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Human Cancer Biology

Clinical Cancer Research

Aldehyde Dehydrogenase 1–Positive Cancer Stem Cells Mediate Metastasis and Poor Clinical Outcome in Inflammatory Breast Cancer

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Abstract

Purpose: To examine the role of cancer stem cells (CSC) in mediating metastasis in inflammatory breast cancer (IBC) and the association of these cells with patient outcome in this aggressive type of breast cancer.

Experimental Design: CSCs were isolated from SUM149 and MARY-X, an IBC cell line and primary xenograft, by virtue of increased aldehyde dehydrogenase (ALDH) activity as assessed by the ALDEFLUOR assay. Invasion and metastasis of CSC populations were assessed by *in vitro* and mouse xenograft assays. Expression of ALDH1 was determined on a retrospective series of 109 IBC patients and this was correlated with histoclinical data. All statistical tests were two sided. Log-rank tests using Kaplan-Meier analysis were used to determine the correlation of ALDH1 expression with development of metastasis and patient outcome.

Results: Both *in vitro* and xenograft assays showed that invasion and metastasis in IBC are mediated by a cellular component that displays ALDH activity. Furthermore, expression of ALDH1 in IBC was an independent predictive factor for early metastasis and decreased survival in this patient population.

Conclusions: These results suggest that the metastatic, aggressive behavior of IBC may be mediated by a CSC component that displays ALDH enzymatic activity. ALDH1 expression represents the first independent prognostic marker to predict metastasis and poor patient outcome in IBC. The results illustrate how stem cell research can translate into clinical practice in the IBC field. *Clin Cancer Res; 16(1); 45–55. ©2010 AACR.*

Inflammatory breast cancer (IBC) is an angioinvasive form of breast cancer associated with a high incidence of early nodal and systemic metastasis. In contrast to the recent decrease in breast cancer incidence in the United States, the annual incidence of IBC continues to increase (1, 2) with an attendant increase in mortality (3). Despite advances in the use of systemic chemotherapy, the prognosis of IBC remains considerably worse than that of other locally advanced breast cancers (1).

Several molecular changes have been described in IBC including RHOC overexpression, hypomethylation of caveolin-1 or caveolin-2 promoters, and deletion of the tumor suppressor WISP3 (4–8). In addition, IBCs have been reported to overexpress E-cadherin/α, β-catenin, and angiogenic factors (4, 7, 9–14). Although each of these genetic changes may contribute to the metastatic nature of IBC, no markers have been described that can predict the development of systemic metastasis or survival in IBC patients. Although ERBB2 expression is associated with aggressive behavior in most breast cancers, this is not the case in IBC (15).

There is increasing evidence that human breast cancers are driven by a tumor-initiating "cancer stem cell" (CSC) component that may contribute to tumor metastasis and therapeutic resistance (16–20). Breast CSCs were initially characterized as $CD44^+/CD24^-/lin^-$ cells that were capable of serial transplantation in nonobese/severe combined immunodeficient (NOD/SCID) mice (21). In addition to these markers, we have recently shown that cells with stem cell properties in both normal and malignant breast samples can be identified by the expression

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Translational Relevance

Inflammatory breast cancer (IBC) carries a poor prognosis and early metastasis. The topic of this work is of major importance because we show that invasion and metastasis in IBC are mediated by a cellular subcomponent with stem cell characteristics expressing the stem cell marker aldehyde dehydrogenase 1 (ALDH1). In addition, we show the clinical relevance of these findings in a series of 109 patients with IBC by showing that the expression of ALDH1 is associated with early metastasis and decreased survival. These results might be important because there are currently no reliable markers that relate to metastasis of IBC. Our work suggests that the stem cell marker ALDH1 could provide such a tool and that ALDH1-positive cancer stem cells play an important role in mediating the clinically aggressive behavior of IBC. This study underlines the importance of the ALDH1 status in translating cancer stem cell research into clinical practice.

of the enzyme aldehyde dehydrogenase 1 (ALDH1). Using flow cytometry and the ALDEFLUOR assay, which measures ALDH activity, we isolated CSCs from primary human mammary carcinomas grown as xenografts in NOD/SCID mice. In addition, ALDH1 immunostaining identified normal and malignant CSCs *in situ* in fixed paraffin-embedded sections (22).

The rare occurrence of IBC as well as the small size of tumor specimens contribute to the difficulties of studying the biology of this disease. This development of an immortalized cell line and xenograft model of human IBC has facilitated studies of IBC biology (23, 24). In the present study, we have used *in vitro* assays as well as mouse models of the SUM149 IBC cell line and early passages of the MARY-X xenograft generated from a primary IBC tumor. We determined whether IBC contains CSCs and whether these cells mediate tumor invasion and metastasis. To investigate the clinical relevance of these findings, we examined the expression of the stem cell marker ALDH1 in tissue sections from patients with IBC. The *in vitro* studies and mouse xenografts provide evidence that the invasive and metastatic behavior of IBC is mediated by an ALDH1-positive CSC component. Furthermore, expression of this stem cell marker was associated with the development of early metastases and poor clinical outcome in IBC patients.

Materials and Methods

Additional data are available in the Supplementary Materials and Methods section.

Cell culture. SUM149, a gift from S. Ethier (Karmanos Cancer Institute, Detroit, MI), is a breast cancer cell line derived from a patient with primary IBC and is obtained from early passages only (<6 mo; ref. 25).[9] SUM149 was grown in adherent conditions using the recommended culture medium (26). MARY-X is a human breast cancer xenograft established by Barskyand collaborators (9) from a patient with IBC and exhibited the phenotype of florid LVI with tumor emboli formation in SCID and nude mice. When cultured *in vitro*, MARY-X gave rise to floating colonies termed spheroids. These primary spheroids could be maintained in suspension culture for periods up to 3 mo (9).

ALDEFLUOR assay and separation of the ALDH-positive population by fluorescence-activated cell sorting. The ALDEFLUOR kit (StemCell technologies) was used to isolate the population with a high ALDH enzymatic activity. SUM149 and MARY-X cells were suspended in ALDEFLUOR assay buffer containing ALDH substrate (BAAA, 1 μmol/l per 1×10^6 cells) and incubated for 40 min at 37°C. In each experiment, a sample of cells was incubated with 50 mmol/L of the specific ALDH inhibitor diethylaminobenzaldehyde (DEAB) as negative control. Flow cytometry sorting was conducted using a FACStarPLUS (Becton Dickinson). ALDEFLUOR fluorescence was excited at 488 nm and fluorescence emission was detected using a standard FITC 530/30 band pass filter. The sorting gates were established using the propidium iodide–stained cells for viability and the ALDEFLUOR-stained cells treated with DEAB as negative controls.

Animal model and test of tumorigenicity. Tumorigenicity of ALDEFLUOR-positive, ALDEFLUOR-negative, and unseparated SUM149 and MARY-X cells was assessed in three independent sets of three NOD/SCID mice. Fat pads were cleared at 3 wk of age before puberty and humanized by injecting a mixture of irradiated and nonirradiated immortalized human fibroblasts (1:1 irradiated/nonirradiated, 50,000 cells/100 μL Matrigel/fat pad) as described in ref. (22).

After sorting, the tumorigenicity of the ALDEFLUOR-positive, ALDEFLUOR-negative, and unseparated populations of SUM149 and MARY-X cells was tested by inoculation of limiting dilutions of cells (50,000, 5,000, and 500 cells) mixed with Matrigel (BD Biosciences; 1:1) and implanted in the cleared humanized mammary fat pads 2 to 4 wk later.

Lentivirus infection. For luciferase gene transduction, 70% confluent SUM149 cells and suspension culture of MARY-X single cells were incubated overnight with a 1:3 precipitated mixture of lentiviral supernatants Lenti-LUC-VSVG (Vector Core) in culture medium. The following day, the SUM149 cells were harvested by trypsin/EDTA and subcultured at a ratio of 1:6, and MARY-X cells were maintained in suspension culture. After 1 wk of incubation, luciferase expression was verified by adding 2 μL D-luciferin 0.0003% (Promega) in the culture medium

[9] http://www.asterand.com/Asterand/human_tissues/hubrcelllines.htm

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and by counting photon flux by device camera system (Xenogen).

Animal model and intracardiac inoculation. Six-week-old NOD/SCID mice were anesthetized with 1.75% isoflurane/air anesthesia, and the left ventricule of the heart was injected with 50,000 or 100,000 cells (ALDEFLUOR positive, ALDEFLUOR negative, unseparated) in 100 μL of sterile Dulbecco's PBS lacking Ca^{2+} and Mg^{2+}. Experiments were done in duplicates for SUM149 and MARY-X.

Bioluminescence detection. After cell inoculations, the animals were screened for metastasis using bioluminescence. Baseline bioluminescence was assessed before inoculation and each week thereafter. Mice were anesthetized with a 2% isoflurane/air mixture and were given a single i.p. dose of 150 mg/kg D-luciferin (Promega) in PBS. Animals were then reanesthetized using a 2% isoflurane/air mixture 6 min after the administration of D-luciferin. For photon flux counting, we used a charge-coupled device camera system (Xenogen) with a nose-cone isoflurane delivery system and a heated stage for maintaining body temperature. Results were analyzed after 2 to 12 min of exposure using Living Image software provided with the Xenogen imaging system. Signal intensity was quantified as the sum of all detected photon flux counts within a uniform region of interest manually placed during data after processing. Normalized photon flux represents the ratio of the photon flux detected each week after inoculations and the photon flux detected before inoculation.

Patients and tissues. IBC patients were selected from computerized clinicopathologic databases of Institut Paoli-Calmettes between 1976 and 2003 as consecutive cases with available paraffin-embedded tumoral specimen. IBC was clinically defined as a T_{4d} tumor (tumor-node-metastasis, Unio Internationale Contra Cancrum), and metastatic patients at time of diagnosis were not included; the presence of dermal lymphatic emboli was not mandatory for IBC definition. One hundred and nine patients with IBC with a median follow-up of 67 mo were included. All details are in Supplementary Materials and Methods.

Immunohistochemistry and antibodies. Expression of ALDH1, BCL2, E-Cadherin, estrogen receptor (ER), MIB1, ERBB2, MUC1, and progesteron receptor (PR) was measured by immunohistochemistry, and expression of CD24 and CD44 was measured by immunohistochemical double staining as well as by flow cytometry. The characteristics of the antibodies used and the details of the technique are listed in Supplementary Table S1 and in Supplementary Materials and Methods. ALDH1 immunostaining was done as described previously (22), using positive external control for each experiment. For each slide, when internal controls (surrounding fibroblasts or histiocytes located in the stroma or between tumoral cells) were not stained for ALDH1, the slide was not included in the study. Results were expressed in terms of percentage (P) and intensity (I) of positive cells as described previously (22). Results were scored by the quick score (Q; $Q = P \times I$). Tumor presenting at least one ALDH1-positive cancer cell was considered as an ALDH1-positive tumor. Scoring was estimated by two independent breast pathologists (ECJ, JJ) and the mean-value was used. Discrepancies were resolved under multiheaded microscope.

Statistical analysis. For *in vitro* experiments and animal models, results are presented as the mean ± SD for at least three repeated individual experiments for each group. Statistical analyses used the SPSS software (version 10.0.5). Correlations between sample groups and parameters were calculated with the Fisher's exact test or the one-way ANOVA for independent samples. A P value of <0.05 was considered significant.

For the IBC retrospective series, data were summarized by frequencies and percentages for categorical variables, and by median and range for continuous variables. No missing data imputation technique was applied. To study the associations among variables, univariate analysis was done using nonparametric Wilcoxon rank-sum test, χ^2 test, or Fisher's exact test when appropriate. Specific survival (SS) and metastasis-free survival (MFS) rates were estimated by the Kaplan-Meier method using the following first event definitions: death by breast cancer for SS and first metastasis recurrence for MFS. Patients without events were censored at the time of last follow-up or at the date of death if they died for other reason than breast cancer. All survival times were calculated from the date of breast cancer diagnosis. Changes in the relative risk of events according to prognostic factors were assessed by using the log-rank test in univariate analysis. Multivariate analysis was done using Cox's proportional hazard models with a backward stepwise selection of variables to minimize the Akaike Information Criterion. All statistical tests were two sided at the 5% level of significance, using the R 2.9.1 software.

Results

Isolation and characterization of an ALDEFLUOR-positive CSC population in SUM149 and MARY-X models of IBC. The enzyme ALDH has been a useful marker for isolating primitive stem cell populations. We have shown previously that normal human mammary stem and progenitor cells as well as transformed tumor-initiating stem cells may be isolated by virtue of their expression of ALDH activity, as assessed by flow cytometry using the ALDEFLUOR assay. To determine whether IBC contains a CSC population, we used two different models: SUM149, a breast cancer cell line derived from a patient with primary IBC (25), and MARY-X, a human IBC xenograft (9). Using the ALDEFLUOR assay, we isolated an ALDEFLUOR-positive component comprising 5.96 ± 2.2% and 7.2 ± 1.5% of the total cell population (Fig. 1A-B and G-H) of SUM149 and MARY-X, respectively. The tumorigenicity of the ALDEFLUOR-positive, ALDEFLUOR-negative, and unseparated populations of SUM-149 and MARY-X cells was tested by inoculation of limiting dilutions of cells (50,000, 5,000, and 500 cells) into mammary fat pads of NOD/SCID mice that were humanized by the introduction of irradiated and nonirradiated human mammary fibroblasts (22, 26). For the two models used,

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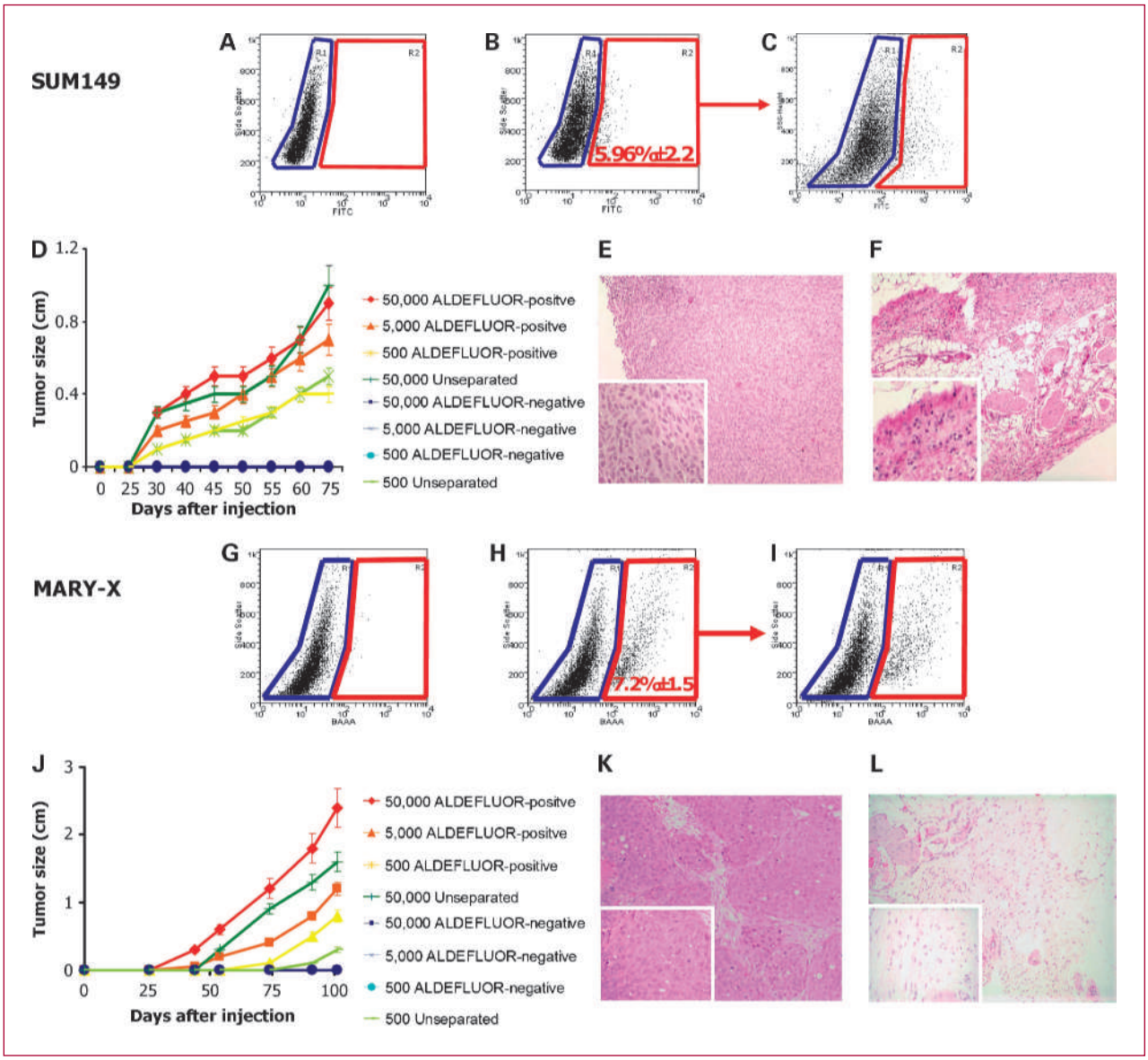

Fig. 1. The ALDEFLUOR-positive cell population of SUM149 and MARY-X cells displays properties of CSCs. Representative flow cytometry analysis of ALDH activity in SUM149 (*A* and *B*) and MARY-X (*G* and *H*) inflammatory breast carcinoma cells. Cells were incubated with ALDEFLUOR substrate (BAAA) and the specific inhibitor of ALDH, DEAB, was used to establish the baseline fluorescence of these cells (*R1*) and to define the ALDEFLUOR-positive region (*R2*; *A* and *G*). Incubation of cells with ALDEFLUOR substrate in the absence of DEAB induces a shift in BAAA fluorescence, defining the ALDEFLUOR-positive population, which represents 5.96 ± 2.2% in SUM149 and 7.2 ± 1.5% in MARY-X of the total population (*B* and *H*). All of the ALDEFLUOR analyses on human breast tumor cells were first gated on propidium iodide–negative cells (viable cells), which represented 99.98 ± 0.0282% (mean ± SD; $n = 7$) of the total population. *C* to *F* and *I* to *L*, in the two models used, only the ALDEFLUOR-positive population was tumorigenic. *C* and *I*, the ALDEFLUOR-positive population was capable of regenerating the phenotypic heterogeneity of the initial tumor after passage in NOD/SCID mice. *D* and *J*, for SUM149 and MARY-X, varying numbers of ALDEFLUOR-positive and ALDEFLUOR-negative cells were injected and tumor growth was measured over a 75-d interval for SUM149 and a 100-d interval for MARY-X. No tumor was detected when 50,000 ALDEFLUOR-negative cells were injected, whereas ALDEFLUOR-positive cells produced tumors that grew at a rate that directly correlated with the number of cells injected. Similar results were observed for SUM149 and MARY-X. *E* and *F*, and *K* and *L*, H&E staining showing presence of tumors at the ALDEFLUOR-positive injection site (*E*, SUM149; *K*, MARY-X) and an absence of tumor at the ALDEFLUOR-negative injection site (*F*, SUM149; *L*, MARY-X).

the fat pads injected with 50,000, 5,000, and 500 ALDEFLUOR-positive cells generated tumors, whereas the ALDEFLUOR-negative cells failed to generate tumors even when 50,000 cells were inoculated (Fig. 1D-F and J-L). A summary of the fat pad injected and the tumor formation in each case is in Supplementary Table S2. H&E staining of fat pad sections confirmed that tumors formed by the ALDEFLUOR-positive cells contained malignant cells with a histology

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similar to the initial tumors (Fig. 1E and K). In contrast, only residual Matrigel, apoptotic cells, and mouse tissue were seen at the sites of the ALDEFLUOR-negative cell injections (Fig. 1F and L). As indicated in Fig. 1D and J, the size and latency of tumor formation correlated with the number of ALDEFLUOR-positive cells injected with 500 ALDEFLUOR-positive cells generating tumors within 30 days for SUM149 and 75 days for MARY-X. To

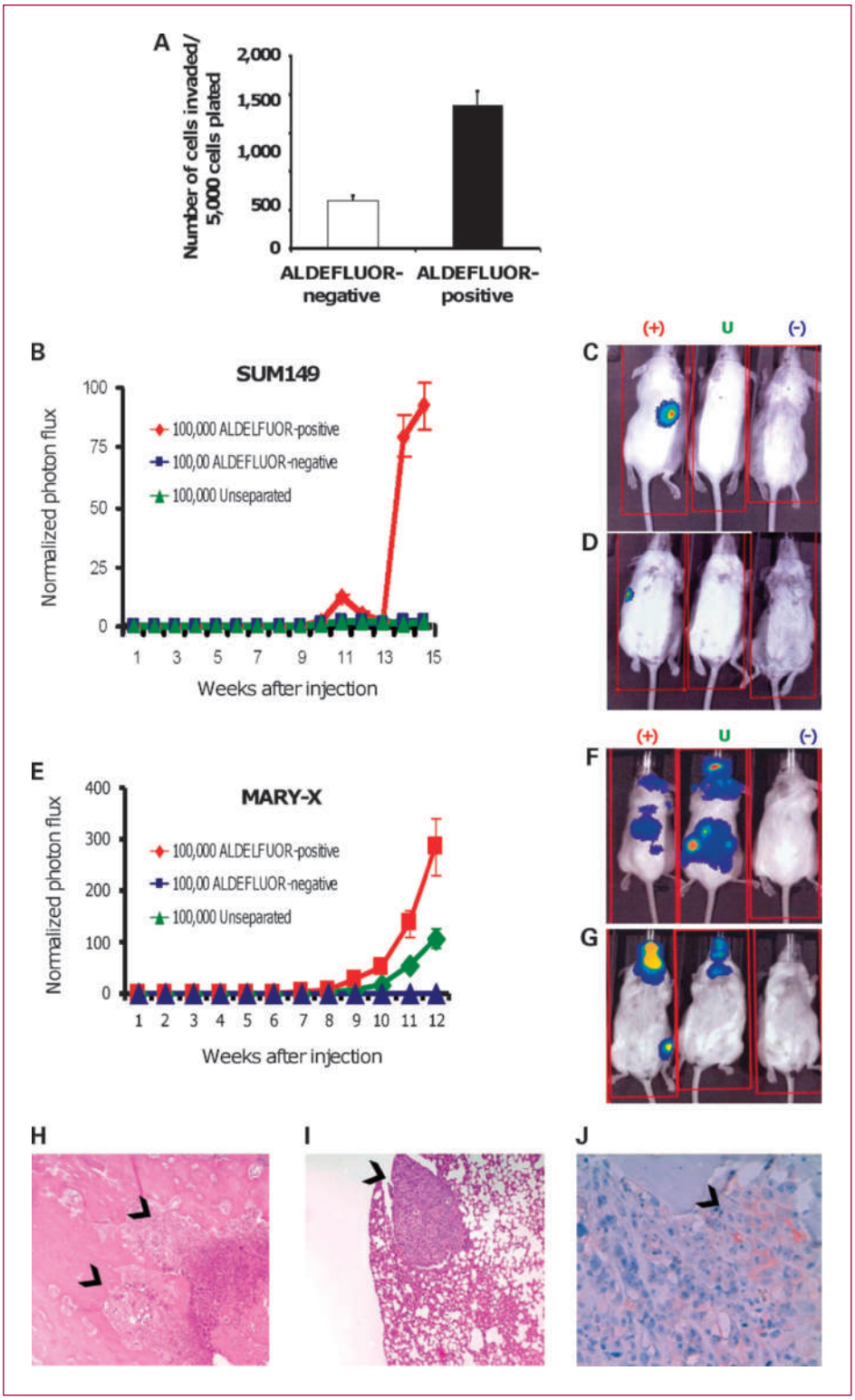

Fig. 2. The ALDEFLUOR-positive cell population of SUM149 and MARY-X cells mediates invasion and metastasis. *A,* the ALDEFLUOR-positive population from SUM149 is associated with greater invasion potential: SUM149 ALDEFLUOR-positive cells showed a 3-fold increase in the Matrigel invasion assay compared with the SUM149 ALDEFLUOR-negative cells ($P < 0.05$). *B* to *I,* using two different models (SUM149 and MARY-X), we showed that only the ALDEFLUOR-positive population displayed metastatic potential. *B* and *E,* quantification of the normalized photon flux measured at weekly intervals following intracardiac inoculations, 100,000 luciferase infected cells from each group (ALDEFLUOR positive, ALDEFLUOR negative, unseparated) and for both models, SUM149 (*B*) and MARY-X (*E*). *C* and *D*, and *E* and *F,* detection of metastasis using bioluminescence imaging software. Mice injected with 100,000 SUM149 or MARY-X ALDEFLUOR-positive cells but not with ALDEFLUOR-negative cells develop systemic metastasis. *H* and *I,* histologic confirmation, on H&E sections, of metastasis in bone and lung resulting from injection of SUM149 ALDEFLUOR-positive cells (*arrows*). Similarly, the presence of metastases was confirmed by histologic inspection in mice inoculated with MARY-X cells. *J,* MARY-X metastasis (spine bone) formed from intracardiac injection of ALDEFLUOR-positive cells contained cells that express (*arrow*) or do not express ALDH1 and recapitulates heterogeneity of the initial tumor.

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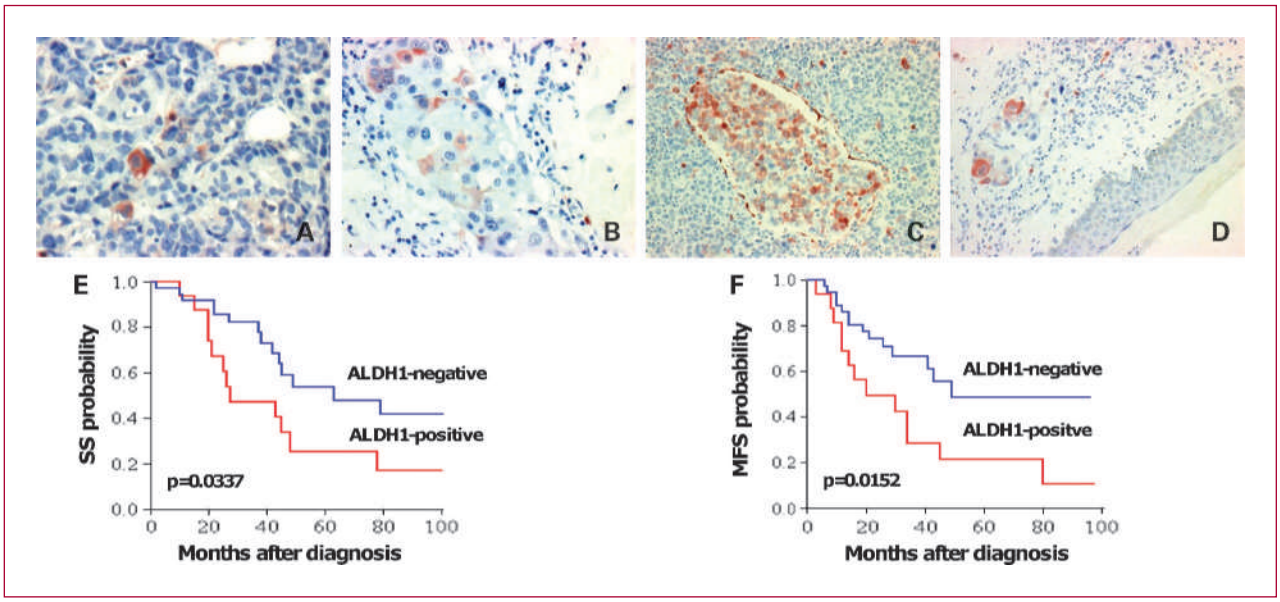

Fig. 3. ALDH1 expression in IBC patient tumors is associated with the development of metastasis and with decreased survival. *A* to *D*, example of ALDH1 expression in a subset of cells in two different IBC samples. *C* and *D*, tumor emboli in dermal lymphatics show cells expressing ALDH1. *E* and *F*, Kaplan-Meier survival curves according to ALDH1 status. ALDH1 expression is associated with decreased SS and MFS.

show the self-renewal potential of the ALDEFLUOR-positive population, we performed three consecutive serial passages in NOD/SCID mice. With each inoculation, the ALDEFLUOR-positive but not ALDEFLUOR-negative cells were able to form tumors even when 500 cells were inoculated. The ability of a few ALDEFLUOR-positive cells to generate tumors that could be serially passaged shows the self-renewal capacity of these cells.

To determine the differentiation capacity of the ALDEFLUOR-positive population, the distribution of ALDEFLUOR-positive and ALDEFLUOR-negative cells was determined at each serial passage. As shown in Fig. 1C and I, ALDEFLUOR-positive cells generated tumors that contained ALDEFLUOR-positive and ALDEFLUOR-negative cells in a similar proportion to that found in the initial tumor. Using the two models, we showed that in addition to self-renewal, ALDEFLUOR-positive cells from SUM149 and MARY-X are able to differentiate, generating a population of ALDEFLUOR-negative nonself-renewing cells.

We next investigated the overlap between the ALDEFLUOR-positive population and the previously described breast CSC phenotype, $CD44^+/CD24^-$ (21). Flow cytometry analysis of the SUM149 tumors showed that the ALDEFLUOR-positive population was enriched in $CD44^+/CD24^-$ cells, with 13.5% of $CD44^+/CD24^-$ cells in the ALDEFLUOR-positive population compared with only 3.02% in the ALDEFLUOR-negative population (Supplementary Fig. S1). Because >98% of MARY-X cells are $CD44^+/CD24^-$, we did not investigate the overlap between the two phenotypes (Supplementary Fig. S1) in this model.

Invasion and metastasis. IBC has a high propensity for the development of lymphagenic invasion and distant metastasis. To determine whether these properties were mediated by the CSC component, we used a Matrigel invasion assay to examine the ability of ALDEFLUOR-positive and ALDEFLUOR-negative SUM149 cell populations to invade. As shown in Fig. 2A, the percentage of ALDEFLUOR-positive SUM149 cells capable of invasion through Matrigel was >3-fold higher than that of the ALDEFLUOR-negative population ($P < 0.05$). To determine the metastatic capacity of these cell populations, we labeled SUM149 and MARY-X cells with a luciferase lentivirus reporter system. The luciferase-labeled cells were sorted using the ALDEFLUOR assay and ALDEFLUOR-positive, ALDEFLUOR-negative, and unseparated cells were introduced by intracardiac injection into NOD/SCID mice. For SUM149 and MARY-X, a suspension of 50,000 and 100,000 cells from each group were injected and the development of metastasis was assessed by noninvasive luciferase bioluminescent imaging quantified by photon flux (27). As shown in Fig. 2B to G, only ALDEFLUOR-positive and unseparated cells formed metastases in this assay. Histologic sections confirmed the presence of bone and lung metastases (Fig. 2H-I) produced from injection of ALDEFLUOR-positive and unseparated cells. Furthermore, vertebral metastasis formed from intracardiac injection of ALDEFLUOR-positive MARY-X cells contained populations of ALDH1-positive and ALDH1-negative cells recapitulating the heterogeneity of the initial tumor.

In contrast, no metastases were detected in mice inoculated with either 50,000 or 100,000 ALDEFLUOR-negative cells. The absence of systemic metastasis was confirmed by histologic examination of sections of the liver, bones,

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brain, and lung tissues. These results suggest that invasion and systemic metastasis in IBC are mediated by cells expressing the stem/progenitor cell marker ALDH1.

ALDH1 expression in a series of human inflammatory carcinomas. We have shown previously that monoclonal antibodies to ALDH1 are able to identify normal and malignant breast stem/progenitor cells in fixed paraffin-embedded sections *in situ* (25). To determine the clinical relevance of expression of this stem/progenitor cell marker, we examined the expression of ALDH1 in a series of 109 IBC patients treated at our institution between 1976 and 2003. ALDH1 was expressed in 34% of tumors with approximately 3% to 5% positively stained cells in these tumors (Fig. 3A). This percentage of ALDH1 expression in this patient population is similar to that found in non-IBC (22). ALDH1-positive cells were also detected in intralymphatic tumor emboli (Fig. 3B-D) consistent with a role for these cells in tumor metastasis. ALDH1 expression correlated with the SBR grade, but there was no correlation with several other clinical and pathologic features including estrogen and progesterone receptors, BCL2, ERBB2, E-cadherin, or MUC1 (Table 1).

Table 1. Correlations between ALDH1 expression and histoclinical factors in inflammatory breast carcinomas

	ALDH1 negative	ALDH1 positive	*P*
	No. of patients (%)		
Age (y)			
<45	11 (21)	9 (31)	NS
≥45	42 (79)	20 (69)	
Axillary lymph node status			
Negative	4 (9)	2 (9)	NS
Positive	40(91)	20 (91)	
SBR grade			
I	2 (4)	1 (3)	0.033
II	17(32)	2 (7)	
III	34 (64)	26 (90)	
BCL2			
Negative	29 (69)	16 (73)	NS
Positive	13 (31)	6 (27)	
$CD44^+/CD24^-$ phenotype			
Absent	16 (33)	5 (28)	NS
Present	33 (67)	13 (72)	
E-cadherin			
negative	20 (51)	10 (56)	NS
positive	19 (49)	8 (44)	
ER			
Negative	13 (42)	15 (71)	NS
Positive	18 (58)	6 (29)	
ERBB2			
0-1	22 (52)	5 (38)	NS
2-3	20 (48)	8 (62)	
Ki67			
≤20	13 (46)	14 (74)	NS
>20	15 (54)	5 (26)	
MUC1			
Negative	21 (48)	8 (53)	NS
Positive	23 (52)	7 (47)	
PR			
Negative	15 (45)	15 (71)	NS
Positive	18 (55)	6 (29)	

Abbreviation: NS, not significant.

To explore another marker of breast CSCs in IBC, and to evaluate its correlation with ALDH expression, we investigated the presence of the $CD44^+/CD24^-$ phenotype in IBC archival specimens. The presence of $CD44^+/CD24^-$ cells was evaluated by immunohistochemistry using double staining on paraffin sections (Supplementary Fig. S2). The $CD44^+/CD24^-$ phenotype was evaluated in 81 cases of the 109 IBC samples, and was present in 51 cases. By comparison with non-IBC tumors, we observed an increase of the percentage of tumors that displayed the $CD44^+/CD24^-$ phenotype in IBC (63% in IBC versus 31% in non-IBC across literature data; ref. 28). This phenotype in IBC was correlated with high proliferation index, with absence of MUC1 expression, and with less lymph node invasion, which differed from previous reports of expression of this phenotype in non-IBC (Supplementary Table S3). More importantly, there was no correlation between the $CD44^+/CD24^-$ phenotype and ALDH1 expression (Table 1).

ALDH1 expression correlates with the development of systemic metastasis and with decreased survival in IBC patients. To determine whether the expression of the stem cell marker ALDH1 correlated with the development of systemic metastasis and survival in IBC patients, Kaplan-Meier survival curves were constructed and compared by log-rank tests. ALDH1 expression correlated with the development of distant metastasis and with decreased survival in these patients. With a median follow-up of 67 months, ALDH1 expression strongly correlated with MFS ($P = 0.0152$) as well as with tumor-specific survival (SS; $P = 0.0337$; Fig. 3E-F). The median MFS was 49 months in patients with ALDH1-negative tumors, compared with 20 months in patients with ALDH1-positive tumors (Table 2). Median SS was 63 months for patients whose tumors did not express ALDH1 compared with only 27 months for patients with tumors expressing ALDH1. Among other parameters tested in univariate analysis, BCL2 expression or ER/PR expression were also correlated with an increased MFS and SS (Table 2). In a multivariate analysis using Cox proportional hazard models, ALDH1 was the only prognostic marker of MFS [$P = 0.0055$; hazard ratio (HR), 2.81; 95% CI (95% confidence interval), 1.355-5.815] and the most powerful prognostic marker of SS (ALDH1: $P = 0.0012$; HR, 2.7; 95% CI, 1.48-4.93) when factors significant in univariate analysis (hormonal receptors and BCL2 expression) were included in the model (Table 3). BCL2 expression was still significant in the model for SS only (BCL2: $P = 0.031$; HR, 0.4; 95% CI, 0.224-0.93). In contrast, the expression of the $CD44^+/CD24^-$ phenotype previously shown to be a

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Table 2. Kaplan Meier univariate analysis of the SS and MFS of 74 IBCs

	No. of patients (%)	5-y SS	*P* of SS	5-y MFS	*P* of MFS
ALDH1					
Negative	37 (70%)	53.69 (36.7-78.6)	0.0337	48.54 (31.2-75.4)	0.0152
Positive	16 (30%)	25.24 (10.2-62.7)		21.09 (7.81-57)	
Age (y)					
<45	22 (30%)	43.87 (25.6-75.2)	NS	28.72 (13.2-62.5)	NS
≥45	52 (70%)	43.43 (30.3-62.2)		44.27 (30.4-64.4)	
Axillary lymph node status					
Negative	5 (7%)	NR	NS	NR	NS
Positive	63 (93%)	40.33 (28.5-57.2)		35.7 (23.9-53.2)	
BCL2					
Negative	35 (66%)	32.02 (17.7-58)	0.00929	36.05 (20.4-63.7)	0.064
Positive	18 (34%)	86.15 (70-100)		63.03 (41-97)	
$CD44^+/CD24^-$ phenotype					
Absent	23 (37%)	41.8 (26.8-65.2)	NS	42.4 (25.1-71.8)	NS
Present	39 (63%)	41 (26.3-63.7)		44.5 (27.4-72.13)	
E-cadherin					
Negative	24 (51%)	50.29 (27.9-90.6)	NS	32.37 (12.5-84)	NS
Positive	23 (49%)	41.09 (24.9-67.8)		41.67 (24.6-70.6)	
ER					
Negative	22 (51%)	11.94 (2.15-66.3)	0.00909	24.24 (8.74-67.3)	0.00454
Positive	21 (49%)	49.26 (28.3-85.7)		35.38 (14.9-83.8)	
ERBB2					
0-1	26 (55%)	59.42 (40.8-86.6)	NS	38.85 (19.9-75.8)	NS
2-3	21 (45%)	47.12 (27.5-80.8)		58.5 (39.9-85.7)	
Ki67					
≤20	20 (51%)	22.79 (8.77-59.3)	0.0797	12.57 (2.22-71.1)	NS
>20	19 (49%)	41.22 (20.5-82.7)		43.2 (21.1-88.6)	
MUC1					
Negative	24 (50%)	68.66 (50-94.3)	NS	53.8 (33.4-86.6)	NS
Positive	24 (50%)	30.41 (14.5-63.8)		29.54 (12.9-67.7)	
PR					
Negative	24 (57%)	15 (4.47-50.3)	0.00377	9.549 (1.6-57.1)	0.00148
Positive	18 (43%)	52.88 (29.5-94.9)		49.33 (23.3-100)	
SBR Grade					
I	3 (4%)	100 (100-100)		50 (12.5-100)	
II	16 (22%)	41.27 (20.1-84.9)	NS	31.25 (11.8-83)	NS
III	54 (74%)	40.01 (27.7-57.8)		42.53 (29.5-61.3)	

Abbreviation: NR, nonrelevant.

CSC marker in non-IBC was not associated with either development of metastasis or patient survival (Supplementary Fig. S3).

Discussion

IBC is among the most angioinvasive and metastatic variants of human breast cancer. Molecular mechanisms have been implicated in IBC clinical aggressiveness. E-cadherin overexpression and dysfunctional, hyposialylated MUC1 may contribute to the metastatic route of IBC (10, 29). The frequent overexpression of RHOC GTPase, hypomethylation of caveolin-1 or caveolin-2 promoters, or deletion of the tumor suppressor WISP3 that belongs to the CCN family illustrates the ability of IBC cells to migrate through putative induction of epithelial to mesenchymal transition process (4–8, 30). Yet, very few markers are available to improve IBC clinical course.

In this study, we have used *in vitro* and mouse models to show that IBCs contain a cellular component, characterized by the expression of ALDH that displays stem cell properties and is able to mediate IBC aggressive behavior.

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ALDEFLUOR-positive cells from IBC cell line and xenograft displayed CSC properties and mediate metastasis. We have previously shown the feasibility of using established cell lines for studies of CSC biology (31). Of note, despite the availability of cell lines representing the different molecular subtypes of breast cancer, the establishment of similar models for IBC has been notoriously difficult. Strikingly, of the 50 cell lines and ~20 xenografts *in vivo* established in the past decades from breast cancers, only SUM149/190, MARY-X, and WIBC-9 were derived from IBC and are currently available to study this type of cancer (9, 24, 32). It highlights the importance of the material available for stem cell studies in IBC. Indeed, tumor xenografting is mandatory to test stem cell properties including tumorigenicity on serial passages and differentiation (16, 22). In that case, the use of primary human breast tumors is not feasible because breast cancer has a well-documented low xenografting rate. Furthermore, due to the small size of tumor specimen available in IBC at time of diagnosis, these questions cannot be addressed for most inflammatory breast tumors. In this study, we used the SUM149 IBC cell line and the MARY-X xenograft, which was established from an IBC tumor without any *in vitro* culture step (9, 13).

We used *in vitro* and *in vivo* experiments to test stem cell properties in cells that are expressing ALDH, an enzyme implicated in retinoic acid metabolism. These "canonical" stem cell properties include self-renewal as shown by tumorigenicity on serial passages, and differentiation as shown by the ability to reconstitute the phenotypic heterogeneity of the initial tumor. Furthermore, we showed that these ALDEFLUOR-positive cells are able to mediate tumor invasion *in vitro* and tumor metastasis in mouse xenografts. These properties recapitulate the aggressive behavior of IBC in patients. This is consistent with the ALDH staining in the tumor emboli described here and by others (33).

Table 3. Cox proportional hazard multivariate analysis in SS and MFS

Cox proportional hazard multivariate analysis in SS (*n* = 77)

Variable	HR (95% CI)	*P*
BCL2 expression		
Negative	1	
Positive	0.457 (0.224-0.93)	0.031
ALDH1 expression		
Negative	1	
Positive	2.7 (1.48-4.93)	0.012

Cox proportional hazard multivariate analysis in MFS (*n* = 61)

Variable	HR (95% CI)	*P*
ALDH1 expression		
Negative	1	
Positive	2.72 (1.322-5.599)	0.069

ALDH1 as an independent prognostic marker in IBC. Although it is clear that IBC has an extremely poor prognosis, there currently are no validated markers that predict outcome in this disease. In fact, well-known markers such as ERBB2 are associated with the aggressive behavior of non-IBC and do not correlate with patient outcome in IBC (15, 34). The antiapoptotic factor BCL2, which expression has been associated with improved SS in non-IBC, is described for the first time as an independent factor for SS in IBC (35). However, BCL2 expression is not significantly associated with longer MFS (Table 2) and is not correlated with the stem cell marker ALDH1 expression (Table 1) or $CD44^+/CD24^-$ phenotype (Supplementary Table S3). In contrast, our work shows that expression of the stem cell marker ALDH1 is associated with development of early metastasis and is an independent prognostic marker for IBC. To our knowledge, ALDH1 is the only independent marker ever described in IBC to predict metastasis. Interestingly, the $CD44^+/CD24^-$ phenotype shown to be associated with CSCs for non-IBC was not associated with outcomes in IBC. This either suggests that ALDH1 and $CD44^+/CD24^-$ are detecting distinct although overlapping cell populations or that the reliability of detection of $CD44^+/CD24^-$ in fixed tumor tissue may be limited (16, 36).

Although cells that expressed ALDH1 have been shown to mediate poor prognosis and metastasis in IBC, the percentage of ALDH1 expression in this patient population is similar to that found in non-IBC and is thus not sufficient to explain by itself the aggressive behavior of IBC. It remains true that expression of ALDH1 is associated with poor outcome and early metastasis in IBC, and we can assume that ALDH-expressing CSC exert different abilities in IBC and in non-IBC. In human hematopoietic system, only a subpopulation of ALDEFLUOR-positive stem cells, expressed CD133, are able to repopulate the murine bone marrow (37). In solid tumor, using a pancreatic cell line xenografted in immunocompromised mice, only a fraction of pancreatic CSCs carry the metastatic potential (38). Consequently, in breast tumor, only a subset of CSC may carry the metastatic potential. Hence, the proportion of metastatic CSC inside the ALDEFLUOR-positive CSC population can differ between IBC and non-IBC and should explain the clinical discrepancies between the two clinical entities. Further studies are needed to isolate potential markers and validate this hypothesis.

Translational perspectives. We have shown that IBC contained a CSC population that expresses ALDH and that ALDEFLUOR-positive IBC cells are highly invasive and mediate metastasis in mice. It is often thought that aggressive cancers may not be organized hierarchically or may have a predominant rather than rare CSC population (39, 40). Our work shows that this is not the case in

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IBC. Moreover, we have shown for the first time that the expression of the stem cell marker ALDH1 is associated with the development of systemic metastasis and decreased survival in IBC patients. Our data also suggest that in addition to mediating metastasis, CSCs also have the ability to reconstitute the tumor heterogeneity of the primary tumor at these metastatic sites.

These results suggest that ALDEFLUOR hierarchy in this subset of breast cancer with respect to its clinical behavior and biological diversity plays an important role in mediating the aggressive course of IBC. Recent preclinical and neoadjuvant clinical studies in non-IBC raised the hypothesis that this hierarchy may affect the clinical management of breast cancer patients. Hence, they have suggested that CSCs in these tumors are relatively resistant to chemotherapy compared with the bulk tumor cell populations (41, 42). Because cytotoxic chemotherapy is the current recommended treatment for IBC (43), it will be a major point to determine whether the CSC components of IBC are also resistant to chemotherapy accounting for poor outcome in these patients. If this is the case, then alternative strategies aimed at targeting this CSC population will need to be developed. Based on the successful clinical application of differentiation therapy in acute promyelocytic leukemia, one of these strategies could be the induction of differentiation (44). This underlines the importance of the ALDH1 status in translating CSC research into IBC clinical practice.

Disclosure of Potential Conflicts of Interest

M.S. Wicha has financial holdings in and is a scientific advisor for Oncomed Pharmaceuticals. The other authors disclosed no conflicts of interest.

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Clinical Cancer Research

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