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Transcription factors associated with epithelial–mesenchymal transition and cancer stem cells in the tumor centre and margin of invasive breast cancer

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ABSTRACT

Although tumor surgery aims for a complete resection respecting tumor-specific safety distance, in many cases the most peripheral part, the invasion front, remains *in situ*. Tumor cells at the tumor margin lose epithelial properties and acquire features of mesenchymal cells. The process of epithelial-to-mesenchymal transition (EMT) has been suggested to be of prime importance for tissue and vessel invasion. Recently, features of EMT were shown to be linked to cells with tumor-founding capability, so- called cancer stem cells (CSC). In this study we show that transcription factors associated with EMT markers Snail, Slug, Twist and Zeb1 are differentially expressed between normal breast epithelium, ductal carcinoma *in situ* and invasive breast cancer. Both invasive and *in situ* carcinoma expressed less Slug and Twist and more Zeb1 compared to normal epithelium. Using fluorescence multi-staining the number of potential CSC among invasive cancer cells varied dramatically depending on the staining combination used (18.5% for $CD44^{+}/CD24^{-}$ and 2.4% for $CD49f^{+}/CD24^{+}$). Interestingly, neither transcription factors associated with EMT nor potential CSC counts varied between tumor centre and invasion front. No association of these features with clinical outcome was detected. Our results suggest that reliable *in situ* markers for EMT are missing for invasive breast cancer. Alternatively, the process of EMT might be activated in tumor cells at the margin as well as the centre. Furthermore, our data show that the bio-markers of CSC detect very variable cell populations within breast cancer, challenging the assumption of a hierarchical organization of CSC in these tumors.

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Introduction

Invasion and molding of distant metastasis are the determining characteristics of malignant tumors and therewith of tumor-related morbidity and mortality. These processes are associated with local detachment accentuated to the tumor margin resembling the invasion front. After detachment, migration and access to lymphatic or blood vessels enable dissemination and readaption of tumor cells to peripheral sites (Alkatout et al., 2008; Nassar et al., 2010; Wodarz and Nathke, 2007). These processes require penetration of basement membrane and extracellular matrix. The corresponding molecular steps are largely unknown. To this end differentiated epithelial tumor cells gain dedifferentiated mesenchymal capabilities through epithelial-to-mesenchymal transition (EMT) and therewith are able to dissociate from each other and migrate (Thiery, 2002). EMT is a substational process during embryology and is induced in terms of carcinoma progression. EMT is associated with a down regulation of E-cadherin as an important factor for tissue stabilization. Therewith it comes to destabilization of cell-cell contacts and detachment of cells from their surroundings. The repression of E-cadherin is triggered by an over expression of certain transcription factors. The proteins Slug, Snail, Twist and Zeb1 have been characterized as transcriptional repressors of E-cadherin and mediators of EMT (Eckert et al., 2011; Elloul et al., 2006; Morel et al., 2008; Moreno-Bueno et al., 2008; Peinado et al., 2007; Thiery, 2002). Recently, an association of EMT with the acquisition of tumor-founding capability in xenograft transplantation models was described. Thus, EMT might be closely linked to features of cancer stem cells (CSC) (Mani et al., 2008).

The assumption that tumors are organized in a hierarchical way composed of CSC capable of founding new tumors after transplantation and their descendants, heterogeneously differentiated cell populations lacking this feature, has been discussed in the past (Alkatout et al., 2008; Hill, 2006). This intratumoral heterogeneity has been first described in leukemia, followed by different solid tumor entities (Lapidot et al., 1994; McDonald et al., 2009; Nassar et al., 2010). Based on their tumor-founding capability, CSC are suspected to be the source of resistance to chemotherapy and radiation and tumor relapses. The percentage of estimated tumor cells with CSC features varies from a

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small percentage up to 25% of all cancer cells (Alkatout and Kalthoff, 2008; Hill, 2006). The proof of "stemness" is based on the ability to reconstitute tumor growth in xenograft models. Different cell surface markers have been used to identify these cells in breast cancer (e.g. $CD44^{high}/CD24^{low}$ or $CD24^{high}/CD49f^{high}$). However, data from malignant melanoma models indicate that this functionally defined "tumor-founding" capability is strongly dependent on the xenograft model used (Al-Hajj et al., 2003; Keller et al., 2010; Lapidot et al., 1994).

Using invasive breast cancer in this study we hypothesized the EMT markers Slug, Snail, Twist, and Zeb1 to express a different marker profile in normal breast tissue compared to breast cancer in immunohistochemical evaluation, and within the tumor a variable expression between tumor centre and invasion front. The aim of our investigation was also to detect potential CSC in normal breast tissue, breast cancer and precursor lesions. The expression patterns $CD44^{high}/CD24^{low}$ or $CD24^{high}/CD49f^{high}$ and the conjunction to EMT were investigated using fluorescence multi-staining. Therewith, flow cytometry-based methods that had been used before for xenograft models were optimally mimicked.

Table 1
Clinicopathologic parameters of the relapse group and non-relapse group. T1 = tumor stage 1, N0 = no nodal metastasis, M0 = no distant metastatsis, <=G2 = grade 2 or 1, ER + (>=3) = estrogen receptor expression >= score 3, PR + (>=3) = progesterone receptor expression >= score 3.

	Relapse group (n = 13)	Non-relapse group (n = 18)
T1	13/13	18/18
N0	10/13	18/18
M0	11/13	16/18
Ductal	8	14
Lobular	2	3
Other histology	3	1
(≤)G2	8/13	12/18
ER + (≥3)	7/13	15/18
PR + (≥3)	6/13	12/18
Her2neu (≥2)	2/13	4/18
Mean age	51	55
Age max.	68	72
Age min.	36	36
Period of follow-up (median in months)	99	54

Patients and methods

Patients were selected among those treated at the breast cancer center of the Department of Gynecology and Obstetrics of the University Hospital Schleswig-Holstein, Campus Kiel in the period July 2008 - September 2009. Informed consent was available for all patients registered at the Breast Cancer Database. Of these registered patients, 13 were selected based on tumor size and availability of high-quality formalin-fixed paraffin-embedded tissue (FFPE). Written informed consent of the original human work that produced the tissue samples was confirmed by the above mentioned ethics committee. All of the selected patients suffered from a local or distant recurrence of the tumor (relapse group). Tumors with a size of less than 2 cm diameter were selected to reliably distinguish between tumor centre and tumor margin/ invasion front on one full slide of the tumor. A second group (non-relapse group) consisted of 18 patients with invasive breast cancer that were matched for histological subtype and tumor stage to the case group. The patients in the non-relapse group did not show local or distant recurrence (median follow-up 50.5 months, range 36 to 132). None of the patients received preoperative radiation or chemotherapy. All patients received appropriate postoperative treatment depending on the stage of the disease, including chemotherapy, radiation and medical anti-estrogen therapy, when indicated. Ductal carcinoma in situ (DCIS) and normal breast tissue adjacent to the tumor or from breast reduction were analyzed as further controls. All carcinomas were classified according to the criteria of the World Health Organization. Staging at the time of diagnosis was based on the TNM system (Tavassaoeli and Davilee, 2003). The clinical parameters of the relapse and non-relapse group are outlined in Table 1.

Tissue micro arrays (TMA)

FFPE specimens were retrieved from the archives of the Department of Pathology. Histological examination was performed with hematoxylin and eosin staining (H&E) and representative areas were selected and assembled in a tissue microarray (TMA) using cores of 1.0 mm diameter and a TMA1 Tissue Arrayer (Beecher Instruments, Sun Prairie, WI, USA). Areas in the tumor center and the invasion front were selected and punched independently, with the distance between both areas being > 2 mm.

Immunohistochemistry for EMT

Three μm sections of the TMA were used for immunohistochemistry. Antigen retrieval was performed with citrate buffer pH6 or pH9 for 3 min by boiling in a pressure cooker. The primary antibody was applied for one hour at room temperature (rabbit polyclonal Snail antibody, 1:100, pH6, Abcam); rabbit polyclonal Twist antibody (1:200, pH6, Abcam); rabbit monoclonal Slug antibody (1:50, pH9, Cell Signaling Technology) and rabbit polyclonal Zeb1 antibody (1:300, pH6 Atlas Antibodies). The secondary antibody (Histofine: Simple MAX PO (Multi) Universal Immuno-peroxidase Polymer produced by Medac) was applied for 30 min at room temperature. The detection was performed using 100 μl/slide Dako DAB. For negative controls, the primary antibodies were omitted. Only nuclear staining was assessed and scored as positive or negative estimating the percentage of positive tumor cells (no positivity, 1–25% positive tumor cells, 25–50%, 51–75%, 76–100%). For all analysis score 0 and 1 were lumped as negative, and score 2–4 as positive. The tissue was analyzed by light microscopy (Zeiss Axiophot, Zeiss GmbH, Jena, Germany) and reviewed by ProCapture software (Mawson Lakes, South Australia).

Immunofluorescence staining for CSC

For the immunofluorescence the Cytokeratin 7 antibody (N-20) (1:100, pH6, Santa Cruz Biotechnology) was used as a marker for epithelial cells. DAPI staining for all cell nuclei was combined with CK7, CD24 and CD44 or CK7, CD49f and CD24. The antibodies, dilutions and antigen retrieval were as follows: CD44 (1:200, pH6, Sigma Aldrich), mouse monoclonal CD24 (Ab-2 (Clone SN3b) 1:50, pH6, Thermo Scientific), polyclonal rabbit CD49f antibody (1:50, pH6, Atlas Antibodies). All antibodies were applied for one hour at room temperature. Secondary donkey anti- rabbit/ mouse/ goat/ rat-antibodies produced by Alexa (1:100, pH6) were performed for one hour in a dark chamber. All slides were analyzed with an Axioplan-2 microscope (Zeiss GmbH, Jena, Germany). Areas considered to be representative of the tumor (reflecting tumor morphology as determined by conventional staining) and of the staining (e.g. providing sufficiently stained positive controls) were selected and photographed with a digital camera SPOT RTTM slider (Diagnostic Instruments Inc., Burroughs,Sterling Heights, MI, USA) and VisisView 1.7.2 software (Visitron Systems, Puchheim, Germany). For each slide and each staining combination four color photos were taken: Cytokeratin 7 to identify epithelial cells, DAPI to identify cell nuclei and the two stem cell marker in combination as described above. Two pictures of each TMA core were taken with 400-times magnification. All Cytokeratin 7 positive cells were counted manually on the pictures for expression of CD44, CD24 and CD49f.

Ethics statement

This study was approved by the Ethical Committee of the Christian-Albrechts-University Kiel, Kiel, Germany (D 426/10).

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