Prevalence of $CD44^+/CD24^{-/low}$ Cells in Breast Cancer May Not Be Associated with Clinical Outcome but May Favor Distant Metastasis

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ABSTRACT

Purpose: **Breast cancer is composed of phenotypically diverse populations of cancer cells. The ability to form breast tumors has been shown by *in vitro*/*in vivo* studies to be restricted to epithelial tumor cells with $CD44^+/CD24^{-/low}$ characteristics. Validation of these findings with respect to detection in clinical samples, prognosis, and clinical relevance is in demand.**

Experimental Design: **We investigated breast cancer tissues for the prevalence of $CD44^+/CD24^{-/low}$ tumor cells and their prognostic value. The study included paraffin-embedded tissues of 136 patients with and without recurrences. In addition, a breast cancer progression array with normal, carcinoma *in situ*, and carcinoma tissues was analyzed. We applied double-staining immunohistochemistry for the detection of $CD44^+/CD24^{-/low}$ cells. Evaluation was by microscopic pathologic inspection and automated image analysis.**

Results: **$CD44^+/CD24^{-/low}$ cells ranged from 0% to 40% in normal breast and from 0% to 80% in breast tumor tissues. The prevalence of $CD44^+/CD24^{-/low}$ tumor cells in 122 tumors was ≤10% in the majority (78%) of cases and >10% in the remainder. There was no significant correlation between $CD44^+/CD24^{-/low}$ tumor cell prevalence and tumor progression. Although recurrences of tumors with high percentages of $CD44^+/CD24^{-/low}$ tumor cells were mainly distant, preferably osseous metastasis, there was no correlation with the event-free and overall survival. There was no influence on the response to different treatment modalities.**

Conclusions: **Our findings suggest that the prevalence of $CD44^+/CD24^{-/low}$ tumor cells in breast cancer may not be associated with clinical outcome and survival but may favor distant metastasis.**

INTRODUCTION

Breast tumors are well known to be composed of phenotypically diverse groups of cells; however, it is unclear which of these cell types contribute to tumor development. In contrast to the hypothesis that all cell populations have the capacity to become tumorigenic through accumulation of mutations, another hypothesis limits this ability to an elite group of cells that share classic features of stem cells such as the ability to self-renew and to differentiate (1). Adult mammary stem cells are thought to be responsible for the massive expansion and differentiation of epithelial tissue during pregnancy and tissue renewal during interpregnancy periods (2). Breast cancer stem cells may share some of these properties, particularly slow cycling time and occasional bursts of proliferative activity. The cancer stem cell concept has been supported by findings in acute myelogenous leukemia with specific cell surface markers (3, 4). Preliminary evidence for cancer stem cells also has been obtained for solid tumors including brain (5) and breast (6) tumors.

In breast cancer, the prospective *in vitro* separation of tumorigenic cells has been recently reported (6). Propagation of human breast tumor cells into the mouse mammary fat pad suggested that breast cancer cells with tumorigenic activity show different cell surface marker expression when compared with nontumorigenic cells. The identified cells strongly expressed the adhesion molecule CD44 together with no or very low levels of the adhesion molecule CD24, referred to as $CD44^+/CD24^{-/low}$ cells. In contrast to other epithelial cells, as few as 200 of these tumorigenic breast cancer cells produced tumors in animals. The $CD44^+/CD24^{-/low}$ cells were shown to resemble normal stem cells with respect to their ability to self-renew, to proliferate, and to differentiate into diverse cell types (6).

The prospective identification of breast cancer stem cells received major attention because of its ultimate implications on breast cancer treatment (7–9). Current breast cancer treatment modalities target proliferating cells, but because breast cancer stem cells are thought to be slowly cycling cells, they may escape present targeted interventions whenever they are not actively proliferating (2, 10). This may be one of the most important reasons behind breast cancer treatment failures and recurrences. It is therefore important to validate the *in vitro*/*in vivo* breast cancer stem cell findings in clinical samples. This will be a critical step toward the development of effective targeted breast cancer treatments; thus far, no data are available on clinical implications of the suggested breast cancer stem cells in clinical samples. We report the identification

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of $CD44^+/CD24^{-/low}$ tumor cells in breast tumor sections by a double-staining immunohistochemistry–based technique and discuss the findings in conjunction with clinical and treatment outcome.

MATERIALS AND METHODS

Study Subjects. We analyzed paraffin-embedded tumor tissues of 136 patients with breast cancer who underwent breast surgery between 1986 and 1997 at Robert-Bosch-Hospital, Stuttgart, Germany. Surgical specimens were obtained before systemic treatment and paraffin embedding was within the framework of diagnostic procedures. Patients' age ranged from 29 to 86 years (mean, 54.6 years). The mean follow-up time was 85.4 (SD, 48) months and the mean time of event-free survival was 71.3 (SD, 53) months. Postsurgery systemic treatment was recorded on a "Yes" or "No" basis and included cyclophosphamide, methotrexate, and 5-fluorouracil (CMF), anthracycline, tamoxifen, or radiotherapy, with many patients having received multiple treatments. Overall, survival included as an event all deaths, whatever the cause. Event-free survival was defined as the time elapsed between excision of the primary tumor to the manifestation of local or distant metastasis or death.

For uniform and simultaneous protein expression analysis of multiple tissue samples, we prepared tissue microarrays that contained cylindrical tissue punches of 1.5-mm diameter from different paraffin-embedded tissues. These tissue cylinders were reembedded in a single paraffin block. As controls, normal epithelial breast tissues of 12 patients in a normal tissue microarray were analyzed. For tumor progression analysis, we used our in-house-developed 96-punch progression tissue microarray with autologous samples of normal, carcinoma *in situ*, and invasive carcinoma of 32 patients with breast cancer. All patient data were fully anonymous.

Immunohistochemistry by Double-Staining Technique. Following breast cancer diagnosis and confirmation of tissue types by H&E staining, immunohistochemistry procedures were done on 3-μm tissue sections with primary monoclonal antibodies for adhesion molecules CD44 (clone 156-3C11, Neomarkers, Fremont, CA) and CD24 (clone 24C02, Neomarkers) using the alkaline phosphatase and anti–alkaline phosphatase (APAAP) as well as EnVision double-staining technique, respectively, in a DAKO autostainer (DAKOCytomation GmbH, Hamburg, Germany). We followed the protocol of Hasui et al. (11) with slight modification using DAKO ChemMate Detection Kits APAAP K5000 and DAKO ChemMate EnVision K5007 (DAKO). Sections were counterstained with hematoxylin for the identification of nuclei.

Control by Single-Staining Immunohistochemistry. To control the reliability of the CD44 and CD24 double-staining, single staining with CD44 and CD24 was done on progression tissue microarray sections (DAKO ChemMate EnVision, K5007). In addition, because in the present study we used a different source for CD44 antibody (Neomarkers) than Al Hajj et al. (ref. 6; PharMingen, San Diego, CA), we confirmed identical immunostaining of CD44 antibody from Neomarkers and PharMingen (clone G44-26) in consecutive tissue sections.

Pathologic Evaluation. Using light microscopy, stained tissue sections were inspected twice by a pathologist (PF) and a trained scientist (BKA) in a blinded fashion. CD44 was identified by red (new fuchsin) and CD24 by brown [3,3′-diaminobenzidine (DAB)] color. Cells with red color staining without much interference from brown color were identified as $CD44^+/CD24^{-/low}$. Percentages of $CD44^+/CD24^{-/low}$ cells were estimated from the entire tumor areas and from punch areas of normal tissue and progression tissue microarrays. Likewise, percentages of $CD24^+$ cells were estimated.

Image Analysis. To control for evaluation bias inherent to subjective pathologic inspection, automatic image analysis with the object (cell)-based image analysis (Cellenger software, DEFINIENS AG, Munich, Germany) was done for 20 randomly selected cases. Digital images were taken from three different tumor areas of each case. The software applies a rule set, which specifies semantic classes, algorithms, and processes to be done on the images, and to identify cells based on morphology, contents, and neighborhood. The rule set further classifies cells according to their own and relative staining intensity with respect to the neighborhood to recognize fuchsin-stained cells ($CD44^+/CD24^{-/low}$) and to avoid false interpretation due to interference with DAB ($CD24^+$). In addition, the total numbers of $CD44^+/CD24^+$tumor cells (fuchsin and DAB stained), $CD44^-/CD24^+$tu-tumor cells (DAB stained only), and $CD44^-/CD24^-$ tumor cells (unstained) were calculated. Results obtained by software analysis were compared with those obtained by pathologic inspection.

Statistical Analyses. Statistical analysis was done using SPSS software version 12.1 (Chicago, IL). Univariate survival analysis was with the Kaplan-Meier method and multivariate survival analysis was calculated by Cox proportional hazard model. Associations between prevalence of $CD44^+/CD24^{-/low}$ tumor cells and clinical parameters were assessed by χ^2 test. All P values resulted from two-sided tests and were considered significant when <0.05.

RESULTS

Identification of $CD44^+/CD24^{-/low}$ Cells. We analyzed CD44 and CD24 antigens in *ex vivo* normal breast epithelial tissues, breast cancer tissues, and a progression series of normal, carcinoma *in situ*, and breast cancer tissues. The normal tissue microarray and first breast cancer series were expected to provide insight into the prevalence of $CD44^+/CD24^{-/low}$ cells in normal breast and breast tumor tissues, whereas the progression tissue microarray series was designed to establish the prevalence of $CD44^+/CD24^{-/low}$ cells in an in-patient tumor progression manner. To unambiguously identify the putative tumorigenic $CD44^+/CD24^{-/low}$ cells we needed to distinguish fuchsin-stained $CD44^+$ cells from double-staining cells labeled with both fuchsin and DAB (Fig. 1*A*, *C*, and *E*). We controlled our microscopic evaluation procedures by image analyses. Identification of $CD44^+/CD24^{-/low}$ tumor cells by image analysis matched those observed by pathologic inspection (Fig. 1*B*, *D*, and *F*). Thus, our double-staining technique and evaluation procedures succeeded in reliable identification of $CD44^+/CD24^{-/low}$ cells.

Prevalence of $CD44^+/CD24^{-/low}$ Tumor Cells in Clinical Samples. In normal tissues, percentages of $CD44^+/CD24^{-/low}$ cells ranged from 0% to 40%. In breast cancer tissues, $CD44^+/$

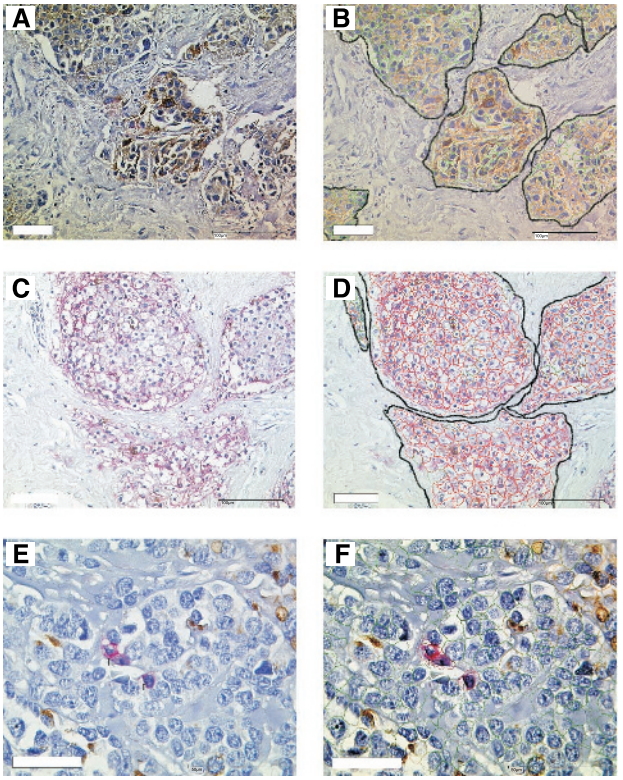

Fig. 1 Double-staining immunohistochemistry of CD44 and CD24 in breast tumor tissue sections. *A* (magnification ×200), *C* (magnification ×200) and *E* (magnification ×600) represent microscopic observations. *A,* absence of fuchsin-stained $CD44^+/CD24^{-/low}$ but presence of DAB-stained $CD24^+$ tumor cells. *C,* area abundant of fuchsin-stained $CD44^+/CD24^{-/low}$ tumor cells selected from one of the rare cases with more than 60% of $CD44^+/CD24^{-/low}$ tumor cells. *E,* presence of three $CD44^+/CD24^{-/low}$ and some DAB-stained $CD24^+$ tumor cells; most tumor cells are negative for both markers. *B, D,* and *F,* automated image analyses of *A, C,* and *E,* respectively. Tumor cells are marked by the Cellenger software with different border lines: red, $CD44^+/CD24^{-/low}$; brown, $CD24^+$; yellow, $CD44^+/CD24^+$; green, $CD44^-/CD24^-$. Tumor areas are selected in *B* and *D* (*black border lines*). *B,* there are mainly $CD24^+$ (*brown*) and $CD44^-/CD24^-$ (*green*) as well as a few $CD44^+/CD24^+$ (*yellow*) cells. *D,* area of mainly $CD44^+/CD24^{-/low}$ (*red*) and a few $CD44^+/CD24^+$ (*yellow*) cells. *F,* area with three $CD44^+/CD24^{-/low}$ (*red*) and few $CD24^+$ (*brown*) cells but a majority of $CD44^-/CD24^-$ (*green*) cells. Comparisons between *A* and *B*, *C* and *D,* as well as *E* and *F* show that image analyses confirm microscopic evaluations.

$CD24^{-/low}$ tumor cells ranged between 0% and 80%. The majority of tumors (78%) displayed ≤10% $CD44^+/CD24^{-/low}$ cells and the remainder contained >10%.

To learn if the percentage of $CD44^+/CD24^{-/low}$ cells increases during tumor progression we analyzed normal, carcinoma *in situ*, and carcinoma tissues from the same patients. Due to loss of tissue punches during experimental procedures, 28 of 32 cases from the progression tissue microarray were evaluated. Tissues of 14 patients (50%) were positive for $CD44^+/CD24^{-/low}$ cells with 12 patients (86%) showing an increased prevalence of $CD44^+/CD24^{-/low}$ cells either in carcinoma *in situ* or in carcinoma. However, we did not observe an increase of $CD44^+/CD24^{-/low}$ tumor cells from carcinoma *in situ* to carcinoma in all cases (data not shown).

Baseline Clinical Characteristics. From the series of 136 patients, 14 cases (10%) were excluded from the analysis due to lack of sufficient tumor area, poor immunohistochemistry staining, or lack of reliable clinical data. Event-free survival and overall survival of 122 patients (90%) are given in Table 1 with respect to histopathologic characteristics and prognostic factors. As expected, nodal status, stage, metastasis, and recurrence had significant influence on the event-free and overall survival (Table 1). Sixty-seven patients (55%) had recurrences and detailed information on recurrences was available for 63 (94%) patients. Sixteen patients (25%) had local, 13 (21%) had local and distant, and 34 (54%) had distant metastases. Distant metastases were located in bone (n = 29), liver (n = 20), lung (n = 17), and other organs (n = 11).

Clinical Significance of the Prevalence of $CD44^+/CD24^{-/low}$ Tumor Cells. There was no significant correlation between the prevalence of $CD44^+/CD24^{-/low}$ tumor cells and event-free or overall survival of patients with breast cancer (Fig. 2*A* and *B*). This was true for cutoff levels from 5% to 50% of $CD44^+/CD24^{-/low}$ tumor cells. Breast cancer patients who developed recurrences did not differ significantly from those without recurrences. Among 15 patients (22%) with recurrences, whose breast tumors contained more than 10% $CD44^+/CD24^{-/low}$ tumor cells, 12 cases (80%) had distant metastasis only (P = 0.04). All 5 cases with more than 20% $CD44^+/CD24^{-/low}$ tumor cells had osseous metastasis (P = 0.02).

No significant differences in the percentage of $CD44^+/CD24^{-/low}$ tumor cells in breast cancer subgroups stratified by histopathologic characteristics and prognostic factors was observed (Table 2). With respect to treatment regimen, the percentage of $CD44^+/CD24^{-/low}$ tumor cells did not show an influence on recurrence, event-free survival, or overall survival. This was true for chemotherapy with cyclophosphamide, methotrexate, and 5-fluorouracil and anthracycline, hormonal treatment with tamoxifen and radiotherapy (Table 3).

DISCUSSION

The present study aimed to determine the diagnostic importance as well as clinical and laboratory feasibility of identifying putative tumorigenic breast cancer stem cells in routine clinical practice. We applied a double-staining immunohistochemistry technique for the identification of $CD44^+/CD24^{-/low}$ tumor cells in paraffin-embedded tissue sections of patients with breast cancer to determine whether the previously *in vitro*/*in vivo* identified putative breast cancer stem cells can be detected in clinical breast cancer samples. To our knowledge, this is one of the first follow-up studies on $CD44^+/CD24^{-/low}$ cancer stem cells and their prevalence in clinical breast cancer samples. Our evaluation did not show an association of the percentages of $CD44^+/CD24^{-/low}$ cells in tumors with the potential to progress or recur, and moreover, had no substantial effect on the event-free survival and overall survival. Moreover, we did not find $CD44^+/CD24^{-/low}$ cells to be associated with a stepwise breast tumor progression. Rather, tumor progression and recurrence were independent from the prevalence of $CD44^+/CD24^{-/low}$ tumor cells. Thus, our *ex vivo*

Table 1 Baseline clinical characteristics of study subjects

Characteristics	*n* (%)	Event-free survival		Overall survival	
		Log rank	*P*	Log rank	*P*
Histology	122	2.99	0.22	2.97	0.23
Invasive ductal	96 (78.7)				
Invasive lobular	14 (11.5)				
Unspecified	12 (9.8)				
Grading*	118	2.55	0.28	1.49	0.48
G1	5 (4.2)				
G2	73 (61.9)				
G3	40 (33.9)				
Tumor size	122	2.20	0.53	4.82	0.19
T1	25 (20.5)				
T2	69 (56.6)				
T3	16 (13.1)				
T4	12 (9.8)				
Nodal status	122	28.38	<0.001	28.32	<0.001
N0	45 (36.9)				
N1	67 (54.9)				
N2-3	10 (8.2)				
Metastasis	122	24.11	<0.001	17.85	<0.001
M0	117 (95.9)				
M1	5 (4.1)				
Stage	122	24.87	<0.001	19.11	<0.001
I	21 (17.2)				
II	69 (56.6)				
III	27 (22.1)				
IV	5 (4.1)				
Menopausal status*	76	0.04	0.85	0.29	0.59
Premenopausal	26 (34.2)				
Postmenopausal	50 (65.8)				
Estrogen receptor*	115	0.03	0.86	0.83	0.36
ER+	73 (63.5)				
ER−	42 (36.5)				
Progesterone receptor*	113	0.82	0.37	0.04	0.84
PR+	66 (58.4)				
PR−	47 (41.6)				
HER-2/*neu**	113	0.82	0.37	1.58	0.21
c-erb-B2+	25 (22.1)				
c-erb-B2−	88 (77.9)				
Recurrence	122	111.68	<0.001	57.44	<0.001
Yes	67 (54.9)				
No	55 (45.1)				

Abbreviations: ER, estrogen receptor; PR, progesterone receptor.
*Number differences reflect missing data.

findings did not support the tumor-progression potential of these cells. However, we observed a high percentage of $CD44^{+}/CD24^{-/low}$ tumor cells in primary tumors of patients with distant metastasis, particularly osseous metastases. This finding may stress the role of CD44 as a homing receptor for distant tissue compartments, a view that is in line with CD44 expression being associated with cell motility through linking with putative actin-binding proteins (12). In addition, it has been shown that CD24 mRNA expression is low in invasive breast cancer cell lines compared with noninvasive cell lines (13).

Our approach differed from that of Al Hajj et al. (6) in that we identified $CD44^{+}/CD24^{-/low}$ tumor cells not in single-cell suspensions but in solid tumor tissues. In contrast to the single-cell *in vitro* approach, the paraffin-embedded solid tissue approach does not require selection of tumor epithelial cells by presence or absence of lineage markers. The former study used the lineage selection to exclude normal human leukocytes, endothelial as well as mesothelial cells, and fibroblasts from the cell suspension for subsequent unanimous assignment of epithelial cell antigens. However, because solid tumor sections display intact morphology, the pathologist can distinguish epithelial from nonepithelial tumor cells under the microscope. Moreover, it is noteworthy that in contrast to the primary tumors investigated by us, all but one tumor investigated in the *in vitro*/*in vivo* study were metastases obtained from pleural effusions. It is therefore possible that detached metastatic cells may display different adhesion properties than tumorigenic cells of the solid primary tumor from which they originated. Although the selection for $CD44^{+}/CD24^{-/low}$ tumor cells from pleural effusions may result in some increased metastatic ability, this may be achieved through improvement of graftability, a view that is supported by our findings of an association with distant, in particular osseous, metastases. Furthermore, the different study outcomes may be attributable to the possibility of the heterogeneity of systemic treatment. Although the primary breast cancer tissues analyzed in the present study were obtained before patients' systemic treatment, there is a chance that the patients with metastatic breast cancer

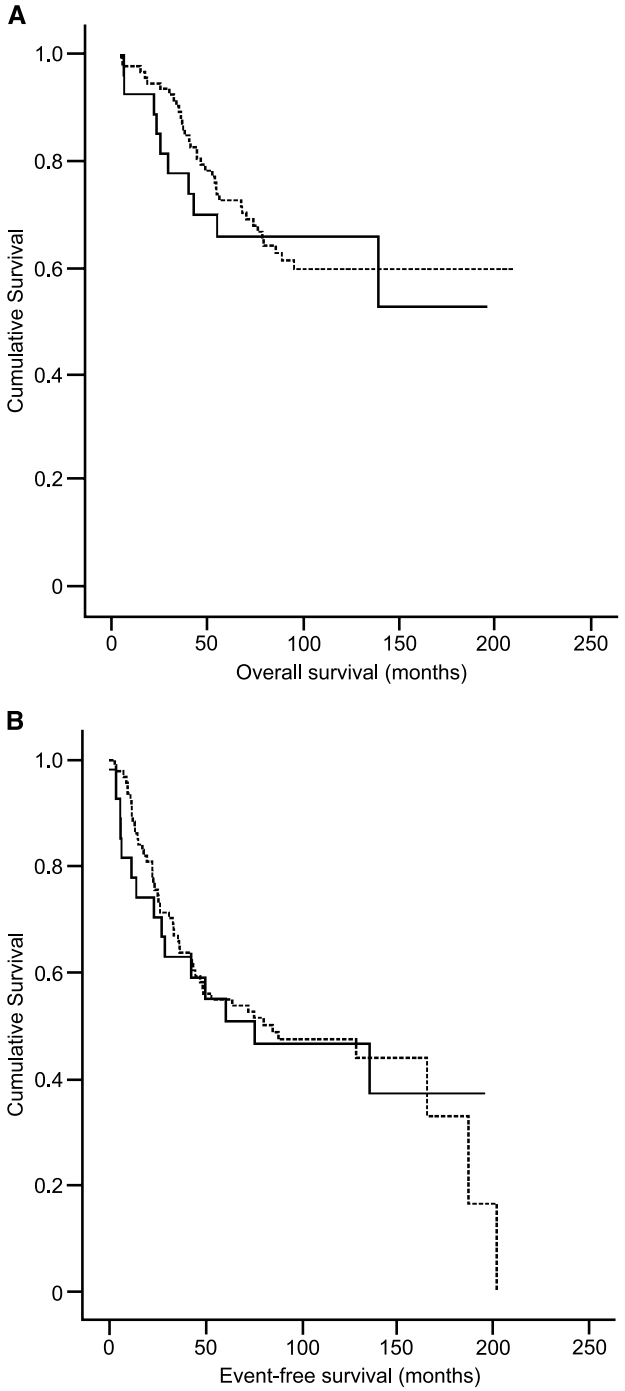

Fig. 2 Clinical correlation of $CD44^+/CD24^{-/low}$ tumor cells. *A* and *B*, univariate survival analysis according to $CD44^+CD24^{-/low}$ tumor cell prevalence. Dotted lines, $CD44^+CD24^{-/low}$ tumor cells ≤10%; solid lines, $CD44^+CD24^{-/low}$ tumor cells >10%. There are no significant differences with respect to (*A*) overall survival ($P = 0.79$) and (*B*) event-free survival ($P = 0.57$).

analyzed in the previous study might have received systemic treatment. Because no treatment information of the $CD44^+/CD24^{-/low}$ tumor cell donors was provided in that study (6) any influence remains elusive.

The prevalence of $CD44^+/CD24^{-/low}$ tumor cells was similar in our *ex vivo* and the published *in vitro*/*in vivo* investigation (6). Our clinical data, however, suggest that the antigenic features of $CD44^+/CD24^{-/low}$ cannot fully describe the tumorigenic properties of breast tumor epithelial cells. Therefore, the suggested $CD44^+/CD24^{-/low}$ tumor cells may rather represent a subclass of tumorigenic cells. This notion is entailed by the previous finding that one out of nine cell isolates did not follow $CD44^+/CD24^{-/low}$-restricted tumorigenicity in animals (6). In that particular case, even $CD24^+$ cells showed the tumorigenic property.

Because tumorigenesis involves complex biological mechanisms, single-cell characteristics may not be sufficient to identify cells with a tumorigenic potential. The previous assignment of tumorigenic cells in breast cancer has been made on the basis of the cell surface marker combination $CD44^+/CD24^{-/low}$; however, its functional relevance in tumorigenesis is not fully understood (2). The putative breast cancer stem cell

Table 2 Prevalence of $CD44^+/CD24^{-/low}$ tumor cells in breast tumors stratified according to clinical characteristics

	$CD44^+/CD24^{-/low}$ tumor cells		
Characteristics	≤10%, *n* (%)	>10%, *n* (%)	*P*
Histology	95	27	0.97
Invasive ductal	75 (78.9)	21 (77.8)	
Invasive lobular	11 (11.6)	3 (11.1)	
Unspecified	9 (9.5)	3 (11.1)	
Grading*	93	25	0.49
G1	3 (3.2)	2 (8.0)	
G2	57 (61.3)	16 (64.0)	
G3	33 (35.5)	7 (28.0)	
Tumor size	95	27	0.24
T1	18 (18.9)	7 (25.9)	
T2	57 (60.0)	12 (44.4)	
T3	13 (13.7)	3 (11.1)	
T4	7 (7.4)	5 (18.5)	
Nodal status	95	27	0.71
N0	34 (35.8)	11 (40.7)	
N1	54 (56.8)	13 (48.2)	
N2-3	7 (7.4)	3 (11.1)	
Metastasis	95	27	0.07
M0	93 (97.9)	24 (88.9)	
M1	2 (2.1)	3 (11.1)	
Stage	95	27	0.08
I	14 (14.7)	7 (25.9)	
II	57 (60.0)	12 (44.4)	
III	22 (23.2)	5 (18.5)	
IV	2 (2.1)	3 (11.1)	
Menopausal status*	59	17	1.00
Premenopausal	20 (33.9)	6 (35.3)	
Postmenopausal	39 (66.1)	11 (64.7)	
Estrogen receptor*	90	25	0.36
ER+	55 (61.1)	18 (72)	
ER−	35 (38.9)	7 (28)	
Progesterone receptor*	89	24	1.0
PR+	52 (58.4)	14 (58.3)	
PR−	37 (41.6)	10 (41.7)	
HER-2/*neu**	88	25	0.06
c-erb-B2+	23 (26.1)	2 (8.0)	
c-erb-B2−	65 (73.9)	23 (92.0)	
Recurrence	95	27	1.0
Yes	52 (54.7)	15 (55.6)	
No	43 (45.3)	12 (44.4)	

*Number differences reflect missing data.

Table 3 Prevalence of $CD44^{+}/CD24^{-/low}$ tumor cells and treatment response

		Recurrence			EFS		OVS	
Treatment	*n*	No, *n* (%)	Yes, *n* (%)	*P*	Log rank	*P*	Log rank	*P*
CMF	50	21	29					
≤10%		15 (71.4)	24 (82.8)	0.49	0.62	0.43	0.51	0.48
>10%		6 (28.6)	5 (17.2)					
Anthracycline	43	12	31					
≤10%		9 (75.0)	25 (80.6)	0.69	0.31	0.58	89	0.35
>10%		3 (25.0)	6 (19.4)					
Tamoxifen	43	17	26					
≤10%		14 (82.4)	18 (69.2)	0.48	0.09	0.27	1.21	0.76
>10%		3 (17.6)	8 (30.8)					
Radiotherapy	70	21	49					
≤10%		18 (85.7)	39 (79.6)	0.74	0.91	0.34	0.47	0.49
>10%		3 (14.3)	10 (20.4)					

NOTE. Treatment groups are selected on a "Yes" or "No" basis with some patients having received multiple treatment.
Abbreviations: EFS, event-free survival; OVS, overall survival; CMF, cyclophosphamide, methotrexate, and 5-fluorouracil.

properties of these cells have been based on experiments within a surrounding murine microenvironment (6). Because in leukemia the cellular milieu of stem cells plays an important role for the leukemogenic process (4), future breast cancer *in vivo* studies may benefit from propagation and validation of breast tumorigenic cells within teratomas derived from the human microenvironment (14).

Altogether, our breast cancer *ex vivo* observation suggests that the relevance of previous *in vitro*/*in vivo* studies to clinical samples and patients is debatable. We may speculate that the putative breast cancer stem cells, although found in the primary tumor, may be more significant in metastases. There might be a possibility that these cells have a tendency to detach early from the primary tumor and leave the mammary gland. Future research should therefore consider the role of the putative breast cancer stem cells in metastases. In light of the importance of stem cells in breast cancer, continuous efforts are needed to recognize additional characteristics for an unambiguous identification of breast cancer stem cells using the baseline information from Al Hajj et al. (6). This will make a very important contribution to the improvement of current therapies and the discovery of novel drug targets in breast cancer. Therefore, it is extremely important to continue efforts on the functional characterization of immunophenotypes of breast tumor cells that drive tumor progression, recurrence, and metastasis.

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Clinical Cancer Research

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