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Original Research

Stem cells in ductal breast cancer: immunohistochemical expression of CD44, CD24, CD133, and ALDH-1 markers in 104 cases

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Summary

Objective: The aim of this study was to evaluate the immunohistochemical expression of stem cell markers CD44, CD24, CD133, and ALDH-1 in breast carcinomas and investigate any correlation with stage, degree of tumor differentiation, cell proliferation index, and hormone receptors status. *Materials and Methods*: One hundred and four cases of invasive ductal breast carcinomas were studied and classified according to grade and stage, hormonal status, and C-erbB2 expression. A semi-automatic Ventana method was used to examine by semi-quantitative method the immunohistochemical expression of anti-CD133, CD24, CD44, and ALDH1 antibodies. *Results:* CD44, CD24, and ALDH1 markers demonstrated a statistically significant correlation with higher disease stage and triple negative cancers (94%-55.5%). CD133 correlated with triple negative cancers (55.5%). No correlation with other clinical data or tumor differentiation was observed. *Conclusion:* Triple negative breast cancers express specific stem cell markers responsible for cell/stroma interaction and this is a useful tool in predicting tumor progression and developing specific targeted therapies.

Key words: Breast cancer; Stem cells; CD44; CD24; CD133; ALDH1.

Introduction

Breast cancer is a complex and heterogeneous disease, consisting of different entities, with variable clinical pictures, histopathological and genetic characteristics, biological behavior, and therapy response [1]. Recent research provides information regarding the presence of stem cell populations within normal and neoplastic breast epithelia, with certain properties that are related to therapy outcome [2] and the tendency for tumor relapse in certain breast carcinomas [3-5].

Stem cells in normal tissues are long-lasting forms that remain in the body for long periods of time and accumulate mutations involved in the transformation of a normal cell into a cell with potential for neoplastic transformation [6, 7]. A large proportion of normal stem cells are found in transient forms, which have an even longer-lifespan and a slow rate of division, showing in certain cases the capacity to multiply indefinitely. These properties are regulated by their immediate microenvironment and are preserved after their neoplastic transformation [7, 8]. There is evidence that a small population of these stem cells survive chemotherapy and are responsible for the 40% recurrence rate of breast carcinomas [2, 9, 10].

Stem cells have been identified in various organs (breast, ovary) and respective tumors by specific receptors located on the cellular surface [5, 7, 11].

The stem cell markers extensively investigated by breast cell cultures and immunohistological methods are the CD44 and CD24 molecules, connected to adhesion molecules [2, 4, 12, 13], the CD133 protein, observed in hemopoietic stem cells [4] and the ALDH-1 enzyme, essential in normal growth and embryogenesis [14-16].

The aim of this study was to evaluate the expression of these markers by immunohistochemical method, in a selected group of infiltrating ductal breast carcinomas and to investigate the correlation with clinical and pathological parameters, such as stage of tumor, degree of differentiation, and hormone receptor status.

Materials and Methods

One hundred and four specimens of invasive ductal breast cancer (NOS) at the Laboratory of Histopathology of University Hospital during a five-year period, with a minimum three-year follow-up available for all cases. All cases were selected among 250 cases of breast cancer studied during this period in total, based on the following criteria: all were solitary tumors of invasive non-

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otherwise specified tumor. Multifocal, bilateral, and various specific type tumors were excluded. Cases were classified according to the histological degree of differentiation, into grade I (well-differentiated), grade II (moderately differentiated), and grade III (poorly differentiated) and staged according to TNM staging system. All cases of breast cancer were evaluated at the time of primary examination for the expression of ER and PR receptor status, the ki-67 proliferation index, and the expression of C-erbB2. Additional clinical data, therapeutic management, and follow-up data of these cases were available from the archives of the corresponding Department of clinical Oncology, where all these patients were treated at. This research was granted permission by the Hospital Research and Ethics Committee -No B-35/27.02.2014, and all cases were coded moreover to ensure patient anonymity.

Additional sections from the archived formalin-fixed paraffin-embedded tissues were examined for the expression of stem cells. A semi-automatic Ventana immunohistochemical method was used, according to manufacturer's guidelines, with anti-CD133, anti-mouse CD24 and CD44, anti-ALDH1 antibodies, Positive and negative controls were examined as well, provided by the manufactures. The evaluation of the staining was done by a semi-quantitative method, by two observers (VD and KA) and was characterized as negative (−) when < 10% of the cells were positive, positive (+) when > 10%of the cells were positive, and strongly positive (++) when > 30%of the cells showed immunoreaction. The location of the stain on the cell membrane or in the cytoplasm was recorded separately. Clinical data (patient's age, location, and size of tumor) were recorded as well.

A software was used for the statistical analysis. The correlation between categorical parameters (stem-cell markers, grade, stage, and receptor expression) was assessed by chi-square, with *p*-value of less than 0.05 considered to be significant.

Results

The clinical and pathological results are as follows: Group A: 21 cases of infiltrating ductal breast carcinomas were Grade 1. The age of patients ranged from 43 to 82 years with an average age of 58 years. The neoplasm was localized mainly at the left breast in 13/21 (62%) cases. The greatest tumor diameter ranged from 0.7 to 6 (mean diameter: 2.2) cm. Positive lymph nodes were observed in 9/21 cases (42%). Nine out of 21 (42%) cases were Stage 1, 7/21, (33%) were Stage 2, and 5/21 (24%) were Stage 3.

In 7/21 (33%) cases, a positive CD44 (+) immunoreaction, localized on the cell membrane of the tumor cells, was observed.In 2/21 cases a focal and faint staining of the cytoplasm was observed as well. In 6/21 cases (28%), a positive CD24 (+) immunoreaction, located on the tumor cell membrane, was observed. The CD133 marker was positive (+) in 4/21 (19%) cases localized on the cell membrane. Normal epithelial cells were negative for these markers. The ALDH-1 marker was positive (+) in 7/21 (33%) cases located in the cytoplasm of neoplastic cells. Normal epithelial cells showed a faint and local immunoreaction. Group B: 45 cases of infiltrating breast carcinomas were Grade 2. Patient's age ranged from 26 to 84 years with an average age of 44 years. The neoplasm was localized mainly in the right breast in 23/45 (51%) cases. The greatest tumor diameter ranged from 0.8 to 4 (average diameter: 3.8) cm. Positive lymph nodes were observed in 22/45 (49%) of the cases. Fifteen out of 45 (33%) cases were Stage 1, 20/45 (45%) were Stage 2, and 9/45 (20%) (45%) were stage 3.

The CD44 marker was positive (+) in 20/45 (44%) of the cases. CD24 was positive (+) in 12/45 (27%) cases. CD133 was positive (+) in 13/45 (29%) cases. The immunostaining was localized on the cell membrane. No normal epithelia were stained. The ALDH-1 marker was positive (+) in 14/45 (31%) cases, and the immunostaining was located in the cytoplasm. Very few normal epithelial cells showed a faint membrane immunoreaction. Group C: 38 cases of infiltrating breast carcinomas were Grade 3**.** The age of patients ranged from 39 to 84 years, with an average age 64 years. The neoplasm was located mainly at the right breast in 20/38 (53%) cases. The diameter range from 1.0 to 7 (average diameter: 2.7) cm. Infiltrated lymph nodes were observed in 18/38 (47%) cases. Eleven out of 38 (29%) of cases were Stage 1,18/38 (47%) were Stage 2, and 9/38 (24%) cases were Stage 3.

CD44 positive (+) immunoreaction was observed in 20/38 (53%) of the cases. CD24 positive (+) immunoreaction was observed in 4/38 (11%) of the cases. CD133 positive (+) immunoreaction was observed in 5/38 (13%) of the cases. Immunostaining in all cases was located on the cell membrane. ALDH-1 positive (+) immunoreaction was observed in 10/38 (26%) cases, located mainly in the cytoplasm. Normal epithelial cells were negative for all markers. The findings are presented collectively in Figure 1.

There were 35 cases of breast carcinomas in Stage 1. CD44 marker was positive (+) in 8/35 (23%) cases. CD24 was positive (+) in 3/35 (9%) cases. CD133 marker was positive (+) in 6/35 (17%) cases. ALDH-1 marker is positive (+) in 6/35 (17%) cases.

There were 46 breast carcinomas in Stage 2. CD44 marker immunoreaction was positive (+) in 21/46 (46%) cases. CD24 was positive (+) in 8/46 (17%) cases. CD133 marker was positive (+) in 11/46 (24%) cases. ALDH-1 marker was positive (+) in 9/46 (19%) cases.

There were 23 cases of breast carcinomas in Stage 3. CD44 score was positive (+) in 18/23 (78%) cases. CD24 marker was positive (+) in 11/23 (48%) cases. CD133 marker was positive (+) in 5/23 (22%) cases. ALDH-1 marker was positive (+) in 17/23 (74%) cases. The findings are presented collectively in Figure 2.

Eighteen out of 104 (17%) examined cases presented characteristics of triple negative breast cancers (estrogen receptor, progesterone receptor, and C-erbB2 negative). Immunohistochemical study showed that, in 17/18 (94%) cases, CD44 (+) immunoreaction was observed, in 7/18 (39%) cases. CD24 (+) reaction was observed in 10/18

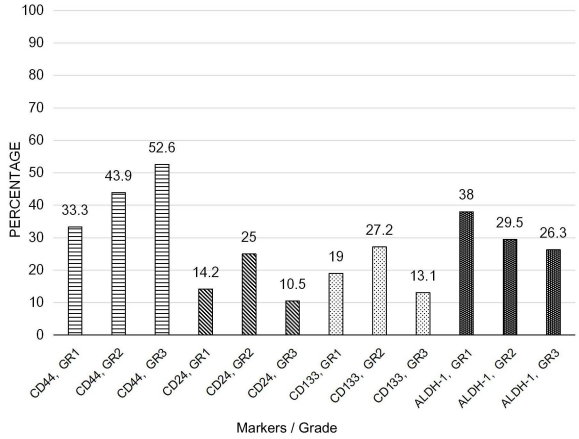

Figure 1. — Immunohistochemical expression of stem cells according to degree of differentiation.

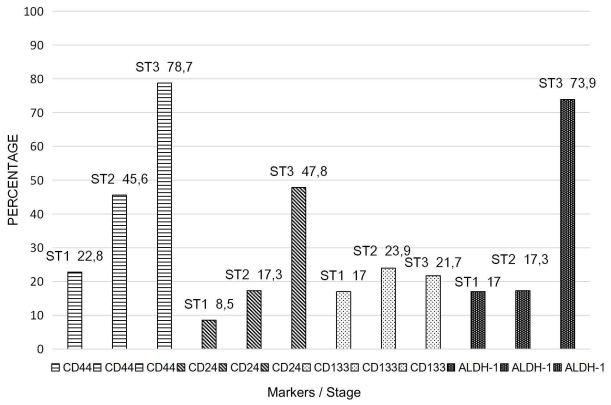

Figure 2. — Immunohistochemical expression of stem cell markers according to (TNM) stage.

(55.55%) cases. CD133 (+) and ALDH-1 (+) immunoreaction was observed. No strongly positive reaction (++) was noted in cases of the present study. The findings are presented collectively in Figure 3.

Statistical analysis of the immunohistochemical results showed that CD44, CD24, and ALDH-1 positive immunoreaction showed significant correlation (p value 0.001-0.002) with Stage 3 of the disease. CD133 was not significantly correlated with Stage (p value 0.180). No significant correlation was observed between the markers and tumor grade, ER(+) status, ki-67 proliferation index, or other clinical parameters, such as the tumor size (TNM staging) or the patient's age. There was a significant inverse correlation between stem markers (+) immunoreaction with negative ER(-) status (p value < 0.005).

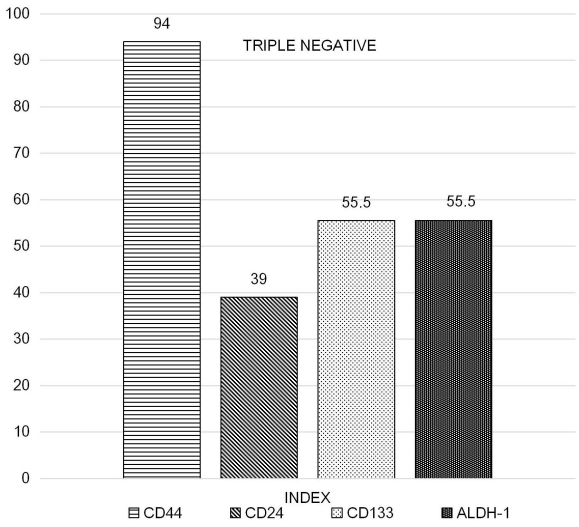

Figure 3. — Immunohistochemical expression of CD44, CD24, CD133, and ALDH1 in triple negative cancers.

Discussion

Breast cancer is a complex and heterogeneous disease to such an extent that it is considered not to be one but various entities, demonstrating different clinical pictures, histopathological and genetic characteristics, response to treatment, degree of relapse risk, and generally different biological behavior [1-3]. Despite this variety, breast cancer cases are classified based on histopathological features such as tumor size, degree of differentiation, lymph node metastasis, and lymphatic infiltration [1]. Pertinent information is collected according to well-established guidelines and used in algorithms, in order to design appropriate treatment for patients.

The complexity and variety of the disease and the necessity for individualized therapy is well-established by the application of high resolution molecular biology techniques, where the presence, absence, and/or amplification of many genes expression in breast cancer is investigated.

According to the molecular classification of breast cancer, the cases are grouped into tumors expressing the gene of estrogen receptor, characterized as luminal cell tumors and are distinguished in type A and type B. [1] Type A strongly expresses the gene estrogen receptor and, to a lesser extent, the Ki-67 cellular proliferation index. Type B tumors express in lesser degree the estrogen receptor gene and a high Ki-67 proliferation index. The luminal type A tumors, are associated with tubular carcinoma, papillary carcinoma, invasive ductal non-further specified carcinoma (NOS), and lobular carcinoma. Type B tumors are related

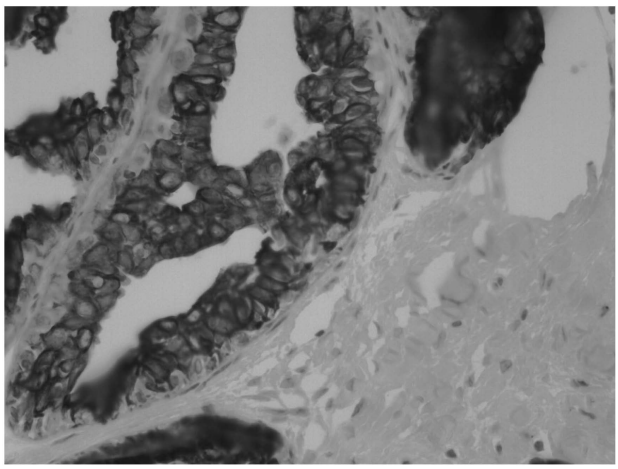

Figure 4. — CD44+ membrane immunoexpression in breast cancer glandular formation (immunostaining ×300).

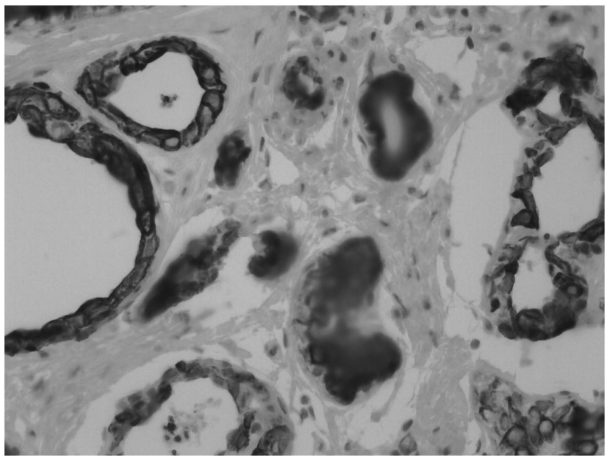

Figure 5. — CD+24 immunoexpression with membrane and cytoplasmic location in breast cancer glands (immunostaining ×240).

to invasive ductal carcinoma (NOS).

Tumors that do not express the ER marker include three groups: The first includes what are described as basal-like tumors, negative for C-erbB2 marker, expressing characteristics of basal/myoepithelial cells of the normal breast (p63 and keratin 5/6(-) immunoreaction). These tumors are histologically classified as high-grade carcinomas (NOS) and metaplastic carcinomas. The second group consists of HER-2 positive tumors, and the third group comprises tumors characterized as "normal breast type".

Evidence regarding the development of breast cancer supports the theory of breast epithelial stem cell existence, which demonstrate functional disorders that are an important step in their malignant transformation. The stem cell neoplastic transformation is caused directly or indirectly by suppressing the defensive mechanisms [2-5, 16]. Expression of these stem cells is the object of various studies, aiming in the development of new breast cancer therapies [9, 10, 16, 17].

The normal mammary gland shows a continuous regeneration of its tissues through controlled cell proliferation A small number of breast epithelial stem cells are recorded as well that proliferate at a slow rate and among them a number of cells are observed that grow rapidly and are called transit amplifying cells [2, 3, 9, 16, 18]. Stem cells have been identified in various organs, primarily in hematopoietic cells, by surface receptors such as CD44, CD24, CD133, ALDH-, and in various combinations of expression [5-7, 17].

The CD44 marker is a family of glycoproteins located on the cell surface of various organs of the body. It consists of an extracellular epitope, a transmembrane domain bound to stromal elements, and a cytoplasmic domain. The cytoplasmic domain is linked to the cell frame. The extracellular domain is linked to the extracellular ground substance, in particular to fibronectin, laminin, collagen fibers, and hyaluronic acid. CD44 is not expressed in normal mammary epithelium and this is in accordance to the present findings. In experimental studies, CD44 has been found to participate in the development of tumors and their metastases as it is shown in Figure 4 [10, 15, 19]. In the present study, CD44 expression showed statistically significant correlation with the disease in Stage 3, characterized by lymph node metastases.It also presents significant correlation with the cell proliferation index Ki-67 and inverse correlation with estrogen receptor expression. In the group of triple negative tumors studied separately, CD44 immunoreaction was expressed in a significantly high proportion (94%). No significant correlation with tumor grade was observed.

CD24 marker is a mucus-type protein molecule with surface cell localization, initially identified in hematopoietic elements of the white line and in hematological neoplasms and various cancers, (lung, pancreatic, ovarian cancer, etc.) [7, 17, 20, 21] This molecule has binding site with P-selectin, which belongs to the cellular cohesion molecules necessary for the coherence of neighboring cells. CD24 is stored in intracellular granules and when is activated is carried on the surface of the epithelial cells. CD24 is not expressed in adult normal tissues, but in neoplastic cells and this is in accordance to the findings of its study. Increased expression of CD24 suggests higher metastatic potential, possibly through augmented cell mobility and disorder of neoplastic cell coherence and epithelial-stromal cohesion [18, 20, 21].

In the present study, a statistically significant correlation was found between the stage of the disease, with its highest expression at an advanced Stage 3 and in the presence of lymph node metastases. The present authors also observed an inverse correlation with positive expression of

estrogen receptors. In triple negative tumors, CD 24+ immune-expression was found in 39% of cases. No significant correlation was found with degree of differentiation or cell proliferation marker. The findings of this study are consistent with the findings of a lung cancer study where it is a poor prognosis indicator associated with metastatic disease and advanced stage. [20, 21, 22]

The CD24 expression was not exclusively in the membrane of the cells of the invasive carcinoma, but in more than 50% of the positive cells it also showed cytoplasmic expression (Figure 5). There are already suggestions for this finding to be considered as a predictor of worst outcome and a tumor with strong positive expression could be an indication for more aggressive treatment [20, 23].

CD133 (PROMININ-1) is known to be a protein found on the cytoplasmic membrane of the cells and present in five transmembrane loci. Its activity remains largely unknown. This protein was first detected in normal human hematopoietic stem cells of many different organs and it is considered to be the marker of cancer stem cells [4]. There is evidence in studies on various cancers, that the expression of CD133 defines presence of clones of neoplastic cells with increased resistance to chemotherapy [4, 16, 26]. In neoplastic cells cultures, CD133 was observed on the cellular membrane and in the lumen of glandular formations, and there is evidence that it is implicated in the onset of carcinoma transformation and tumor recurrence.

In the present study there was no statistically significant correlation between the expression of CD133 and the degree of tumor differentiation, the disease stage or in the cell proliferation index, and this is in agreement with other studies [14, 16]. Moreover a significant inverse correlation between expression of estrogen receptors was found regardless of degree of differentiation and was observed in 56% of triple negative tumors examined.

A recent experimental study has shown that using paclitaxel nanoparticles against CD133 + cells inhibited the relapse or also tumors growth in cell cultures of glandular cancer cells, (CD133-targeted nanoparticles CD133NPs method). This method may also be useful in the future treatment of breast cancer [24-26].

The ALDH-1 marker is an enzyme responsible for the oxidative conversion of retinol to retinoic acid. This substance is important for the normal growth and homeostasis of tissues of various organs and essential for embryogenesis. Disorders of ALDH-1 expression are considered as promoters of neoplastic transformation of cell groups into various organs (pancreas, large intestine, mammary, and brain cells. These cell groups present stem cell features and form "cellular orbs" [14, 28]. In mammary cell cultures, this marker is expressed by cellular orbs showing neoplastic transformation [6, 10, 11].

In this study there was no statistically significant correlation between ALDH-1 expression and the degree of tumor differentiation or the stage of disease. There was an inverse correlation with ER expression and it was expressed in 55.55% of cases of triple negative cancers.

Cancer stem cells are considered one of the most important causes of breast cancer recurrence [2, 6]. Radiation therapy and chemotherapy treatments [17, 19, 24] have good results in the elimination of a large proportion of breast tumor cells, but appear to fail significantly to eliminate cancer stem cells, probably because of deregulation of genome repair mechanisms [18, 23, 27]. The study of stem cancer cells in breast [9, 21] as well as other organs [11, 12, 16] provide valuable information for targeted therapies, used already in certain cancers [19, 28].

Conclusion

The CD44 marker has a statistically significant correlation with a greater disease stage (78.3%) and presence of lymph node metastases. Greater is the negative expression of estrogen receptors, especially in triple negative cancers it is expressed in an extremely high percentage (94%). The CD24 marker has a statistically significant correlation with more and advanced stage of the disease, the greater the negative expression of estrogen receptors; in triple negative cancers it is expressed in 39% of cases.

The CD133 marker is not significantly related to stage of the disease. It is correlated with negative expression of estrogen receptors, and is greater in triple negative cancers, expressed in 55.55% of cases.

The ALDH-1 marker is not related to disease stage and in triple negative cancers is expressed in 55.55% of cases. No statistically significant correlation with the degree of tumor differentiation was observed ($p>0.005$) for the stem cell markers examined.

In conclusion, immunohistochemical investigation of CD44, CD24, CD133, and ALDH-1 markers of stem cancer cells considered responsible for the metastatic potential of tumors, provides valuable information on their prognosis, their metastatic potential, and future treatment.

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