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PRECLINICAL STUDY

Detection and clinical relevance of hematogenous tumor cell dissemination in patients with ductal carcinoma in situ

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Abstract Hematogenous tumor cell dissemination is a crucial step in systemic disease progression and predicts reduced clinical outcome in breast cancer patients. Only invasive cancers are assumed to shed tumor cells into the bloodstream and infiltrate lymph nodes. However, recent studies revealed that disseminated tumor cells (DTCs) may be detected in bone marrow (BM) of patients with preinvasive lesions, i.e., ductal carcinoma in situ (DCIS). The purpose of this analysis was to examine the incidence and clinical value of DTC detection in a large series of patients with pure DCIS. 404 patients treated for DCIS at the University Hospital Tuebingen, Germany were included into this analysis. BM was analyzed by immunocytochemistry (pancytokeratin antibody A45-B/B3) using ACIS system (Chromavision) according to the ISHAGE evaluation criteria. Sentinel nodes were analyzed in 316 patients by step sectioning and hematoxylin–eosin staining. DTCs were detected in 63 of 404 patients (16 %). No correlation was observed between BM status and tumor size, grading, histology or Van Nuys prognostic index. In two cases, metastatic spread into lymph nodes was observed; isolated tumor cells were found in one patient. After a median follow-up of 45 months (range 3–131 months), 3 % of BM positive patients died compared to 1 % of BM negative patients ($p = 0.254$). Relapse of any kind was observed in 7 % of patients with DTCs vs. 5 % of patients without DTCs ($p = 0.644$). The differences in overall ($p = 0.088$) and disease-free survival ($p = 0.982$) calculated by log-rank test were not statistically significant. Tumor cell dissemination may be detected in patients diagnosed with DCIS. Whether these cells disseminate from real preinvasive mammary lesions or represent the earliest step of microinvasion, remains unclear. A longer follow-up may be necessary to accurately assess clinical value of these cells in DCIS patients.

Keywords Ductal carcinoma in situ · Breast cancer · Disseminated tumor cell · Bone marrow · Survival

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Abbreviations

ADH	Atypical ductal hyperplasia
BM	Bone marrow
CK	Cytokeratin
CTC	Circulating tumor cell(s)
DIN	Ductal intraepithelial neoplasia
DCIS	Ductal carcinoma in situ
DTC	Disseminated tumor cell(s)
HE	Hematoxylin–eosin
ITC	Isolated tumor cell
SN	Sentinel node
SLNB	Sentinel lymph node biopsy
VNPI	Van Nuys prognostic index

Introduction

The past two decades have seen increasingly rapid advances in the field of tumor cell dissemination in patients with solid tumors. Disseminated tumor cells (DTCs) are routinely detected in the bone marrow (BM) of 20–40 % non-metastatic breast cancer patients; their presence strongly predicts poor clinical outcome [1]. Recent evidence suggests that the ability of cancer cells to enter circulation and persist at secondary homing sites is acquired at much earlier time point during disease progression than initially assumed [2]. Current hypotheses place the onset of tumor cell spread at small clinically undetectable invasive lesions. However, animal model studies demonstrated that tumor cells can disseminate even from earliest epithelial alterations, such as ductal carcinoma in situ (DCIS) [2]. These findings challenge the prevailing view that preinvasive lesions are unable to cause metastatic growth since the tumor, limited to epithelial layer, does not reach blood or lymphatic vessels.

DCIS is defined as a proliferation of malignant cells without invasion across the basement membrane [3]. The incidence of DCIS has increased in the past years due to widespread introduction of mammography screening programs, accounting for 10–15 % of all newly diagnosed breast cancers [4, 5]. According to epidemiological data, breast cancer specific mortality in DCIS patients is estimated at 1–2 % despite optimal removal of the primary tumor [6]. Further, (micro-)metastases in axillary lymph nodes are detected in 1–6 % DCIS patients [7–9]. We previously reported that DTCs may be detected in BM of patients diagnosed with pure DCIS [9].

These observations prompt several questions: Do in situ carcinomas have the ability to shed tumor cells into the blood circulation? Are tumor cells derived from preinvasive lesions clinically relevant? Is DTC presence a result of a (micro)invasive lesion missed by standard pathological workup of the specimen? Additional assays, such as sentinel node biopsy (SLNB) or DTC detection, might have the potential to identify patients with such occult microinvasion.

In this study, we aimed to evaluate the prevalence and clinical value of DTC status in a large group of 404 patients diagnosed with pure DCIS at the Comprehensive Cancer Center, University of Tuebingen, Germany.

Materials and methods

After written informed consent BM samples were intraoperatively obtained from 404 primary DCIS patients who were treated at the Department of Gynecology and Obstetrics (Comprehensive Cancer Center, University Hospital of Tuebingen, Germany, a certified and multidisciplinary breast cancer center) within the period of March 2003–December 2012. Patients with DCIS larger than 2 cm (based on mammography) received a SLNB as a routine procedure in accordance with current treatment guidelines. None of the patients had history of cancer. This analysis was approved by the ethics committee of the University of Tuebingen (502/2010A). Following data were documented for each patient: age, menopausal status, grading, tumor size, hormone receptor status, and Van Nuys prognostic index (VNPI, a score for predicting the risk of local recurrence, based on tumor size, margin width and pathological criteria).

Immunocytochemistry

DTC detection was performed as described in detail previously [9]. Tumor cell isolation and detection was performed based on the recommendations for standardized tumor cell detection [10]. Briefly, 10–20 ml BM was aspirated from posterior iliac crest into syringes containing heparin anticoagulant general anesthesia using Jamshidi's technique immediately prior to begin of breast surgery [11]. All samples were processed within 24 h. BM aspirates were separated by density centrifugation using Bicoll (density 1,077 g/ml, Biochrom, Germany). Mononuclear cells were collected from the interphase layer and were spun down onto a glass slide (Hettich cytocentrifuge, Germany) (10^6 MNC/spot) and air-dried overnight at room temperature. For detection of cytokeratin(CK)-positive tumor cells, slides were fixed in 4 % neutral buffered formalin for 10 min and rinsed in PBS.

Staining of slides and DTC identification

Automatic immunostaining was performed on the DAKO Autostainer using the monoclonal mouse antibody A45-B/

B3 (Micromet, Germany) and the DAKO-APAAP detection kit (DakoCytomation, Denmark) according to the manufacturer's instructions. The A45-B/B3 antibody is directed against common CK epitopes including the heterodimers 8/18 and 8/19. The malignant breast cell line MCF-7 was used as a positive control. Leukocytes of a healthy volunteer served as negative control. In addition, isotype matched myeloma protein conjugated to FITC was included as negative staining controls (Sigma, Deisenhofen). For each patient 2×10^6 cells were analyzed on two slides. Analysis was performed on the Automated Cellular Imaging System (ACIS, ChromaVision Medical Systems, San Juan, Capistrano, CA). Details of this system have been described in detail elsewhere [12]. Criteria for detection of DTCs were based on the recommendations of the European ISHAGE Working group for standardization of tumor cell detection and the consensus statements [10, 13].

Histopathological evaluation of sentinel lymph nodes and primary tumors

Preparation of sentinel nodes (SNs) was conducted as described previously [9]. Briefly, SN were sliced in 2 mm intervals, fixed in 10 % buffered formalin and embedded in paraffin. At least three step sections in 500 μm intervals were cut, stained with routine hematoxylin–eosin (HE) stain and evaluated for metastasis by light microscopy by an experienced pathologist. In equivocal cases suspicious areas were immunostained with anti-CK–antibody (AE1/AE3). Primary tumors were sampled for histology in at least 0.5 cm intervals. Sections were evaluated by HE-staining. In 35 % of the cases, we found areas suspicious for invasion. Specifically, these cases showed cancerisation of lobules or involvement of small glandular structures in areas of sclerosing adenosis. Tissue sections of these areas were submitted for immunohistochemical staining with an antibody against smooth muscle myosin heavy chain or basal CKs (CK 5/6 or CK 5/14). These antibodies specifically stain the myoepithelial cell layer surrounding non-invasive intraductal carcinomas. Therefore, lack of this staining would indicate invasive growth. All primary tumors were examined by an experienced pathologist; cases with lymph node metastasis were evaluated by at least two independent pathologists.

Statistical analysis

Chi-squared test and Fisher's exact test were used to evaluate the relationship between DTC detection and clinicopathological factors. For the survival analysis, we considered in separate analyses the following primary end points: (1) death and (2) relapse, defined as distant or local disease recurrence, or both. Survival intervals were measured from the time of BM aspiration to the time of death or of the first clinical, histological, or radiographic diagnosis of relapse. We constructed Kaplan–Meier curves and used the log-rank test to assess the univariate significance of the parameters. All reported p values are two-sided. Statistical analysis was performed by SPSS, version 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Patients characteristics

404 patients diagnosed with pure DCIS were included into the analysis. Clinicopathological data are summarized in Table 1. 20 % of tumors were ≤15 mm, 33 % between 16 and 40 mm, and 47 % measured ≥41 mm. With regard to grading, the majority of patients presented with ductal intraepithelial neoplasia (DIN) grade 3 (44 %) and DIN 2 (38 %), followed by 18 % with DIN 1c. 75 % of the tumors were ER-positive and 69 % showed PR-positivity. Median age of all patients was 55 years. The distribution of

Table 1 Clinical characteristics of patients

	n (%)	DTC positive (%)	*p* value
Total	404	63 (16)	
Menopausal status			0.475
Premenopausal	112 (28)	21 (19)	
Postmenopausal	290 (72)	42 (14)	
Histology			0.857
DIN 1c	61 (18)	11 (18)	
DIN 2	124 (38)	19 (15)	
DIN 3	146 (44)	22 (15)	
Tumor size			0.231
≤15 mm	74 (20)	7 (10)	
16–40 mm	125 (33)	23 (18)	
≥41 mm	176 (47)	28 (16)	
ER status			0.464
Negative	98 (25)	12 (12)	
Positive	298 (75)	49 (16)	
PR status			0.927
Negative	122 (31)	19 (16)	
Positive	272 (69)	42 (15)	
Van Nuys prognostic index			0.218
1–4 points	44 (16)	5 (11)	
5–7 points	148 (54)	20 (14)	
8–9 points	80 (29)	17 (21)	

BM bone marrow, *DIN* ductal intraepithelial neoplasia, *ER* estrogen receptor, *PR* progesterone receptor

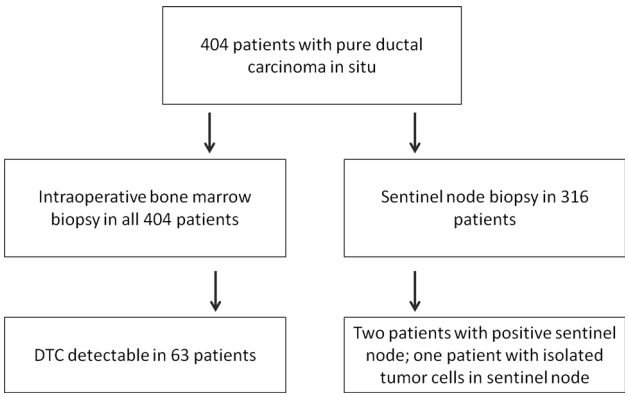

Fig. 1 Patient distribution diagram according to the Recommendations for Tumor Marker Prognostic Studies (REMARK)

patients is summarized in a Recommendations for Tumor Marker Prognostic Studies (REMARK) diagram (Fig. 1) [14].

DTC detection in DCIS patients

BM aspiration was conducted in general anesthesia immediately before begin of surgery. DTCs were detected in 63 of 404 (16 %) of patients. Figure 2 shows a typical DTC from a breast cancer patient. The number of detected DTCs ranged from 1 to 3 per 2×10^6 mononuclear cells. 11 % of patients at VNPI Group I were BM positive compared to 14 and 21 % at VNPI Group II and III, respectively ($p = 0.218$). Median age of patients with DTC was 54 years compared to 56 years in the DTC negative group. No correlation was observed between DTC detection and established clinicopathological factors (Table 1).

Lymph node metastasis in DCIS patients

316 of 404 patients (78 %) underwent SLNB. In two (1 %) patients metastatic spread into lymph nodes was observed (histology of the primary tumor in both cases: DIN 2, tumor size: 6.0 and 4.5 cm, respectively). One patient with a large (>10 cm) DIN 1c lesion presented with isolated tumor cells (ITCs) in one of four removed SN (Fig. 3). All these patients were DTC negative. Additional sectioning of the primary tumor was performed in all three cases but did not reveal invasive cancer.

DTC detection and survival

A follow-up was available in 356 patients. Only these patients were included into survival analysis. Median follow-up was 45 months (range 3–131 months). Six deaths and 20 relapses of any kind occurred during follow-up (Table 2). A relapse was defined as diagnosis of distant metastasis (eight patients) and/or local recurrence (invasive recurrence in seven cases, non-invasive recurrence in nine cases) and/or contralateral cancer (three patients). 3 % of BM positive patients died compared to 1 % of BM negative patients. The rate of relapse of any kind was 7 % in patients with DTC vs. 5 % in DTC negative cases. The differences in overall survival (OS) and disease-free survival (DFS) calculated by log-rank test were not statistically significant (OS: $p = 0.088$, DFS: $p = 0.982$).

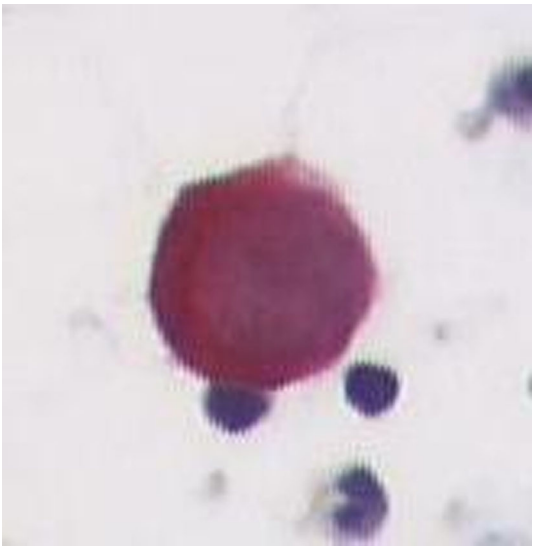

Fig. 2 Disseminated tumor cell with typical cytomorphology and staining pattern (positive cytokeratin staining, large nucleus, high nuclear to cytoplasmic ratio, nucleus partially covered by cytokeratin staining, nucleus granular; immunocytochemistry using A45-B/B3 pancytokeratin antibody)

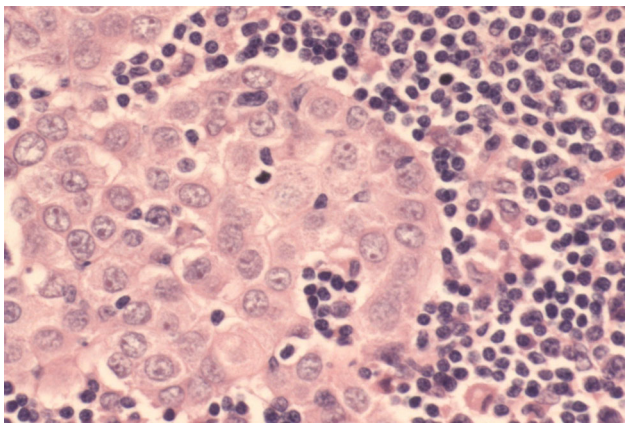

Fig. 3 Micrometastasis in a lymph node of a patient diagnosed with pure DCIS. Additional sectioning of the primary tumor did not reveal an invasive focus

Table 2 Presence of DTCs and survival in DCIS patients

	Death	Any relapse	Distant metastasis	Local recurrence	Contralateral cancer
DTC positive $n = 58$	2 (3 %)	4 (7 %)	2 (3 %)	3 (5 %)	0 (0 %)
DTC negative $n = 298$	4 (1 %)	16 (5 %)	6 (2 %)	13 (4 %)	3 (1 %)
Total	6 (2 %)	20 (6 %)	8 (2 %)	16 (4 %)	3 (1 %)

Discussion

Tumor cell dissemination in DCIS

Metastatic spread of cancer cells into the blood circulation and throughout patient's body had been classically regarded as a relatively late event in the evolution of malignant tumors. According to the research results of the last decades, however, hematogenous tumor cell dissemination may occur at the earliest stages of malignant disease, possibly long before the tumor becomes clinically apparent [15]. So far, the exact time point of the onset of such spread remains under discussion. Theoretically, preinvasive lesions of the breast are not able to shed tumor cells; DCIS is generally assumed to have excellent survival rates reaching 100 %. This simplistic view of DCIS lesions has been challenged by the observation that 1–3 % of patients with pure DCIS present with metastatic spread to the axillary lymph nodes at time of diagnosis and a proportion of patients will be diagnosed with distant relapse years after successful primary treatment of DCIS [6, 16].

This is the largest study so far on hematogenous and lymphatic tumor cell dissemination in DCIS. We evaluated BM samples and SNs from 404 patients; primary lesions were examined by extensive step sectioning to exclude the possibility of microinvasion. DTCs were detected in the BM of 16 % of patients. This is in accordance with previous smaller studies (Tables 3, 4). Most authors used immunocytochemical detection of DTCs using A45-B/B3 antibody against common epitopes on several CKs including CK 8, 18, and 19. Sanger et al. analyzed BM aspirates from 19 patients with pure DCIS as well as seven patients with microinvasive breast cancer using two antibodies against different CKs (A45-B/B3 and AE1/AE3 against different CKs such as CK 1–8, 10, 14, 15, 16, 19) [17]. DTCs were detected in 21 % of DCIS patients, as opposed to 57 % of patients with microinvasion. Only one study reported on circulating tumor cells (CTCs) in patients with preinvasive breast lesions. Franken et al. examined CTCs in peripheral blood of DCIS patients using the FDA-approved CellSearch system [18]. CTCs were detected in nine out of 48 patients (19 %). However, authors also reported a surprisingly high (15 %) CTC positivity rate in

Table 4 Axillary node involvement in patients with ductal carcinoma in situ

References	No. of patients with SLNB	SN positive (%)
Present study	316	2 (1)
Intra et al. [8]	854	12 (1)
Intra et al. [33]	223	7 (3)
Banys et al. [9]	221[a]	2 (1)
Cox et al. [34]	195	26 (13)
Kelly et al. [35]	134	3 (2)
Mabry et al. [7]	171	10 (6)
Katz et al. [36]	110	8 (7)
Zavagno et al. [37]	102	1 (1)

SN sentinel node, *SLNB* sentinel lymph node biopsy

[a] These patients were included into present analysis as well

Table 3 Hematogenous tumor cell dissemination in patients with pure DCIS

Author	No. of patients	Method	Blood/BM	DTC/CTC positive (%)
Present study	404	ICC (CK; A45-B/B3)	BM	16
Banys et al. [9]	266[a]	ICC (CK; A45-B/B3)	BM	13
Franken et al. [18]	48	CellSearch	Blood	19[b]
Sanger et al. [17]	19	ICC (CK; A45-B/B3 and AE1/AE3)	BM	21
Husemann et al. [2]	39	ICC (CK; A45-B/B3)	BM	13

BM bone marrow, *CK* cytokeratin, *CTC* circulating tumor cells, *DTC* disseminated tumor cells, *ICC* immunocytochemistry

[a] These patients were included into present analysis as well

[b] ≥1 CTC (no cut off)

patients with benign breast tumors and patients with DCIS and benign disease were excluded from survival analysis.

Progression from non-invasive lesions to invasive breast cancer

The concept of an early stage in tumor progression which is not yet able to produce metastasis developed in the first half of the twentieth century with the introduction of the term "in situ *carcinoma*" [19, 20, 21]. The malignant potential of in situ lesions varies strongly; lobular carcinoma in situ is widely regarded as an indicator for increased risk of future malignancy, while DCIS is considered a direct anatomical precursor of invasive cancer and represents a truly preinvasive lesion [22]. With the development and wide introduction of high-quality mammography, DCIS is the most rapidly growing subgroup of breast carcinomas, representing 10–25 % of all newly diagnosed breast cancer cases. It is generally accepted that DCIS may progress to invasive disease and thus require therapeutic intervention.

Factors determining the ability of non-invasive DCIS lesion to progress to invasive breast cancer remain yet to be cleared. According to genomic-based studies, DCIS is a genetically advanced disease with distinct patterns of genomic alterations, such as loss of heterozygosity (LOH) at chromosome 16q in low-grade DCIS and 13q loss and high level amplifications of 17q12 and 11q13 in high-grade DCIS [23]; chromosomal instability occurs thus before histological cancer invasion [24].

Husemann et al. chose an animal-based model that mimics progression of human breast cancer to examine tumor cell dissemination from preinvasive lesions [2]. Interestingly, DTCs became detectable in BM of transgenic mice at an early stage of the disease when extensive histopathological analysis of the breast tissue revealed areas of atypical ductal hyperplasia (ADH) but no higher grade lesion. However, electron microscopy could clearly show the invasion of epithelial cells through the basement membrane within ADH lesion. The malignant nature of detected DTCs was confirmed as proof of principle by single-cell comparative genomic hybridization.

In the metastatic cascade, two key steps are the intravasation of cells from the primary lesion and their successful extravasation at secondary sites. In theory, these cells may become fully malignant during this process and form metastasis independently from the primary lesion and even before the transition from non-invasive to invasive lesion at the primary site has taken place. Data from animal-based models suggest that such a phenomenon is indeed possible. Podsypanina et al. showed that tumor cells injected into the circulation of engineered mice are able to bypass transformation at the primary site and form metastatic lung noduli upon oncogene induction [25]. These findings suggest that normal untransformed cells are able to persist in the blood circulation and secondary sites and form metastasis independently of the progression at the primary site.

Clinical relevance of DTC detection in DCIS

In our study, presence of tumor cells in BM of DCIS patients was not associated with clinical outcome. Hypothetically, if single tumor cells are able to leave the primary lesion and reach secondary sites, such as BM, an occult (micro-)invasive focus must be assumed, even if the pathological workup of the tumor confirms the diagnosis of pure DCIS. Whether such new diagnostic information is of clinical relevance, remains unclear. Upgrading of the T stage from Tis to T1mi does not necessarily change patient's expected prognosis since microinvasive breast cancer has excellent survival rates, comparable to pure DCIS [26–30]. Conclusive data on the prognostic impact of microinvasion are pending. Parikh et al. presented long-term follow-up (median: 9 years) of 72 patients with microinvasive breast cancer and 321 patients with pure DCIS; patients in both groups had favorable outcomes, reaching 10-year OS rate of 93.2 % in case of pure DCIS and 95.7 % for microinvasive cancer ($p = 0.95$) [27]. Microinvasion did not predict for worse breast relapse-free survival either (89.0 vs. 90.7 %, respectively, $p = 0.36$).

Theoretically, omission of a microinvasive focus may result in undertreatment; however, the optimal management of microinvasive breast cancer is as yet undefined. Data about the epidemiology and clinical relevance of this entity has been so far limited by its uncommon incidence and lack of standardized clinical trials. With regard to local therapy, breast-conserving surgery followed by radiotherapy is generally considered an oncologically safe approach for both pure DCIS and microinvasive cancer, simple mastectomy being the alternative for patients with large lesions. The overall incidence of axillary lymph node metastasis is higher than in pure DCIS and estimated at 2–10 % [27, 29, 31, 32]; therefore, in contrast to pure DCIS, most guidelines accept SLNB as a standard procedure for axillary staging in patients with microinvasion. The need for adjuvant systemic therapy in microinvasive breast cancer remains controversial since there have been no clinical trials specifically addressing the role of endocrine, cytotoxic or targeted therapy in this subgroup of patients. Possible clinical consequences of tumor stage upgrading from Tis to T1mi are discussed in Table 5. Hypothetically, presence of DTCs might serve as an additional risk indicator and be helpful in discussing adjuvant endocrine therapy with patients with pure DCIS.

Table 5 Management of pure ductal carcinoma in situ and microinvasive breast cancer

	Pure DCIS	Microinvasive breast cancer	Clinical consequence of microinvasion
Prognosis	Excellent	Excellent [26–30]	None
Breast surgery	Breast-conserving surgery plus radiotherapy is a safe option with low rates of local recurrence; importance of negative margins	Breast-conserving surgery plus radiotherapy is a safe option with low rates of local recurrence; importance of negative margins	None
Axillary staging	No routine axillary lymph node dissection; SLNB may be discussed in high-risk patients (i.e., large lesions, high grade, comedo-type necrosis)	SLNB is generally recommended; No routine axillary lymph node dissection	Yes; SLNB in case of microinvasion; impact on survival unclear [27]
Radiotherapy	Recommended after breast-conserving surgery; no benefit from additional boost	Recommended after breast-conserving surgery; data on tumor bed boost in T1mi limited; boost may be discussed (impact on survival not demonstrated)	Addition of boost radiation in case of microinvasion possible; impact on outcome unclear
Endocrine therapy[a]	Optional in some patients; tamoxifen therapy lowers local recurrence risk without a proven overall survival benefit [38]	Generally recommended in hormone receptor positive disease; however, untreated patients have excellent outcomes	Possible; recommendation toward endocrine therapy in case of microinvasion
Chemotherapy[a]	Not recommended	Not recommended	None
Trastuzumab therapy[a]	Not recommended	Generally not recommended, HER2 status of microinvasive lesion has no impact on outcome [30]	None

SLNB sentinel lymph node biopsy

[a] Recommendations may change in case of lymph node involvement

Conclusions

ITCs may be detected either in BM or axillary lymph nodes of a significant proportion of patients diagnosed with pre-invasive lesions of the breast. It is not yet clear whether these cells derive from an occult microinvasive focus within DCIS or whether epithelial cells are able to spread from truly preinvasive lesions. Prognostic relevance of DTC detection seems limited since both pure DCIS and microinvasive breast cancer have excellent outcomes. A deeper understanding of earliest stages of tumor progression is necessary; further studies on molecular and functional characteristics of DTCs are needed to understand the phenomenon of tumor cell dissemination. Longer follow-up may be required to fully assess the clinical relevance of DTCs in DCIS.

Conflict of interest The authors declare that they have no conflict of interest.

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