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The SOX2 Status of Disseminated Tumor Cells in Breast Cancer Patients Treated With Neoadjuvant Chemotherapy

NATALIA KRAWCZYK[1], KATHRIN JANOWSKI[2], MAGGIE BANYS-PALUCHOWSKI[3,4], ANNETTE STAEBLER[5], HANS NEUBAUER[1], CHRISTOPH MEISNER[6], ANDREAS HARTKOPF[2], SARA BRUCKER[2], DIETHELM WALLWIENER[2] and TANJA FEHM[1]

[1]*Department of Obstetrics and Gynecology, University of Duesseldorf, Duesseldorf, Germany;*
[2]*Department of Obstetrics and Gynecology, University of Tuebingen, Tuebingen, Germany;*
[3]*Department of Obstetrics and Gynaecology, University Hospital Schleswig-Holstein, Campus Lübeck, Lübeck, Germany;*
[4]*Medical Faculty, University of Duesseldorf, Duesseldorf, Germany;*
[5]*Department of Pathology, University of Tuebingen, Tuebingen, Germany;*
[6]*Institute for Clinical Epidemiology and Applied Biometry, University of Tuebingen, Tuebingen, Germany*

Abstract. *Background/Aim: Detection of disseminated tumor cells (DTCs) after systemic treatment predicts poor prognosis in breast cancer patients. The aim of our study was to assess the expression of stem-cell marker SOX2 on DTCs and in the primary tumor of patients treated with neoadjuvant chemotherapy (NAT). Materials and Methods: In 170 DTC-positive patients after NAT an additional slide of bone marrow aspirate was stained by double immunofluorescence to detect SOX2-positive DTCs. The SOX2 status of the primary tumor was assessed using the same antibody. Results: The SOX2-status of DTCs was determined in 62 patients and 20 of those (32%) had SOX2 positive DTCs. The SOX2 status of DTCs was not associated with any of the clinicopathological factors. A total of 36% of the patients with a SOX2-negative tumor showed SOX2-positive persistent DTCs. Conclusion: SOX2-positive DTCs can be detected in breast cancer patients after NAT, even in patients with SOX2-negative primary tumors. This suggests that these populations may have evolved independently of each other.*

In the past decade, neoadjuvant therapy (NAT) has become a standard approach in breast cancer (BC) management and is recommended if chemotherapy is indicated based on clinical characteristics and tumor subtype (1). The original aim of NAT was to reduce the size of inoperable or large tumors, thus allowing complete surgical removal and, in some cases, breast conservation (2). However, potential advantages of NAT reach beyond tumor size reduction and include *in vivo* evaluation of tumor sensitivity and identification of non-responders, who can be spared of the unnecessary toxicity of ineffective therapy (3, 4). Moreover, residual tumor burden after NAT is an indicator of unfavourable outcome in most subtypes of BC and may guide the choice of further post-neoadjuvant treatment strategies (5, 6).

While NAT can induce a pathological complete response (pCR) in up to 60% of BC patients, predicting the long-term survival benefit, a relevant proportion of BC patients still suffer from distant recurrence during follow up (6). The presumed pathophysiology of metastatic relapse is based on an early haematogenous spread of cells from the primary tumor. These isolated tumor cells can be detected in peripheral blood (circulating tumor cells, CTCs) or bone marrow (BM) (disseminated tumor cells, DTCs) of patients with most solid malignancies. In breast cancer, presence of CTCs and DTCs has been confirmed as an independent unfavourable prognostic factor for overall survival (OS) and disease-free survival (DFS) (7-11). CTCs/DTCs are nowadays assumed to be a surrogate marker for minimal residual disease (MRD) and their eradication is one of key goals of systemic treatment in non-metastatic BC (12, 13). Since DTCs can persist in secondary homing sites after completion of cytotoxic treatment, their further characterisation aiming at identifying new therapeutic targets is of high clinical interest.

There is a growing body of evidence that tumor progression and metastasis formation can be traced to a small subpopulation of tumor cells with stem-like features, usually

Correspondence to: Dr. Natalia Krawczyk, MD, Department of Obstetrics and Gynaecology, University of Duesseldorf, Moorenstr. 5, 40225 Duesseldorf, Germany. Tel: +49 0211/8117501, Fax: +49 0211/8118483, e-mail: Natalia.krawczyk@med.uni-duesseldorf.de

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referred to as cancer stem cells (CSCs) (14, 15). Several studies have shown that these cancer-initiating or stem-like cells persist beyond treatment with cytotoxic agents, suggesting the development of effective mechanisms of chemoresistance (16-19). In this context, it has been hypothesized that at least some DTCs are in fact CSCs. Several studies reported that DTCs with stem-like phenotypes can be detected in the BM of primary BC patients (20-22). Moreover, the presence of stem-like DTCs was shown to predict unfavourable prognosis (22). However, the stem-like features of DTCs persisting beyond neoadjuvant chemotherapy have been scarcely investigated so far (21, 22).

Sex-determining region Y (SRY)-Box2 (SOX2) is a key member of the SOX transcription factor family and an essential embryonic stem cell marker able to induce pluripotency in human somatic cells (23, 24). An important role of SOX2 as a stem cell marker in different human malignancies including breast cancer has been reported previously (25-29). A high expression of SOX2 has also been demonstrated in breast cancer cells that have acquired chemoresistance (30). The aim of this study was to assess the expression of SOX2 in DTCs persistent after NAT in a large cohort of patients with primary non-metastatic breast cancer and to compare it with clinicopathological factors as well as the SOX2 status of the primary tumor.

Patients and Methods

A total of 170 primary breast cancer patients treated from 2001 to 2011 at the Department of Obstetrics and Gynecology, University of Tuebingen, Germany were eligible for this analysis. Non-metastatic BC (T1-T4, N0-3, M0) patients, who received intraoperative BM biopsy and were DTC-positive after completion of NAT were included into the study. Patients with history of any malignancy were excluded. This study was approved by the Ethical Committee of the University of Tuebingen (307/2012R). Patient characteristics are shown in Table I. Pathological complete response (pCR) was defined as the absence of residual invasive cancer in the breast and negative lymph node status after NAT (ypT0/ypTis ypN0). The flow chart of the study is shown in Figure 1.

Collection and analysis of bone marrow. Between 10 and 20 ml of BM were aspirated intraoperatively from the anterior iliac crest under general anaesthesia and processed within 24 hours. All specimens were obtained after written informed consent from patients. This study was approved by the local ethical committee (307/2012R). BM samples were separated by density centrifugation over Ficoll (Biochrom, Germany) with a density of 1.077 g/ml. If necessary, red blood cells were lysed with lysis buffer (155 mM NH4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA pH 7.2). Using a cytocentrifuge (Hettich, Tuttlingen, Germany), 1×10^6 mononuclear cells were spun onto a glass slide and dried at the room temperature, overnight. For each patient, 2×10^6 cells were analyzed and the remaining slides were stored at –20˚C. Slides were than fixed in a 0.5% neutral buffered formalin solution for 10 min and were rinsed in phosphate-buffered saline. Automatic immunostaining was performed on the DAKO autostainer using the monoclonal mouse A45–B/B3 Pan-cytokeratin antibody (Micromet, Munich, Germany), and the DAKO-APAAP detection kit (DakoCytomation, Glostrup, Denmark) according to the manufacturers' instructions. Slides were automatically scanned using the ACIS™ imaging system (ChromaVision, Medical Systems Inc., San Juan, Capistrano, CA, USA) and evaluated based on the recommendations for standardized tumor cell detection as described previously (31, 32). In a subset of DTC-positive patients one additional slide per patient was analysed by immunofluorescence double staining for the presence of SOX2-positive DTCs (1×10^6 cells per patient). Control cytospins with SOX2-positive HT-29 cells were prepared, stored and fixed in the same way.

Immunofluorescence staining of SOX2. One additional slide was thawed at room temperature in a humid chamber for approximately 20 min. After an initial washing step with PBS (Sigma, Munich, Germany), cells were permeabilized with 0.1% Triton X-100 for 12 min and after being washed three times, blocked with normal donkey serum (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:10 dilution for 30 min. The automated double immunofluorescence staining procedure was performed on the DAKO Autostainer using the polyclonal goat Sox2-antibody (R&D Systems, Inc., Minneapolis, MN, USA) at a 1:50 dilution for 60 min. Cytospins were simultaneously incubated with fluorescein isothiocyanate (FITC) conjugated pan-cytokeratin antibody (C11) (1:500, Sigma, Munich, Germany) for 30 min. Secondary detection was performed with a donkey anti-goat antibody, labelled with Alexa Fluor 594 (1:400, Invitrogen Molecular Probes, Carlsbad, CA, USA) for 30 min. Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA) was used to stain nuclei. Preparations of the colorectal cancer cell line HT-29 mixed with PBMCs from a healthy volunteer served as a positive control for CK and SOX2 staining. For the SOX2 negative control, all conditions were kept the same, except that the primary antibody was omitted. Additionally, cytospins of PBMCs with no added tumor cells served as a negative control for both. Positive and negative control staining is demonstrated in Figure 2.

Fluorescence microscopy. Slides were manually analysed for the presence of tumor cells using a computerised fluorescence microscope Axioplan 2 (×40 oil immersion objectives, Carl Zeiss Micro Imaging GmbH, Göttingen, Germany). To screen for SOX2-positive DTCs a single-pass filter for individual fluorochromes, FITC, Texas Red or DAPI, and a triple-pass filter for (FITC/TRITC/DAPI) were used. Immunostained cells were evaluated based on the morphological criteria of the International Society of Hematotherapy and Graft Engineering Working group for standardisation of tumor cell detection and the consensus statements (33, 34). Cytokeratin-positive cells that contained an intact nucleus (DAPI positive) were identified as DTCs. DTCs with either moderate or intense staining of the nucleus were considered SOX2 positive. Slides were evaluated by two, or in doubtful cases, three independent investigators (TF, KJ and HN).

Immunohistochemical staining of the primary tumor. Immunohistochemical analysis was performed either on core biopsies or surgical resection specimens according to the method described previously by our group (35). Staining was performed on 3 to 5 μm thick sections using DAB Map Detection Kit and heat-induced antigen retrieval (HIER). The polyclonal goat SOX2 antibody (R&D Systems, Inc.) was diluted 1:40 in DISCOVERY Antibody Diluent

Table I. *Clinical data of all patients included into the trial.*

	n N=170 (%)
Total	170
Menopausal status	
Premenopausal	86 (51)
Postmenopausal	84 (49)
Tumour size before NAT	
cT1	2 (1)
cT2	82 (48)
cT3	43 (25)
cT4	38 (22)
unknown	5 (3)
Tumor size after NAT	
ypT0/ypTis	44 (26)
ypT1	66 (39)
ypT2-4	60 (35)
Nodal status before NAT	
Negative	49 (29)
Positive	117 (69)
Unknown	4 (2)
Nodal status after NAT	
ypN0	89 (52)
ypN+	81 (48)
Pathologic response	
pCR	38 (22)
non-pCR	132 (78)
Histology	
Ductal	141 (83)
Lobular	26 (15)
Others	3 (2)
Grading	
I/II	114 (67)
III	56 (33)
ER status	
Negative	73 (43)
Positive	97 (57)
PR status	
Negative	46 (27)
Positive	124 (73)
HER2 status	
Negative	133 (78)
Positive	37 (22)
IHC subtype	
HR+/HER2–	92 (54)
HR+/HER2+	19 (11)
HR–/HER2+	18 (11)
TNBC	41 (24)

ER: Estrogen receptor; HER2: human epidermal growth factor receptor 2; PR: progesterone receptor; IHC: immunohistochemistry; TNBC: triple negative breast cancer, NAT: neoadjuvant treatment; pCR: pathological complete response.

(Ventana) and applied according to the manufacturer's instructions. Secondary detection was performed with a rabbit anti-goat antibody (Jackson ImmunoResearch, Inc., West Grove, PA, USA) at a 1:200 dilution. 3,3'diaminobenzidine (DAB) was used as a chromogen. Finally, the slides were counterstained with haematoxylin and

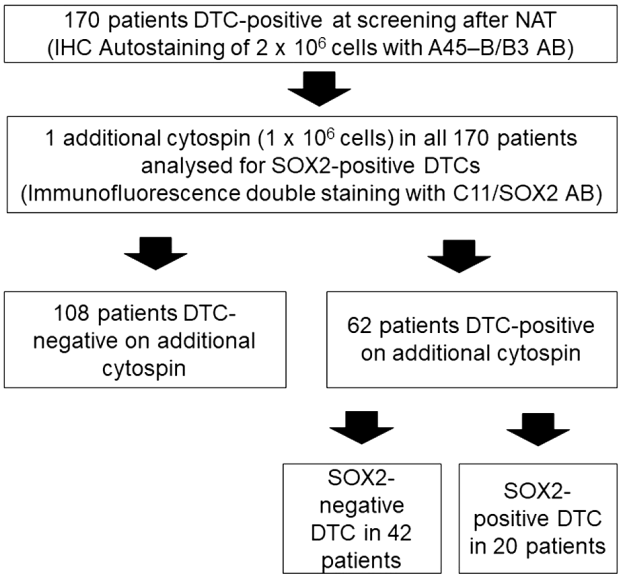

Figure 1. *Study flow chart. DTC: Disseminated tumor cell, AB: antibody, IHC: immunocytochemistry, NAT: neoadjuvant therapy.*

mounted for examination. For assessment of the SOX2 status, the percentage of cells with nuclear reactivity (score 0: none, 1: >0% <10%, 2: >10% < 50%, 3: ≥50% <90%, 4: ≥90%) was determined according to the score published by Pham *et al*. (36). Tumors with a score of 1 or more were considered SOX2 positive.

Statistical analysis. A chi-squared test was used to evaluate the relation between SOX2-positive DTCs and/or primary tumor and clinicopathological factors. Statistical analysis was performed by SPSS, version 24 (SPSS Inc., Chicago, IL, USA). Values of $p<0.05$ were considered statistically significant.

Results

Patients' characteristics. A total of 170 primary BC patients were included in the analysis. The clinical data of patients are listed in Table I. 86 out of 170 (51%) patients were premenopausal. The most common histological tumor type was invasive ductal carcinoma (83%). Estrogen and progesterone receptor (ER, PR) status were positive in 57% and 73% of these patients, respectively. 37 patients (22%) had HER2-positive tumors. All patients were treated with NAT. 38 out of 170 (22%) patients achieved pathological complete response (pCR).

SOX2 status of DTCs after NAT. SOX2 status of persistent DTCs was determined in 62 patients after NAT. Among these 62 patients, SOX2-positive DTCs were detected in 20 cases (32%; Table II, Figure 3). No significant correlation was observed between SOX2 status of DTCs and any

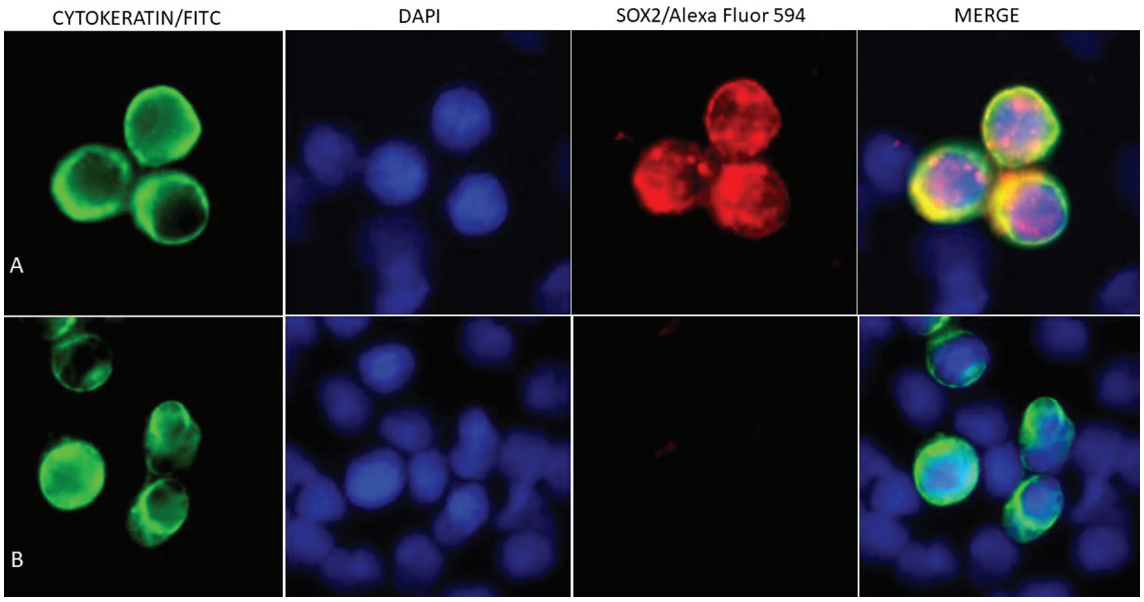

Figure 2. *SOX2 control staining. (A) Positive control staining (HT-29 cells). (B) Negative control staining (HT-29 cells, primary antibody omitted) (×63 oil immersion objective).*

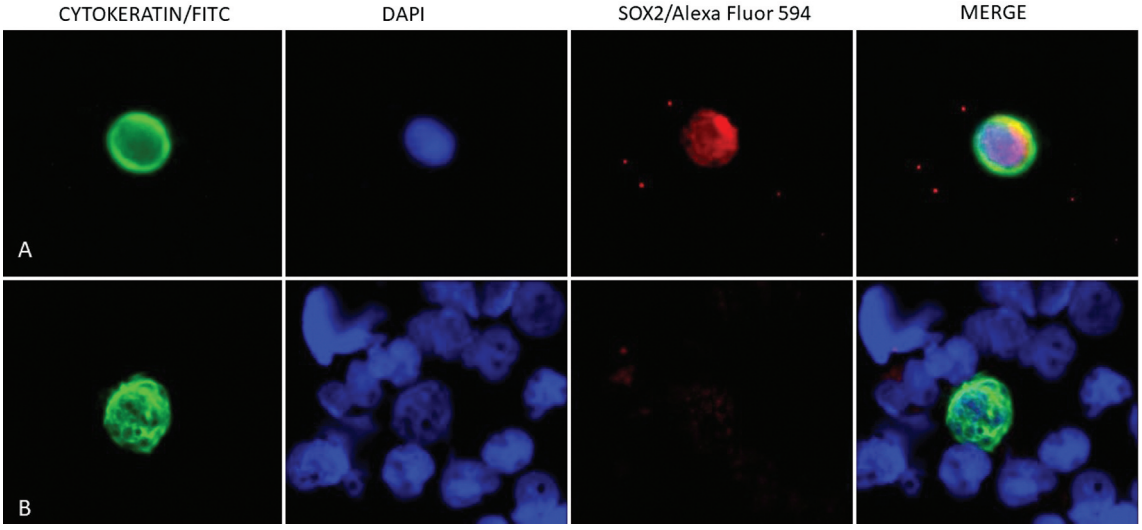

Figure 3. *SOX2 staining of DTCs in primary breast cancer patients. (A) SOX2-positive DTC. (B) SOX2-negative DTC (×63 oil immersion objective).*

clinicopathological characteristics. SOX2 status of DTCs persisting beyond NAT was not associated with pathological response to treatment.

Expression of SOX2 in the primary tumor. Primary tumor tissue was available for immunohistochemical determination of SOX2 status in 38 patients prior to systemic treatment and in 27 patients after NAT. The tumors were SOX2-negative in most cases [30 out of 38 (79%) before and 18/27 (67%) after NAT, respectively]. No correlation could be found between the SOX2 status of primary tumor (pre- and post-therapeutic) and any of the established prognostic factors. Neither was the SOX2 status of primary tumor associated with response to NAT. In 17 patients, the SOX2 status has been assessed on persistent DTCs and the primary tumor

Table II. *Clinical data of 62 patients included in further analysis of SOX2-status of DTC.*

	n (%)	SOX2-positive DTCs (%)	*p*-Value*
Total	62 (100)	20 (32)	
Menopausal status			1.0
Premenopausal	31 (50)	10 (32)	
Postmenopausal	31 (50)	10 (32)	
Tumor size before NAT			0.76
cT1	1 (2)	0 (0)	
cT2	27 (43)	8 (30)	
cT3	13 (21)	6 (46)	
cT4	18 (29)	5 (28)	
unknown	3 (5)	1 (33)	
Tumor size after NAT			0.45
ypT0/ypTis	16 (26)	4 (25)	
ypT1	24 (39)	10 (42)	
ypT2-4	22 (35)	6 (27)	
Nodal status before NAT			0.94
Negative	14 (23)	4 (29)	
Positive	45 (73)	15 (33)	
Unknown	3 (5)	1 (33)	
Nodal status after NAT			0.47
ypN0	30 (48)	11 (37)	
ypN+	32 (52)	9 (28)	
Pathologic response			0.55
pCR	12 (19)	3 (15)	
non-PCR	50 (81)	17(27)	
Histology			0.53
Ductal	52 (84)	18 (35)	
Lobular	8 (13)	2 (25)	
Others	2 (3)	0 (0)	
Grading			0.95
I/II	40 (65)	13 (32)	
III	22 (35)	7 (32)	
ER status			0.79
Negative	20 (32)	6 (30)	
Positive	42 (68)	14 (33)	
PR status			0.59
Negative	13 (21)	5 (38)	
Positive	49 (79)	15 (31)	
HER2 status			0.36
Negative	45 (73)	13 (29)	
Positive	17 (37)	7 (41)	
IHC subtype			0.22
HR+/HER2–	34 (55)	10 (29)	
HR+/HER2+	11 (18)	5 (45)	
HR–/HER2+	6 (10)	2 (33)	
TNBC	11 (18)	3 (27)	

*Chi-squared test. ER: Estrogen receptor; HER2: human epidermal growth factor receptor 2; PR: progesterone receptor; IHC: immunohistochemistry; TNBC: triple negative breast cancer; NAT: neoadjuvant treatment; pCR: pathological complete response.

before systemic treatment and showed a concordance rate of 59% (Table III). The SOX status of the primary tumor before and after NAT was evaluated in 18 patients and was concordant in 78% of cases (p=0.045, Table IV).

Discussion

Disseminated tumor cells persisting beyond cytotoxic treatment predict impaired survival in primary breast cancer patients (10, 11, 37). These cells are currently assumed to serve as a surrogate marker of minimal residual disease and their eradication is considered to be a main target of systemic therapy. However, about a half of DTC-positive BC patients remain tumor-free during a follow up period of over 10 years (7, 38). This phenomenon may be explained by the so-called "metastatic inefficiency". According to this hypothesis, only a small population of DTCs is able to persist and subsequently cause tumor growth in secondary sites (39, 40). One theory presently under discussion is the hypothesis that some of these cells undergo the process of epithelial-mesenchymal transition (EMT) that increases their invasiveness and leads to acquisition of stem-cell features (17, 41, 42). These cancer stem cells can evade systemic treatment and are thought to play a major role in the metastasis cascade (18, 42). In this context, we assessed the expression of the stem cell marker SOX2 on DTCs persisting in the BM of BC patients after NAT.

In 170 patients with persistent DTCs after completion of neoadjuvant therapy, an additional bone marrow cytospin was analyzed. In 62 cases, at least one DTC could be found and these patients were included in further analysis of the SOX2 status. Why some of the additional cytospins contained no DTCs can be explained by several factors, such as the freezing and thawing process of the slides, staining of only one additional slide (1×10^6 cells per patient) compared to two slides (2×10^6 cells per patient) analyzed in the routine IHC staining as well as different assays (IHC *vs.* immunofluorescence) and the different anticytokeratin anibodies used (A45-B/B3 *vs.* C11).

To assess SOX2 status on persistent DTCs, we developed a double immunofluorescence staining assay based on cytokeratin positivity and morphological criteria according to the Consensus Recommendations for Standardized Tumor Cell Detection (34). 32% of DTC-positive patients had at least one SOX2 positive tumor cell in BM. This is, to the best of our knowledge, the largest study demonstrating that DTCs persistent after NAT express a stem cell associated feature and the first evaluating SOX2 expression on DTCs in BC patients. Reuben *et al*. have analyzed DTCs in 30 BC patients after NAT in terms of stemness and found epithelial CD44+CD24low cells in 57% of these patients (21). Similar to our observations, a detection of potential CSC in BM was not associated with response to NAT. In another study by Giordano *et al*., 18 of 26 patients (69%) had potential CSCs in BM after NAT (22). The same detection method, a multi-parameter flow cytometry, was used in both trials (21, 22) which might explain the much higher CSC positivity rates compared to our study. Further, both trials used

Table III. *SOX2 status of persistent DTCs and primary tumor before NAT.*

	SOX2-status	DTC		Total (%)
		SOX2 negative (%)	SOX2 positive (%)	
PT	SOX2 negative (%)	9 (53)	5 (29)	14 (82)
	SOX2 positive (%)	2 (12)	1 (6)	3 (18)
	Total (%)	11 (65)	6 (35)	17 (100)

PT: Primary tumor; DTC: disseminated tumor cell; NAT: neoadjuvant treatment.

Table IV. *SOX2 status of the primary tumor before and after NAT.*

	SOX2-status	PT pre-NAT		Total (%)
		SOX2 negative (%)	SOX2 positive (%)	
PT post-NAT	SOX2 negative (%)	11 (61)	3 (17)	14 (78)
	SOX2 positive (%)	1 (5.6)	3 (17)	4 (22)
	Total (%)	12 (66)	6 (34)	18 (100)

PT: Primary tumor; NAT: neoadjuvant treatment.

ALDH/CD44/CD24 and not SOX2 as CSC marker, making a direct comparison of our studies difficult.

Our analysis demonstrates that some of the cells detected in secondary homing sites after NAT may exhibit a stem-like phenotype. Tumor initiating-capacity on the one hand and ability to elude cytotoxic therapy and persist in a quiescent and/or dormant state on the other hand, are the features postulated to account for chemoresistance and metastatic potential of CSCs (43). A high expression of SOX2 has been indeed demonstrated in BC cell lines known for their cross-resistance to taxanes, anthracyclines and cisplatin (30). Furthermore, SOX2 expression has been linked to tamoxifen resistance in BC (44) and was shown to significantly affect adhesion properties of BC cells (45). SOX2 was also recently shown to mediate proliferation and dissemination in lung cancer cells resistant to tyrosine kinase inhibitors (46). The CSC hypothesis is supported by the phenomenon of tumor cell dormancy, clinically well-known in BC patients, who can experience a relapse after a very long period, sometimes up to 25 years, without evidence of the disease (47, 48). In concordance with this clinical observation isolated tumor cells have been detected in the blood of asymptomatic BC patients up to 22 years after primary surgery (49). However, these persistent cells have not been analyzed in terms of stem cell-like features in any of the available studies.

While studies on the expression of SOX2 on DTCs are missing, data on the SOX2 expression in primary BC tissue have been reported previously (35, 50) In our cohort, 21% of patients have SOX2-positive tumors prior to NAT and 33% of tumors were SOX2-positive after NAT. This is in line with our earlier analysis demonstrating a SOX2 tumor positivity rate of 28% (24/86 patients) (35), compared to 16.7% (33/198 patients) reported by Rodriguez-Pinilla *et al*. (50). The fact that the SOX2 positivity rate of the primary tumor in our cohort was higher after NAT than prior to the systemic therapy is consistent with the reported phenomenon that CSC frequency increases in BC tissue after cytotoxic treatment (18, 51). Recently, chemotherapy was shown to induce BC stemness in a xenograft mouse model (52). A direct comparison of SOX2 status between pre- and post-therapeutic tumor tissue was possible in 18 patients (Table IV). In this group, the SOX2 status remained the same in most patients, with only one patient acquiring SOX2 positivity and three patients converting from positive to negative SOX2 status.

In 41% of analyzed patients, the SOX2 status of primary tumor before NAT differed from the SOX2 status of persistent DTCs. A positive SOX2 status of DTCs was observed in 36% of patients (5 of 14 cases) with SOX2-negative tumors (Table III). A discrepancy between tumor and (persistent) DTCs regarding other phenotypic features has been described in previous studies (53-55), showing that MRD cells may evolve independently from the primary tumor. This observation is consistent with the parallel tumor progression model proposed by Klein *et al*. (56).

Another aspect evaluated in our study was the correlation of SOX2 status of primary tumors/DTCs and other clinical-pathological factors. Previously published studies reported a significant association between SOX2 positivity and higher grading, nodal positivity and poor prognosis (57-59). In contrast, no correlations were observed in our study, possibly due to the fact that the SOX2 status of both the tumor and DTCs was only available in a small proportion of patients.

Limitations of our Study

Even though DTC detection based on their epithelial and morphological features is considered standard, the lack of single-cell molecular analysis confirming tumor origin and SOX2-positivity of these cells at the genomic level may be considered a potential limitation of our study. Furthermore, analysis of a whole BM suspension (approx. 5-10 ml), aspirated from each patient instead of one cytospin with 1×10^6 cells per patient would possibly provide higher DTC numbers and result in higher numbers of DTCs available for analysis of the SOX2 status. Further trials implementing molecular characterization of single DTCs as well as analysis of other stem cell-associated markers are necessary to confirm the stem-like character and to establish the DTCs' clinical relevance.

Conclusion

In the present study, we demonstrated that DTCs with stem-like phenotype can persist after neoadjuvant treatment in a relevant number of breast cancer patients. SOX2-positive DTCs were detected in patients with SOX2-negative primary tumors, suggesting that these populations may have evolved independently of each other. Stem-like character of minimal residual disease should be further evaluated using molecular analyses in future studies.

Conflicts of Interest

The Authors declare that there are no conflicts of interest.

Authors' Contributions

NK performed the data analysis and drafted the manuscript; KJ performed the IF experiments and collected the data; MBP made a substantial contribution to interpretation of the data and helped to draft the manuscript; AS performed and evaluated the IHC experiments of the primary tumors; HN coordinated the study and reviewed the manuscript; TF designed the study, made substantial contributions to interpretation of the data and reviewed the manuscript; HA, BS, WD and MC were involved in interpretation of the data, drafting of the manuscript or revising it. All Authors read and approved the final manuscript.

Availability of Data and Materials

The data that support the findings of this study are available from the authors upon reasonable request and with permission from Tanja Fehm.

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