies with a larger patient cohort.

Conclusion

We found concomitant and increased expression of especially Nanog as well as CD44, CD133, and Oct3/4 in advanced-stage BCs, but decreased expression of HLA-G and HLA-E compared to non-malignant breast biopsies. These findings suggest that malignant breast tumors might have CSC-like cells and that these cells might play a role in the occurrence of malignant behavior. For proliferation and tumor formation, immune evasion is essential for both of malignant and benign tumors. Higher expression of HLA-G and HLA-E might be an indication that non-malignant tumors are more immune-evasive than malignant tumors. However, further prospective studies are needed to confirm our findings.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Celal Bayar University School of Medicine (19.11.2014/No: 20478486-383).

Informed Consent: Informed consent is not obtained due to the retrospective nature of this study.

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