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Prognostic impact of the cancer stem cell related markers ALDH1 and EZH2 in triple negative and basal-like breast cancers

MARINA DE BROT*, RAFAEL M. ROCHA†, FERNANDO A. SOARES† AND HELENICE GOBBI*

*Laboratory of Breast Pathology, Faculty of Medicine, Universidade Federal de Minas Gerais, Belo Horizonte, and †Department of Anatomic Pathology, Hospital AC Camargo, Sao Paulo, Brazil

Summary

Aims: We assessed the expression of ALDH1 and EZH2, cancer stem cell (CSC) related markers, in triple negative and basal-like breast cancers, investigating their association with clinicopathological features and outcome.

Methods: Clinicopathological data were obtained from 140 cases of triple negative breast cancer. A tissue microarray was constructed and immunohistochemistry for ER, PR, HER2, ALDH1, EZH2, CK5, CK14, EGFR, p63, caveolin, and p53 was performed. Tumour cell and stromal expression of ALDH1 were evaluated. Multivariate analysis was conducted, including all significant variables.

Results: The majority of triple negative breast cancers were invasive ductal carcinomas of no special type (NST) (116/140). Tumour cells exhibited cytoplasmic expression of ALDH1 in 26 of 140 cases, while stromal expression was detected in 117 of 140 cases. Tumour cell expression did not correlate with any of the parameters. Conversely, stromal expression was associated with better overall survival (p=0.044). Assessment by Cox Regression Model showed a HR of 2.80 (HR = 1/0.357 = 2.80; 95%CI 0.178–0.714; p = 0.004) for breast cancer death when ALDH1 was not found in the stromal compartment of tumours, independent of age, histological type/grade, nodal status, stage, relapse, and expression of basal markers. High EZH2 expression was noted in 120 of 140 triple negative breast cancers and was not associated with other variables. Basal-like cancers comprised 75% (105/140) of triple negative breast cancers. Interestingly, we found association between EZH2 and CK14 expression (p = 0.041).

Conclusions: ALDH1 expression is frequent in tumour-associated stromal cells of triple negative breast cancer and is associated with better outcome. Tumour microenvironment should be considered when studying prognostic impact of CSCs in breast cancer.

Key words: ALDH1, basal-like breast cancer, breast neoplasms, EZH2, follow-up, immunohistochemistry, neoplastic stem cells, survival, triple negative breast cancer.

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BACKGROUND

Heterogeneity is an acknowledged phenomenon in solid tumours, as they are composed of a range of phenotypically diverse malignant cells.[1–3] Indeed, the concept that cancers contain a subset of cells similar to epithelial stem cells was proposed several years ago, along with emerging studies suggesting that some malignancies may obey a cancer cell hierarchy similar to that observed in normal tissues.[4–6] According to the controversial 'cancer stem cell hypothesis', cancer cells are hierarchically organised, and transformation from their own progenitor stem cells results in carcinogenesis, tumour growth and spread.[7–9] It is postulated that cancer stem cells (CSCs) possess self-renewal capacity, slow cell division, and the ability to generate a differentiated progeny, paralleling the properties of normal stem cells.[6,10,11] This subpopulation of cancer cells correspond to a very small percentage of cells in solid tumours which would give rise to the diversity of differentiated cells that comprise the bulk of the tumour.[6,11,12] Such cells were also thought to exhibit distinct properties compared to the rest of the cells in a given tumour.[13] Moreover, CSCs would hold selective resistance to radio- and chemotherapy.[4,10,14] Relapse of cancer and treatment failure could consequently echo the intrinsic quiescence and drug resistance of CSCs.[15–17]

Over the last few years, CSCs have been identified in different human cancers including primary and metastatic breast carcinomas, in addition to a number of established breast cancer cell lines.[9,11,18] Mammary stem cells and breast CSCs have been purified in *in vitro* culture systems by cell surface antigen identification.[18,19] Although considerable progress has been made towards identification of human mammary stem cells, the exact phenotype of these cells still remains poorly defined.[5,9,11,20] The CD44 cell-surface marker has been used to identify putative cancer stem cells in breast cancer. It has been shown in xenograft models that a CD44+/CD24− cell population meets the criteria for CSCs. Flow cytometry, followed by cell sorting, developing mammospheres and injecting a small number of these CD44+/CD24− breast tumour cells into nude mice, resulted in tumour growth.[18,21] Some studies have observed that ER+ breast cancer cell lines lack a CD44+/CD24− population of cells and that these cells appear to be restricted to mesenchymal/triple negative cell lines.[21,22] The CD44+/CD24− phenotype has also been shown to be enriched in basal-like breast tumours.[23] In addition, reports have confirmed poor prognosis of CD44+/CD24− expressing tumours.[21,23] Other cell surface markers have been linked to a CSC phenotype and were found in various subsets of breast cancer cells, including alpha 6 integrin, CD133, and beta 1 integrin/CD29.[21,24]

It is probable that CSCs have a phenotype defined by the cell of origin (stem cells or early progenitor cells) and by the oncogenic events that contribute to transformation.[25] A candidate marker of CSCs is aldehyde dehydrogenase 1 (ALDH1), an enzyme responsible for oxidising intracellular aldehydes and crucial during embryogenesis.[26] ALDH1 has been reported to

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have a role in early differentiation of stem cells, converting retinol to retinoic acid.[27] Increased ALDH1 activity has been demonstrated in human haematopoietic stem/progenitor cells and in stem cell populations in multiple myeloma.[28] In a study by Ginestier *et al.*,[29] the Aldefluor assay was utilised to show that cells with ALDH1 enzymatic activity isolated from normal human breast have phenotypic and functional properties of mammary stem cells. Interestingly, a small number of Aldefluor positive cells were capable of generating tumours in animal models.[29] They have also demonstrated that both normal and malignant human mammary stem cells may be identified *in situ* by immunohistochemistry.[29] In prior studies of human breast cancers, ALDH1 expression has been related to poor clinical outcome, absence of oestrogen and progesterone receptors, and expression of basal cytokeratins.[29–31] Moreover, some studies have shown that expression of ALDH1 is not restricted to epithelial cells but it has also been observed in stromal cells.[29,31,32] A study by Resetkova *et al.*[31] showed that not only has ALDH1 expression been identified in the tumoural stroma, it was associated with better clinical outcome in two cohorts of triple negative breast cancers.

Recently, it has been proposed that an aggressive secondary cancer stem cell population, the so-called 'tumour-initiating cancer cells', arises from a primary cancer stem cell population through acquisition of additional genetic mutations and drives cancer progression.[33] Chang *et al.*[33] reported that over-expression of EZH2, essential in stem cell self-renewal, is linked to the regulation and growth of breast tumour initiating cells. EZH2 is a Polycomb group (PcG) protein homologous to Drosophila Enhancer of Zeste which has been associated with breast cancer aggressiveness and poor outcome.[34,35] Novel research by Chang *et al.* identified that EZH2 expression mediates down-regulation of DNA damage repair. They have also tested an anti-cancer drug, sorafenib, which eliminates EZH2-promoted breast cancer stem cells.[33] These findings shed light on the value of EZH2 as a target for breast cancer therapy.

There is limited literature on expression of stem cell related markers in triple negative breast cancer, a subgroup that lacks expression of oestrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor type 2 (HER2).[36] Triple negative breast cancer shares morphological and genetic abnormalities with basal-like breast cancer, a subgroup of breast cancer primarily defined by gene-expression profiling.[36,37] Basal-like breast cancer has been said to express cytokeratins typical of basal cells and other non-luminal (basal) genes.[38,39] Although heterogeneous, triple negative and basal-like breast cancers typically occur in younger women and are associated with a range of adverse biological features including high grade, high mitotic count and p53 positivity.[40,41] While responsive to conventional chemotherapy, triple negative and basal-like breast cancers tend to relapse and metastasise early and have a worse prognosis than other tumour subtypes.[39,42] Thus, both subgroups represent high risk cancers that lack the benefit of specific treatment and for which we have no known targeted agents.[39,43] A recent preclinical study has shown that a multiple tyrosine kinase inhibitor, dasatinib, has a more potent antitumour effect on triple negative/basal-like breast cancer cells with a significant loss of putative cancer stem cell population.[44] Here, we sought to assess the potential utility of ALDH1 and EZH2 as prognostic markers in triple negative and basal-like cancers. We examine the expression pattern of ALDH1 and EZH2, investigating their association with clinical parameters, pathological variables, and basal-like markers. Furthermore, we describe the presence and significance of ALDH1 positive cells in the stroma of triple negative invasive breast carcinomas.

METHODS

Cases for this study were drawn from 2235 patients submitted to surgical treatment from 1985 to 2006 at the A. C. Camargo Cancer Hospital (São Paulo, Brazil), and at the Federal University of Minas Gerais (UFMG) Clinic Hospital (Belo Horizonte, Brazil). Cases were selected based on the availability of clinical data and paraffin blocks; original histological diagnosis of invasive breast carcinoma; and a previous pathology report showing negativity for ER, PR and HER2 (triple negative cancer). Formalin fixed, paraffin embedded (FFPE) tumours of 140 patients were obtained from the archives of the Pathology Department. Lack of expression for ER, PR, and HER2 was confirmed by a new immunohistochemical study.

This study was approved by the Research Ethics Committee of our institution under protocol number ETIC 466/06.

Clinicopathological data

Patients' clinical history and tumour characteristics were retrieved from the medical records and correlated with ALDH1 and EZH2 expression. The assessment included date of diagnosis; age at initial diagnosis; race; menopausal status; family history of breast cancer; nodal status; and pathological stage at diagnosis [2009 American Joint Committee on Cancer (AJCC) TNM Staging System]. Survival data were comprised of survival time, disease-free interval, date and type of relapse, development of distant metastasis, date and cause of death. Follow-up data were available for 132 patients, and the follow-up period ranged from 1 to 148 months (median 39 months; mean 51 months).

All original haematoxylin and eosin (H&E) stained sections of representative tumour blocks were reviewed in detail and histological type and grade were re-evaluated by a single pathologist (MDB).

Our triple negative cohort consisted of 140 women (mean age 55 years; range 32–86 years) with invasive breast carcinoma (116 invasive ductal carcinomas of no special type, 6 carcinomas with medullary features, 5 metaplastic carcinomas, 4 micropapillary carcinomas, 4 papillary carcinomas, 3 apocrine carcinomas, 1 medullary carcinoma, and 1 adenoid cystic carcinoma).

Construction of the tissue microarray

The tissue microarray (TMA) was constructed by extracting 1.0 mm diameter cores of histologically confirmed invasive breast carcinoma from the original paraffin blocks using a tissue core extractor (Beecher Instruments, USA) and re-embedding these cores into a gridded paraffin block. One such recipient paraffin block was constructed, containing two tissue cores from each selected tumour. Control tissue cores from normal liver were placed at two ends of this recipient paraffin block for orientation. Slides containing sections of a positive breast tumour were included in all batches as external control. Two cores were taken from tumour dense areas from the original biopsy block from each of the 140 patients. Each tissue core was assigned a unique TMA location, and was entered into an Excel database. After construction, 4 μm tissue sections were cut using a microtome and adhered to Fisher SuperFrost Plus glass slides.

Immunohistochemistry and scoring

Sequential slides from the TMA were deparaffinised in xylene, rehydrated in graded alcohol and stained with commercially available antibodies: ER (Novocastra, UK), PR (Novocastra), HER2 (Novocastra), ALDH1 (Epitomics, USA), EZH2 (Zymed, USA), cytokeratin 5 (NeoMarkers, USA), cytokeratin 14 (Biogenex, USA), EGFR (Zymed), p63 (Dako, USA), caveolin (Epitomics), and p53 (Dako). Antigen retrieval varied according to the primary antibody following the supplier's specifications. Negative controls were produced by omitting the primary antibody. All primary antibodies used, clones, dilutions, and suppliers are shown in Table 1. All immunohistochemistry (IHC) procedures were performed on a Link 48 Autostainer (Dako) using the Flex Plus visualisation system as per manufacturer's specifications. Stains were visualised using diaminobenzidine (Dako) and haematoxylin (Dako) counterstain.

Firstly, slides were stained for ER, PR, and HER2 to confirm the triple negative diagnosis. Then, basal cytokeratins (cytokeratins 5 and 14) and other