Cancer stem cells biomarkers in triple negative invasive carcinoma of the breast and associated in situ lesions

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Abstract

Triple negative breast cancer (TNBC) [negative for expression of estrogen and progesterone receptors (ER, PR) and HER2/neu protein] represent a subtype of breast cancer associated with poor prognosis and highly aggressive behavior. The characterization of stem cell in this type of carcinoma could determine the appearance of new ideas concerning origin and evolution. There is an impressive amount of data in the literature related to TNBC and a growing interest for stem cell research during the past years, but there are no data concerning the genetic characterization of stem cells in the context of cell biology of TNBC as compared with associated DCIS. We performed immunohistochemical studies for the expression and distribution of several stem cell-related antigens, focusing on the association of TNBC with DCIS and comparing the presence of stem cells in the invasive and in the in situ component. Optimization of detection, identification and characterization of tumorigenic breast cancer stem cells might permit further identification of targeted treatment.

Keywords: stem cells, breast cancer, triple negative.

Introduction

Triple negative breast cancer (TNBC) [negative for expression of estrogen and progesterone receptors (ER, PR) and HER2/neu protein] represent a subtype of breast cancer associated with poor prognosis and highly aggressive behavior – younger age at diagnosis, high grade, large tumor size, aggressive relapse. They represent about 10–20% of all breast carcinomas, depending on the thresholds of tests, but much higher proportion of all breast cancer mortality [1].

Targeted therapies are available in hormone positive or Her2-positive tumors, but to this day, there is no specific therapy for triple negative breast cancer. TNBC are primarily comprised of a molecularly distinct subtype of breast cancer, the basal-like subtype, but the two entities do not overlap in all cases [2]. The identification of basal like subtype is based on gene expression profiles identified by microarray analysis, which is not clinically available and the immunohistochemical surrogate profiles described by Nielsen et al. [3] categorize TNBC as a proxy for the basal-like subtype. But, this is still an immunophenotypic description, based on the presence of basal cytokeratins and/or myoepithelial markers and a discordance of up to 30% has been described between the two groups [4].

Cancer stem cells are supposed to be tumor cells expressing CD44”CD24low that exhibit aldehyde dehydrogenase activity (Aldefluor+) [5]. While the origin and identity of breast cancer stem cells/progenitors is contentious, treatment-resistant cells survive and propagate only because aberrant and potentially targetable signaling pathways are recruited [6]. In addition to pathways known to regulate self-renewal of normal stem cells (Wnt, Notch, Hedgehog), there could be others implicated in the regulation of normal and malignant breast stem cell self-renewal. They also show a signature accounting for 186 differentially expressed genes (invasiveness gene signature, IGS) mainly involved in the IkB1 NF-kB (nuclear factor of kappa light polypeptide gene enhancer in B-cells 1) and RAS/MAPK (mitogen-activated protein kinase) pathways and in the epigenetic control of gene expression [7].

Despite the interest shown to stem cells recently, there are little data concerning the genetic characterization of stem cells in the context of cell biology of TNBC and associated ductal in situ carcinomas (DCIS).

The aim of this study was to analyze the breast cancer stem cells from primary triple negative tumor and associated DCIS and their many interrelationships, as it is unknown whether there are distinct types of cancer stem cells that correspond to different subtypes of breast cancer.

Materials and Methods

In this study, we enrolled 30 breast cancer patients with known triple negative status. All cases were invasive ductal carcinomas and associated an in situ component. Cases were selected from the cases of “Victor Babeș” National Institute for Research and Development in Pathology and Biomedical Sciences, Bucharest, Romania, and Hematoxylin–Eosin (HE) slides were assessed according to World Health Organization classification criteria. All specimens were also characterized by routine breast cancer immunohistochemistry panel (ER, PGR, c-erbB-2, Ki67) and only cases with no expression of hormone receptor or c-erbB-2 in the invasive component were included. For the hormone receptors, we used a cut-off of 1% tumor cells in distinguish ‘positive’ from ‘negative’ cases was ≥1% ER/PR positive tumor cells. c-erbB-2 expression was assessed using the HercepTest (DAKO):
• No staining is observed or membrane staining is observed in less than 10% of the tumor cells: score 0;
• A faint/barely perceptible membrane staining is detected in more than 10% of the tumor cells. The cells are only stained in part of their membrane: score 1+;
• A weak to moderate complete membrane staining is observed in more than 10% of the tumor cells: score 2+;
• A strong complete membrane staining is observed in more than 10% of the tumor cells: score 3+.

Scores of 0 or 1+ were considered negative, 2+ was equivocal, and 3+ was positive. For all 2+ cases, we performed CISH (chromogenic in situ hybridization) and only cases with no amplification were accepted.

All cases were stained for EGFR and CK5/6. Further on, we added several stem cell-related antigens CD24, CD44, ALDH1, SOX2, Nanog, Musashi. Semi-quantitative scoring systems were applied for all these markers.

Several patterns of staining were accepted for these antibodies and both the extent and intensity of immunopositivity in the cell membrane and cytoplasm were considered:
• Single isolated cells;
• Small clusters;
• Majority of cells strongly stained (rare).

For the assessment of CD24 and CD44, we applied the scoring system of Honeth et al. [8]:
• 0 – 0% positive tumor cells;
• 1 – 1% to 10% positive cells;
• 2 – 11% to 50% positive cells;
• 3 – 51% to 75% positive cells;
• 4 – 76% to 100% positive cells.

ALDH1 expression was assessed in the stroma and the cytoplasm of the tumor cells, disregarding any nuclear staining and classified as positive or negative.

## Results

The 30 patients included in the study group were all females aged between 30 and 69-year-old. The histological profile of the cases showed invasive ductal carcinoma (Figure 1) with associated in situ lesions (Figure 2). The in situ component varied from focal to extensive. We considered the in situ carcinoma as being extensive if more than 25% of the tumor mass was non-invasive (six cases). Histological grading of the cases showed a predominance of high-grade tumors (G3) in 17 cases. Only one cases was graded G1, while the remaining cases (12) were G2. Lymph node metastases were described in 15 cases.

All cases were triple negative on immunohistochemistry for ER, PGR and c-erbB-2. In order to correctly identify tumor cells and separate them from inflammatory cells, we performed a cytokeratin (AE1/AE3) staining (Figure 3) on all cases.

When applying the profile ER-, PR-, Her2/neu-, EGFR+ (Figure 4) and/or CK5/6+ describing basal like carcinomas, we identified 13 cases fulfilling all these conditions.

A method considered to be reliable in identifying cancer stem cells is applying the phenotype CD44+CD24−low and ALDH1+. We considered cases of CD24 staining included in score 0 and 1 to be negative, nine and respectively one cases, and cases of CD44 staining included in score 2, 3, and 4 to be positive (two, seven and six cases) (Table 1).

### Table 1 – CD24/CD44 profile of tumor cells

<table>
<thead>
<tr>
<th>Biomarker/Score</th>
<th>CD24</th>
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<tr>
<td>0</td>
<td>9</td>
<td>8</td>
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CD24 expression was identified mainly in the cytoplasm (Figures 5 and 6), while CD44 stained mainly in the membrane (Figures 7 and 8). The CD44+CD24−low was identified in 15 cases.

ALDH1 expression was identified in 11 cases, only in the invasive component, with various degrees of intensity of the cytoplasmic reaction (Figure 9). In some cases, there was moderate expression of the stromal component. The normal breast tissue present in some sections showed light staining in the ductal cells. There was no staining in any of the in situ lesions. All ALDH1 cases were high-grade tumors, node positive.

The phenotype CD44+CD24−low and ALDH1+ is considered the most accurate method to identify breast CSC. When we applied this formula to our study group the number of cases dropped down at 9 (30%).

SOX2 and Nanog are pluripotency-associated transcription factors, involved with self-renewal of undifferentiated embryonic stem cells.

SOX2 (short for sex determining region Y – box 2) encodes a member of the SOX (SRY-related HMG-box) family of transcription factors, thus having a vital role in embryonic development. Nanog overexpression enhances proliferation and is correlated with the stimulation of growth and metastasis of breast cancer cells.

SOX2 immunostaining showed cytoplasmic positivity in six cases (Figure 10), with no nuclear positivity and was expressed in DCIS and low-grade breast cancer.

On the other hand, Nanog was positive in the cytoplasm of invasive cancer cells in four cases and showed no positivity in in situ lesions.

Musashi serves as a switch for two cell-signaling pathways that control cell growth and is important to the development of many cancers, expressed in particularly in aggressive tumors. We only encountered low expression (Figure 11) in two cases and only the invasive component. Both cases were node positive.

## Discussion

Breast cancer is the most common malignancy in women and the second leading cause of cancer-related death, although advances in the therapeutic strategies have been made. This might be because the biology of breast cancer remains poorly understood and the multiple treatment options do not target cancer stem cells.

The cancer stem cell hypothesis proposes that tumors arise in either normal stem or progenitor cells through the dysregulation of self-renewal processes. The identification of factors required for their survival and self-renewal of cancer stem cells (like markers involved in regulatory signaling pathways) could highlight the
molecular aberrations critical for tumor growth and improve TNBC therapy.

Stem cells are cells with the capacity to self-renew as well as to generate daughter (progenitor) cells that can differentiate into multiple cell lineages. The cancer stem cell model proposes that tumors, like normal tissues, are organized in a cellular hierarchy. The ‘differentiated’ cancer cells may have high, but not unlimited proliferation potential. The ‘cancer stem cells’ are the only cells with unlimited proliferation potential and therefore capable of driving tumor growth and metastasis [9]. The cancer stem cell hypothesis proposes that tumors arise in either normal stem or progenitor cells through the dysregulation of self-renewal processes. This results in tumors that are driven by a cellular subcomponent that retains stem cell properties [10].
Figure 7 – In situ ductal carcinoma of the breast: positive reaction for CD44, 400×.

Figure 8 – Invasive ductal carcinoma of the breast: intensive positive reaction for CD44, 400×.

Figure 9 – ALDH1-positive invasive ductal carcinoma of the breast, 200×.

Figure 10 – Cytoplasmic positivity for SOX2 in an invasive ductal carcinoma, 400×.

Figure 11 – Low expression of Musashi in tumor cells, 400×.

Triple negative breast cancer (TNBC) (negative for expression of estrogen and progesterone receptors (ER, PR) and HER2/neu protein) represent a subtype of breast cancer associated with poor prognosis and highly aggressive behavior. Patients with TNBC do benefit from chemotherapy, but there is need of therapies that are less toxic, reduce the risk of disease progression, and are more targeted to this patient population. Targeting cancer stem cells could have an important impact in these patients [11]. There is a significant increase in presence of stem cell markers in triple-negative as compared to non-triple-negative breast cancers [12].

Breast tumors are comprised of phenotypically diverse populations of cells. Recent evidence suggests that only a certain type of tumor cells among these, the cancer stem cells or cancer initiating cells, harbor tumorigenic potential. The hypothesis that the initiation of malignancy has to take place in cancer stem cells derives from the observation that it often takes many months or years for the promotion stage of carcinogenesis to occur, suggesting that the cell that suffers such lesions must necessarily stay viable over a long period. Such cells have been identified for hematological malignancies, brain tumors and breast cancer. For breast cancer, such cells (putative breast cancer stem cells or breast cancer-initiating cells) have been identified to have a CD44^+CD24^−/low phenotype, CD44 is a cell adhesion molecule known to be expressed in most cell types and has been associated with stem cells in normal breast tissue. It is a cell surface receptor associated with the tumorigenicity and metastasis of breast cancer and elevated expression of this receptor is currently acknowledged as one marker discriminating the tumorigenic stem cell breast cancer cell population from the non-tumorigenic cells [14].

CD44 showed intense membrane positivity in the
invasive and in situ component. This pattern of staining is in accordance to the data of the literature [15]. CD24 showed cytoplasmic positivity, but membrane positivity has also been described, sometimes present in normal breast cells [15].

By many standards, the algorithm CD44^CD24^low is considered one of the most valuable cancer stem cell markers [16].

The CD44^CD24^low phenotype is associated with cancer initiation, being 1000 times more tumorigenic than CD24^high/CD44^+ cells [17].

The CD44^CD24^low phenotype was present in 50% of the cases showing that most cases presented very rarely positivity/negativity for both markers at the same time. All 13 cases showing the “basal like” immuno-phenotype were CD44^CD24^low, as previously described by Honeth et al. (2008) [8].

The presence of the phenotype CD44^CD24^low ALDH1-positive was identified in 30% of cases. Data from the literature vary in the percentage of cases showing this phenotype, to as little as <1% [18]. We presume that the higher number identified in our study is due to the selection of a special subtype of breast carcinomas.

Although other studies found no correlation between ALDH1 positivity and morphological indicators of bad prognosis, in our study the positive cases presented with lymph node metastases. This correlation was also described by Yoshioka et al. [19].

In a study investigating the ALDH1 expression in benign and malignant lesion of the breast, TNBC had the highest prevalence of positivity [20].

Nanog is described as a pro-carcinogenic protein in many solid cancers [21] and SOX proteins are expressed during early embryogenesis but also an early role in breast carcinogenesis and high expression could correlate with metastatic potential types [22].

There is little data in the literature concerning the role of SOX2 and Nanog in triple negative breast cancer. They are both transcription factors required for the renewal of embryonic stem cells. In our study, only some cases were positive and we found an association during the positivity of these markers and the degree of positivity of CD24, as previously described by Azzam et al. [23]. Most of the cases were CD44^CD24^low+ and fewer CD44^CD24^low-. None of the SOX2 positive cases showed node or distant metastasis but the tumors had little follow-up as they were recent cases.

We only identified cytoplasmic positivity for SOX2 and registered the cases as positive, although there are studies in the literature that take into consideration only the nuclear staining.

To our knowledge, there is no study investigating the expression of Musashi in triple negative breast cancer and its role in the etiology and progression of these diseases is unknown, but there is a study [24] that associates Musashi expression with bad prognosis. In our group, all positive cases were invasive, aggressive tumors, node positive, suggesting its role in the progression of breast cancer.

The identification of factors required for their survival and self-renewal of cancer stem cells (like markers involved in regulatory signaling pathways) could highlight the molecular aberrations critical for tumor growth and improve TNBC therapy.

Conclusions

One of the most important things to keep in mind is that TNBC and basal-like breast cancer overlap but are not identical. TNBC is generally initially chemosensitive but latter on becomes aggressive with a characteristic relapse pattern. This is why the characterization of CSC could open the doors for the development of novel targeted therapies against a subtype of very aggressive tumors, non-responsive to current chemotherapy regimens.

Acknowledgments

Study conducted with the support of Project “Triple negative breast cancer, associated in situ ductal carcinoma, cancer stem cells – a triple connection?”, PN-II-RU-PD-2011-3-0248.

References


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Received: March 20, 2014
Accepted: June 26, 2014