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Original contribution

Reduction of CD44$^+$/CD24$^-$ breast cancer cells by conventional cytotoxic chemotherapy☆

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Summary Breast cancer cells with the CD44$^+$/CD24$^-$ phenotype have been associated with stem cell properties. To analyze effects of cytotoxic chemotherapy on these cells, we examined a series of 50 breast carcinomas before and after neoadjuvant chemotherapy with epirubicin/cyclophosphamide using double immunofluorescence. Before treatment, an average of 4.4% of the tumor cells displayed a CD44$^+$/CD24$^-$ phenotype. However, after chemotherapy, the frequency of CD44$^+$/CD24$^-$ cells dropped to 2% (P = .008). To test this unexpected finding, we analyzed a second collective of 16 patients that preoperatively had received either 4 cycles of doxorubicin/pemetrexed, followed by 4 cycles of docetaxel or 4 cycles of doxorubicin/cyclophosphamide, followed by 4 cycles of docetaxel with similar results (8.7% CD44$^+$/CD24$^-$ cells on average before and 1.1% after chemotherapy). In addition, no association was observed between the frequency of CD44$^+$/CD24$^-$ cells and the response to chemotherapy or patient survival. However, patients with tumors containing high numbers of CD44$^+$/CD24$^-$ cells more frequently developed bone metastases in the course of disease. In conclusion, our findings challenge the proposed role of CD44$^+$/CD24$^-$ cells as cancer stem cells in tumor resistance to chemotherapy as they apparently are not selected by conventional cytotoxic agents.

1. Introduction

Over the past years, the concept of tumor stem cells has gained major attention. Initial observations that large amounts of unselected tumor cells are needed to induce growth of novel tumors following xenograft transplantation suggested that only a small fraction of cells (ie, tumor stem cells) yielded sufficient regenerative capacity [1]. This finding was reproduced with tumors spontaneously arising in mice rendering potential influences of an anti-host immune response less likely. Furthermore, the number of cells needed for successful transplantation was reduced by repeated passaging within animals indicating a potential selection for tumor stem cells [2].

In human primary malignancies, the best data supporting the stem cell concept exist for leukemia in which individual tumor cells containing surface markers (CD34$^+$/CD38$^-$) similar to normal bone marrow stem cells have been identified. Transplantation of these CD34$^+$/CD38$^-$ cells into immunodeficient mice led to the initiation and sustained growth of the neoplastic infiltrate in vivo while the bulk mass of non-CD34$^+$/CD38$^-$ cells could not regrow the leukemia [3,4]. As most leukemias show at least some tendency towards maturation into (atypical) blood cells or their

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precursors, this finding resembles the situation in normal bone marrow with stem cells regenerating the normal peripheral blood cells.

In solid tumors, stem cells have been a lot more elusive, for most organs or tissues no nonneoplastic adult stem cell population expressing specific surface markers has been demonstrated. For normal human mammary gland epithelia, a stem cell or precursor phenotype with high expression levels of cytokeratin 5/6 without cytokeratin 8/18 or smooth muscle actin has been suggested [5], but these data have not been reproduced by other authors [6] and no corresponding subset of cells has been reported for the majority of breast carcinomas. Recently, a subpopulation of human breast cancer cells expressing the CD44 surface antigen and showing little or no CD24 expression has been associated with stem cell qualities. Using cells derived from either primary tumors or pleural effusions that had been propagated in immunocompromised mice, Al Haji et al [7] could demonstrate that cells displaying a $CD44^+/CD24^-/low$ phenotype had the capacity to regrow tumors following transplantation into recipient mice at relatively low numbers while cells with a different phenotype were nontumorigenic. In addition, breast cancer cells grown as floating clusters (so called mammospheres) displayed a $CD44^+/CD24^-$ phenotype, had the capacity to adhere and differentiate towards epithelial or myoepithelial cells after addition of fetal bovine serum and were tumorigenic following injection into SCID mice [8].

Tumor stem cells are not only characterized by an increased regenerating ability, but are also believed to preferentially escape conventional cytotoxic chemotherapy and represent the origin of tumor recurrences. In the present study, we have examined the frequency of $CD44^+/CD24^-$ breast cancer cells in primary breast carcinomas treated using neoadjuvant chemotherapy.

2. Material and methods

2.1. Patients and tumor samples

Initial studies were performed on a series of 50 consecutive patients treated using 6 cycles of neoadjuvant epirubicin/cyclophosphamide (EC) between 1995 and 1999 and for which paraffin embedded tissue of the core biopsies before chemotherapy and the final resection specimen (after chemotherapy) were available. Tumors were classified according to the World Health Organization [9], staging was performed following the TNM guidelines (fourth edition). Expression of estrogen and progesterone receptors as well HER2 status were evaluated immunohistochemically at the time of diagnosis using standard procedures. Response to chemotherapy was classified as proposed by Sinn et al [10] (grade 0, no effect; grade 1, resorption and tumor sclerosis; grade II, minimal focal invasive residues of $\leq$5 mm; grade III, only noninvasive tumour residues; grade IV, no viable tumor cells detectable). Only grade IV regression was considered to represent a pathological complete response. Slides and tissue blocks from all cases were retrieved from the archives of the Institute of Pathology of the Heidelberg University.

To test whether the findings are influenced by the type of neoadjuvant chemotherapy, we analyzed a second series of 16 patients who had been treated in a randomized trial and had received either 4 cycles of doxorubicin/pemetrexed, followed by 4 cycles of docetaxel (AP-Doc; 10 patients) or 4 cycles of doxorubicin/cyclophosphamide, followed by 4 cycles of docetaxel (AC-Doc, 12 patients).

Except for the cases with pathological complete response, representative tissue blocks of the surgical specimen containing residual tumor cells were selected after review of the original slides. The analyses were approved by the Ethics Committee of the Heidelberg University in accordance with the Helsinki recommendations.

2.2. CD44/CD24 double immunofluorescence staining

Four-micrometer tissue sections were cut from the paraffin-embedded tissue of core biopsies and surgical specimen, dewaxed, and rehydrated. After heat-induced epitope retrieval using citrate buffer (pH 6.1, DakoCytomation, Hamburg, Germany), slides were allowed to cool down, rinsed briefly in phosphate-buffered saline (PBS) and preadsorbed using bovine serum albumin (0.1% wt/vol in PBS). Incubation with primary and secondary antibodies was performed at room temperature for 30 minutes with washes in PBS in between. First, a mouse IgG1 anti-CD44 antibody (1:50, clone 156-3C11, Neomarkers/Medac, Hamburg, Germany) was applied and detected using a Cy3-conjugated sheep anti mouse antiserum (1:300, Jackson Immunoresearch, West Grove, PA). In a second step, a mouse IgM κ anti-CD24 antibody (1:25, clone SN3b, Neomarkers/Medac) was applied and detected using a biotinylated goat anti μ-chain antibody (1:300, Jackson Immunoresearch) and fluorescein-streptavidin (1:200, Roche, Mannheim, Germany). Slides were covered with a fluorescence mounting medium containing di-amidinophenylindole (Antifade, Axxora, Lörrach, Germany). Slides were viewed and photographed on an epifluorescence microscope (BX40, Olympus, Hamburg, Germany) equipped with a 100 W high-pressure mercury lamp and a color charged-coupled device camera. To compensate for a possible heterogeneous distribution of $CD44^+/CD24^-$ cells within the tumors, the frequencies of these cells characterized by an intensive red (Cy3) membrane staining in the absence of any green (fluorescein) membrane or cytoplasmic labeling were estimated in 5 to 10 high-power fields (depending on the amount of tissue containing vital tumor cells) by two observers (S.A. and N.W.) followed by averaging of the results.

2.3. Bright field immunostains and morphometry

To verify immunofluorescent detection and quantification of $CD44^+/CD24^-$ breast cancer cells, the core biopsies of the first 17 patients in the EC-treated series were analyzed in parallel using bright field double-stains combined with morphometric image analysis. In brief, deparaffinized and rehydrated slides were pretreated as described above, followed by sequential detection of CD44 and CD24 using the Envision G2 Doublestain system (DakoCytomation) according to the manufacturers instructions. The CD44 antibody (1:150) was detected using alkaline phosphatase/permanent red, CD24 (1:50) was labeled by peroxidase/3,3'-diaminobenzidine (DAB). For each slide, three representative medium-power fields (20× objective) were photographed and imported into the ImageJ software [11]. Quantification of $CD44^+/CD24^-$ cells was performed using the cell-counter function of the particle analysis module to allow for morphological differentiation of tumor cells from non-tumorous stromal cells.

2.4. Statistical analysis

Data were analyzed using the R software package (version 1.7.1, http://www.r-project.org). For count data, Fisher's exact test (2-sided) was used, continuous data were analyzed using the two-sided Wilcoxon rank sum test. Differences before and after neoadjuvant chemotherapy were calculated using the paired Wilcoxon signed rank test. The Kaplan-Meier method was applied to calculate survival rates. Univariate survival data were tested for significance using the Mantel-Haenszel log rank test. *P* values less than .05 were considered significant.

Table 1 Clinicopathologic characteristics for EC- and AP/AC-Doc–treated cases

	EC-treated cases	AP/AC-Doc–treated cases
Total no. of cases	50	16
Average age	53 (range, 33-70)	51 (range, 38-67)
Histology		
Invasive ductal carcinoma	40	14
Invasive lobular carcinoma	10	2
Grading		
G1	4	1
G2	25	11
G3	21	4
Clinical tumor stage		
T1	4	2
T2	27	11
T3	12	2
T4	7	0
unknown		1
Hormone receptors		
ER+	39	12
PgR+	29	11
HER2 overexpression	19	4
Triple negative	5	2
Regression after chemotherapy		
None (score 0)	21	4
Minor (score 1)	23	4
Marked (score 2+3)	6	4
Complete (score 4)	0	4
LN metastases (after CHT)		
No	19	9
Yes	31	5
Unknown	0	2

Abbreviations: ER, estrogen receptor; PgR, progesterone receptor; LN, lymph node; CHT, chemotherapy.

3. Results

The clinicopathologic characteristics of the tumors examined in both series are summarized in Table 1. In the series of EC-treated patients, a total of 29 cases (58%) showed histological signs of tumor regression following chemotherapy; in 6 patients (12%), only minimal residual disease was left. No complete responses were observed. Overall response to the AP/AC-Doc therapy regime was better with 4 (25%) of 16 tumors showing a complete response and another 4 cases displaying a high degree of regression. Tumor patients preoperatively treated with EC chemotherapy were followed for an average of 41 months (range, 8-82 months). During this interval, 18 patients (26%) developed distant metastases, of which 13 died during the follow-up time. Distant tumor spread most frequently involved bone (10 cases) followed by liver (8 cases), brain (5 cases), lung, pleura, and distant lymph nodes (3 cases each). Using univariate analysis, tumor grade and the presence of lymph node metastases were associated with a shorter overall survival ($P = .002$ and $P = .008$, respectively), high-grade tumors also had a significantly lower progression-free survival ($P < .0001$).

$CD44^+/CD24^-$ cells were detected in core biopsies and surgical specimen using double immunoflourescence staining. In most cases, these cells were disseminated in a single cell manner throughout the tumors in varying frequencies. However, in other tumor samples, nests or clusters of $CD44^+/CD24^-$ cells were identified. In addition, small mononuclear cells (macrophages or lymphocytes) staining positive for CD44 and lacking CD24 were frequently observed in close association with the tumor cells. However, using careful morphologic evaluation and comparison with parallel sections stained using hematoxylin and eosin in difficult cases, these cells were well distinguished from the tumor cells. Fig. 1 shows representative staining examples. Depending on tumor cell size and overall cellularity, morphometric analysis included an average of 578 tumor

cells (range, 183-1259). Comparison of fluorescent detection with bright-field double stains combined with morphometric cell counting showed overall very similar results and a high correlation (r = 0.956, Pearson correlation, see Fig. 2).

In the EC series, $CD44^+/CD24^-$ cells comprised an average of 4.4% of the total tumor cell mass in the core biopsies before chemotherapy (range, 0-50%). One case lacking any detectable CD44 or CD24 staining in either tumor or non-tumor cells was assumed not informative; in 9 cases (18%), high numbers of $CD44^+/CD24^-$ cells (arbitrarily defined as ≥10% of tumour cells) were observed. Comparison with the clinicopathologic characteristics of the cases revealed no association with tumor stage, grade, patient age or hormone receptors. However, high numbers of $CD44^+/CD24^-$ cells were significantly more frequent in tumors overexpressing the HER2 oncogene (7 [37%] of 19 HER2 overexpressing tumors compared with only 2 of the remaining 29 cases with known HER2 status [7%], P = .019), none of the triple-negative tumors contained more than 10% $CD44^+/CD24^-$ cells. Regarding the follow-up data, no association with progression-free or overall survival was observed for the tumors containing larger numbers of $CD44^+/CD24^-$ cells (Fig. 3C). However, these cases showed an association with the development of bone metastases (4 [44%] of 9 informative cases compared with 5 of the remaining 40 tumors (12.5%), P = .046] while tumor spread to liver, brain or other organs did not display significant differences in either group.

Following EC-based chemotherapy, the frequency of $CD44^+/CD24^-$ tumor cells decreased from an average of 4.4% to only 2.0% (P = .0079, paired Wilcoxon test) which was not due to reduced staining sensitivity in resection specimen as the chronic inflammatory infiltrates associated with the chemotherapy-induced changes frequently included

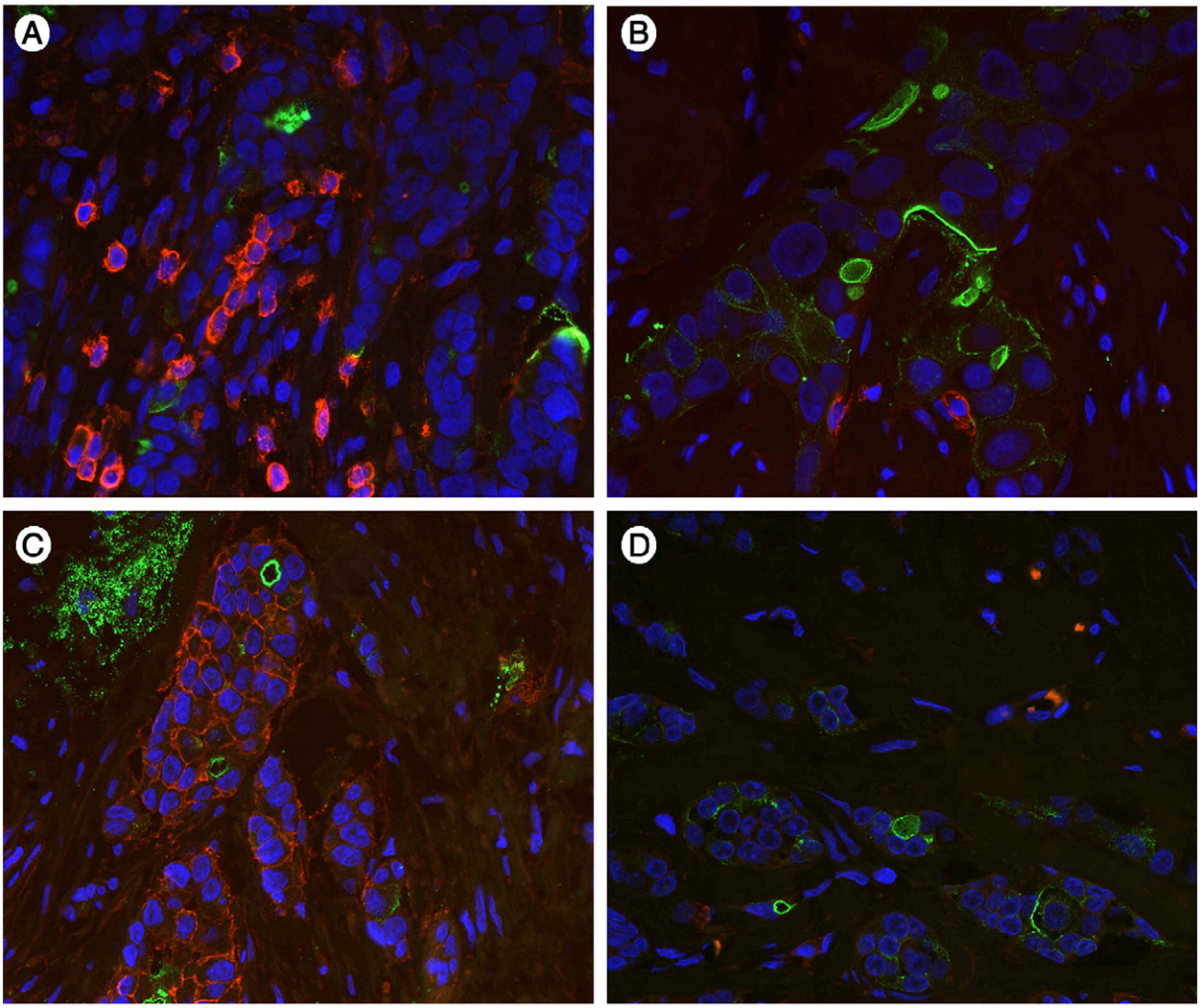

Fig. 1 CD44/CD24 double immunofluorescence. CD44 is detected using Cy3 (red), CD24 is labeled using fluorescein (green). A and B, The same tumor before (A) and after chemotherapy (B). On the left side (A, before CHT) several $CD44^+$,$CD24^-$ cells are seen. After therapy (B), only individual stromal cells are labeled for CD44 while the tumor cells show an occasional positivity for CD24. C, Tumor biopsy taken before chemotherapy with AC-Doc under which the tumor underwent complete regression. In this case, a high number of $CD44^+/CD24^-$ cells are present. D, Another tumor treated with AC-DOC with only little regression. In this case, several $CD24^+$ positive cells but only few $CD44^+/CD24^-$ cells are seen.

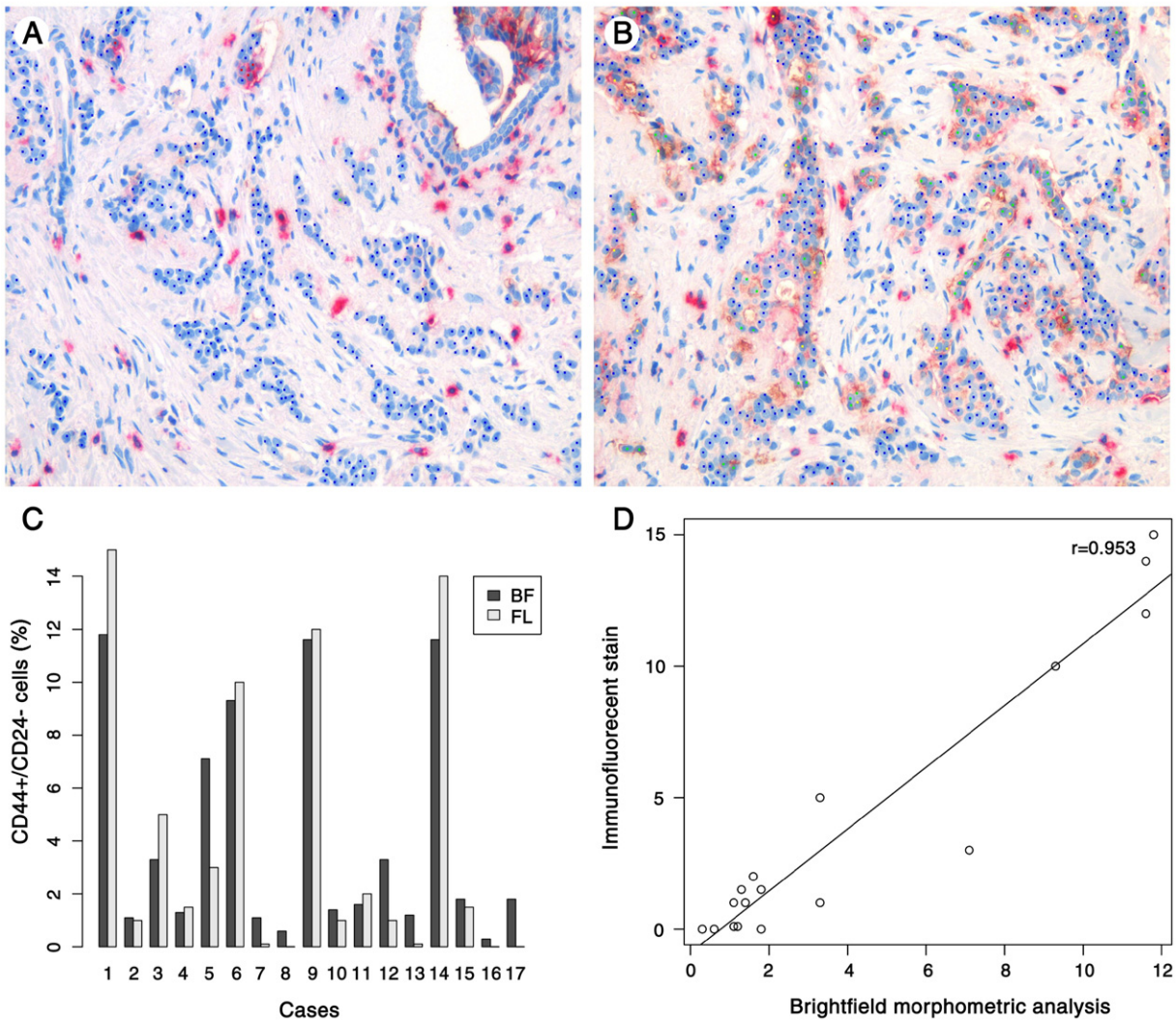

Fig. 2 A, Bright field doublestains for the detection of CD44 (red) and CD24 (brown) were performed to verify $CD44^+/CD24^-$ cell quantification and analyzed morphometrically (B). The results showed a high overall homology (C) as well as a good correlation (D, correlation coefficient $r = 0.956$). Abbreviations: BF, bright field stains followed by morphometric analysis; FL, fluorescent detection of $CD44^+/CD24^-$ cells.

$CD44^+/CD24^-$ mononuclear cells (Fig. 1). In the AP/AC-Doc treated cases, a similar reduction of $CD44^+/CD24^-$ cells from an average of 8.7% before chemotherapy to 1.1% after therapy was observed (Fig. 3A). Owing to the limited number of cases, this difference was significant only when comparing both groups as a whole ($P = .007$, Wilcoxon rank test) but not on a case-by-case basis ($P = .10$, paired Wilcoxon test). An association of the number of $CD44^+/CD24^-$ cases with the response to chemotherapy was seen neither for the EC series nor for the cases treated with AP/AC-Doc (Fig. 3B).

In the core biopsies taken before chemotherapy, approximately 15% (range, 0%-80%) of the tumor cells showed a $CD44^-/CD24^+$ phenotype. This frequency did not change significantly following cytotoxic therapy (14% $CD44^-/CD24^+$ cells on average in the resection specimen, range, 0%-70%, $P = .286$, paired Wilcoxon test). There was also no association between the number of $CD44^-/CD24^+$ cells and response to chemotherapy. Tumors containing a higher number of $CD44^-/CD24^+$ cells (≥10% of the tumor cells) showed a somewhat decreased overall survival (Fig. 3D), but that failed to reach significance ($P = .106$). Coexpression of CD44 and CD24 was mainly seen in individual cells of cases containing higher numbers of cells positive for one (or both) markers. The number of $CD44^+/CD24^+$ cells also was not associated with survival or response to cytotoxic treatment.

4. Discussion

Until a couple of years ago, solid tumors were believed to consist of a relatively homogeneous population of neoplastic cells having the same potential to sustain tumor growth,

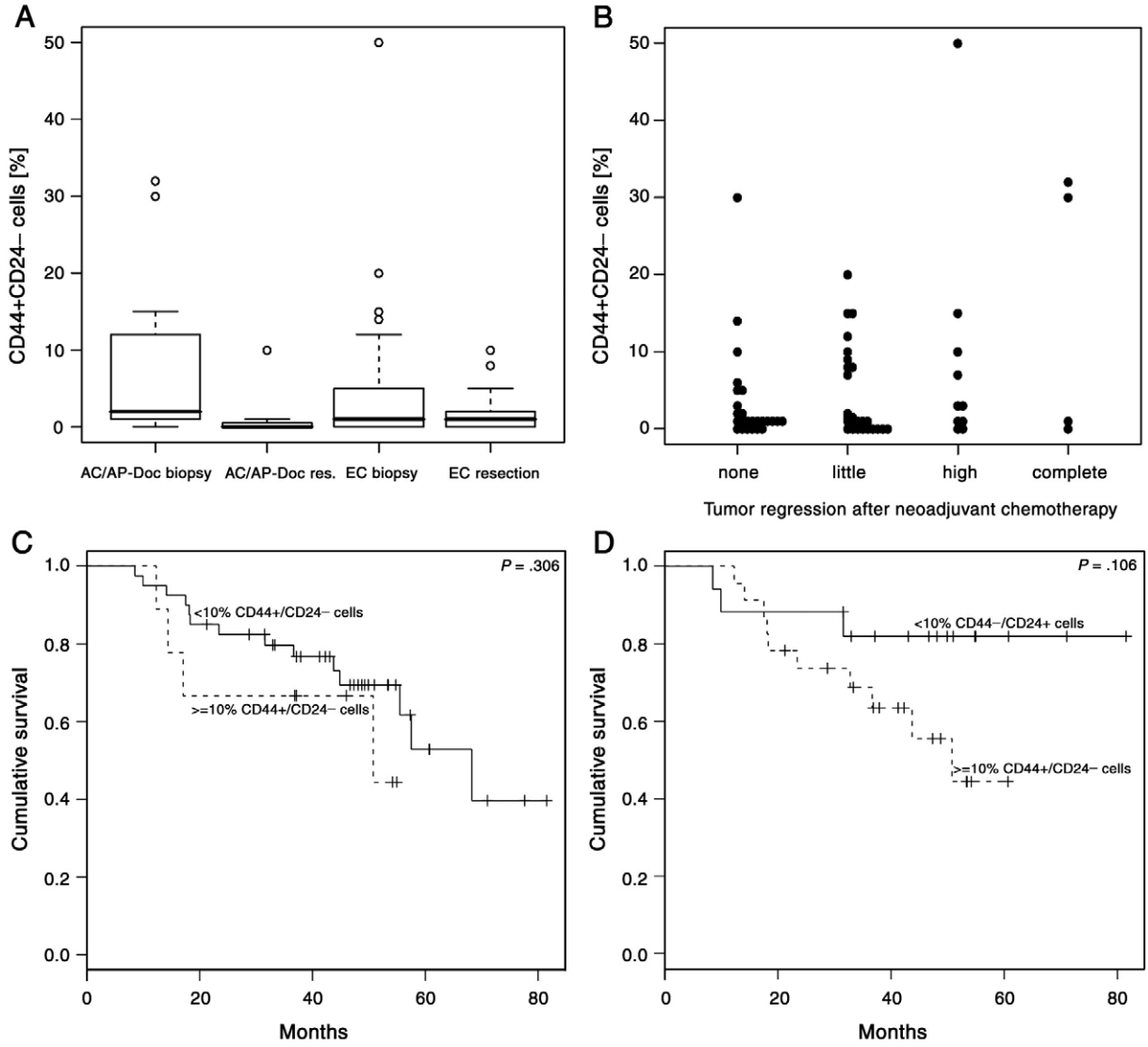

Fig. 3 A, Frequency of $CD44^+/CD24^-$ breast cancer cells before and after neoadjuvant chemotherapy with AC/AP-Doc and EC. B, The number of $CD44^+/CD24^-$ cells was not associated with response to neoadjuvant chemotherapy. The legend translates into the following regression scores: none, score 0; minor, score 1; high, score 2 and 3; complete, score 4. C and D, Overall survival stratified by the number of $CD44^+/CD24^-$ cells (C) or $CD44^-/CD24^+$ cells (D).

invasion, metastasis and recurrence. However, based on observations in leukaemic cell populations, the existence of individual tumor cells that have the capacity to initiate new tumor cell proliferations, the so-called cancer stem cells, has been proposed. This theory has major clinical implications not only regarding metastatic tumor spread but also in the tumor regrowth following radiation or systemic antitumor chemotherapy. Conventional cytotoxic agents do not selectively target tumor stem cells, but often are most efficient on proliferating cells and are thought to miss relatively quiescent tumor cell subpopulations such as the proposed cancer stem cells [12].

Using breast cancer xenografts, a $CD44^+/CD24^-$ tumor cell population has been identified that in contrast to the bulk mass of other tumor cells has the capacity to regenerate the carcinoma following transplantation into immunodeficient mice [7]. Two previous studies using double-staining immunohistochemistry have examined the presence of $CD44^+/CD24^-$ tumor cells in clinical breast cancer specimen and reported frequencies ranging between 0 and 80% [13,14] with most cases containing less than 10% $CD44^+/CD24^-$ cells. Using the same primary antibodies in an immunofluorescence assay, we observed similar frequencies of $CD44^+/CD24^-$ cells (4.4% on average in the EC cases and 8.7% in the AP/AC-Doc-cases). In concordance with both previous studies, there was no relation between the number of $CD44^+/CD24^-$ cells and patient survival. However, cases with high numbers of $CD44^+/CD24^-$ cells showed a significant association with the development of bone metastases [13], a finding that we could reproduce with our data and that is

further supported by the observation that most disseminated tumor cells in the bone marrow of breast cancer patients show the same immunophenotype [15].

Among the most important qualities attributed to cancer stem cells is their relative resistance to conventional cytotoxic therapy, which may be explained by their quiescent behavior. After chemotherapy, tumor recurrences are believed to descent from the surviving stem cells. Given the previously postulated role of $CD44^+/CD24^-$ cells as breast cancer stem cells, we had expected the relative frequency of these cells to increase in partially responding tumors following chemotherapy as most of the proliferating other tumor cell compartment should have been eliminated by the treatment. However, surprisingly, we observed a decrease of $CD44^+/CD24^-$ cells from 4.4 to 2% following chemotherapy with EC. Treatment-specific influences were excluded by analyzing an independent collective that had received a different chemotherapy regime (AP/AC-Doc). This finding is contrary to data recently published by Li et al [16], indicating that conventional cytotoxic therapy with AC leads to an increase of $CD44^+/CD24^-$ cells, whereas additional treatment using lapatinib, a combined inhibitor of type 1 tyrosine kinase receptors including epidermal growth factor receptor (EGFR) and HER2 successfully targets these cells in HER2 positive breast cancer and leads to a high rate of more than 60% near complete or complete remissions in the treated breast cancer patients. However, as the authors state that the epithelial cell content within tumors remained unchanged in both treatment groups before and after chemotherapy, these data are difficult to interpret in the knowledge that overall reduction of the number of (epithelial) tumor cells and the corresponding increase of stromal fibrosis, fibroblasts and inflammatory cells is the key feature of response to cytotoxic therapy (which is also reflected by the various classification systems for tumor response to neoadjuvant treatment [17]). Furthermore, Li et al used flow cytometric analysis to quantify $CD44^+/CD24^-$ cells, a method that may be influenced by the content of fibrous tissue when used with tumor samples that need to be disintegrated and that does not allow a distinction of labeled invasive tumor cells from non-cancerous breast epithelia or residual in situ carcinoma cells.

Although our data provide evidence against a stem cell role of $CD44^+/CD24^-$ breast cancer cells, the expression of these molecules, both of which have been linked to cellular adhesion and metastasis [18-20], might also be associated with interactions with the tumor cell microenvironment. In fact, especially for CD44, a hyaluronate receptor, several splice variants with different expression levels in epithelial cells of different organ systems have been observed [21]. Although CD44 positivity may actually favor adhesion in the bone marrow as suggested by our and previous data [13,22], loss of CD44 has been associated with metastases to the lung in a breast cancer animal model [23]. As cellular adhesion and microenvironment in a breast cancer xenograft model do not necessarily represent the situation in breast cancer patients, the capacity of $CD44^+/CD24^-$ tumor cells to regrow transplanted tumors in the mammary fat pad of immunocompromised mice may as well simply confer an ability of the cells to adhere and survive in the injection place rather than undergo apoptosis. Various influences of tumor microenvironment on survival, proliferation and cell death have been documented [24] that may act as confounding factors in the analysis of cell adhesion molecules and tumor stem cell qualities.

In conclusion, we analyzed the frequency of $CD44^+/CD24^-$ breast cancer cells in 2 different series of patients treated with neoadjuvant chemotherapy and observed a significant decrease following treatment. Although this finding does not rule out the possible existence of breast cancer stem cells, it challenges the role of $CD44^+/CD24^-$ cells as these cells apparently are not selected by conventional cytotoxic agents and thus are less likely to give rise to breast cancer recurrences. Future studies will need to verify these findings in larger series and in cases treated with different chemotherapy regimes.

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