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ALDH$^+$/CD44$^+$ cells in breast cancer are associated with worse prognosis and poor clinical outcome

Yan Qiu [a,b], Tianjie Pu [a,b], Peng Guo [a], Bing Wei [b], Zhang Zhang [b], Hongying Zhang [b], Xiaorong Zhong [c], Hong Zheng [c], Lina Chen [d], Hong Bu [a,b], Feng Ye [a,*]

[a] Laboratory of Pathology, West China Hospital, Sichuan University, Chengdu 610041, China
[b] Department of Pathology, West China Hospital, Sichuan University, Chengdu 610041, China
[c] Cancer Center and Laboratory of Molecular Diagnosis of Cancer, State Key Laboratory of Biotherapy, National Collaborative Innovation Center for Biotherapy, West China Hospital, Sichuan University, Chengdu 610041, China
[d] Department of Pediatrics, West China Second University Hospital, Sichuan University, Chengdu, Sichuan Province, China

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ABSTRACT

Background: Breast cancer stem cells (BCSCs) play essential roles in tumor metastasis and contribute to remarkably negative clinical outcomes. Recently, aldehyde dehydrogenase (ALDH) and CD44 positivity (ALDH$^+$/CD44$^+$) was identified as a marker of BCSCs in vitro/in vivo studies. The aim of this study was to evaluate the prevalence of ALDH$^+$/CD44$^+$ cells in breast cancer and the association of these two markers with clinicopathological features and clinical outcomes.

Materials and methods: We investigated the prevalence of ALDH1A3$^+$/CD44$^+$ cells in a cohort of 144 formalin-fixed, paraffin-embedded (FFPE) breast cancer tissues. The tissues were stained for ALDH1A3 and CD44 by single and dual immunohistochemistry (dIHC). The associations among the prevalence of ALDH1A3$^+$/CD44$^+$ cells, the clinicopathological features and the clinical outcomes of the patients were also analyzed.

Results: ALDH1A3$^+$/CD44$^+$ cells were present in 39 patients (27.1%). By the Mann–Whitney U test, the Pearson Chi-square test or Fisher's exact test, it was demonstrated that the prevalence of ALDH1A3$^+$/CD44$^+$ cells was closely correlated with larger tumor size ($p = 0.001$), nodal metastasis status ($p = 0.043$), more advanced clinical stage ($p = 0.021$) and distant metastasis after initial surgery ($p = 0.001$). In a univariate survival analysis, the presence of ALDH1A3$^+$/CD44$^+$ tumor cells had a significant negative association with both disease-free survival (DFS) and overall survival (OS) ($p_{DFS} < 0.001$; $p_{OS} < 0.001$). The negative clinical outcomes in ALDH1A3$^+$/CD44$^+$ tumors were further confirmed by a multivariate analysis using Cox proportional hazard models ($p_{DFS} < 0.001$, HR = 3.155; $p_{OS} = 0.001$, HR = 3.193). This was also true with respect to the clinical treatment regimens of chemotherapy ($p_{DFS} < 0.001$; $p_{OS} = 0.001$), radiotherapy ($p_{DFS} = 0.004$; $p_{OS} = 0.004$), and endocrine therapy ($p_{DFS} < 0.001$; $p_{OS} < 0.001$).

Conclusion: In summary, our results indicate that the prevalence of ALDH1A3$^+$/CD44$^+$ tumor cells in breast cancer is significantly associated with worse prognostic factors and favors a poor prognosis.

1. Introduction

Breast cancer is currently considered to include heterogeneous tumors with diverse biological behaviors, clinical outcomes, and responses to treatment (Jemal et al., 2011). Despite various therapies that have been applied to treat breast malignancies, breast cancer is still the leading cause of death for women worldwide (Jemal et al., 2010). In recent years, increasing evidence has suggested that only a small subset of cells, which are defined as breast cancer stem cells (BCSCs), is responsible for the heterogeneity (Ginestier et al., 2007), aggressive behavior and therapeutic resistance in breast cancer (Lee et al., 2011; Velasco-Velázquez et al., 2012). Therefore, CSCs are potential prognostic indicators in patients with breast cancer (Dalerba et al., 2007; Tsang et al., 2012).

During the past decade, much effort has been expended to define biomarkers that may enable the identification of CSCs. Various proteins such as CD44, CD24, ALDH1, CD133, CD49 and EpCAM have been identified as biomarkers of breast CSCs, which have been found to demonstrate CSC characteristics in breast neoplasm (Visvader and Lindeman, 2008; Vieira et al., 2012). To identify CSCs with greater specificity, the use of the co-expression of markers such as CD44/CD24 (Al-Hajj et al., 2003; Abraham et al., 2005), CD49/EpCAM (Ghebeh et al., 2013), and CD44/ALDH (Croker et al., 2009) has been advocated. However, the ideal combination of markers has not yet to be confirmed for the identification of CSCs that are capable of the initiation and metastasis of

* Corresponding author.
E-mail address: fengye@scu.edu.cn (F. Ye).

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tumors. Croker and his colleagues demonstrated that high ALDH expression and CD44 expression in cells of mice and in vitro experiments enhanced the invasion and metastatic ability of the tumor cells (Croker et al., 2009) and that the inhibition of ALDH activity could reduce the resistance of $ALDH^{hi}CD44^{+}$ cells to chemotherapy and radiotherapy (Croker and Allan, 2012). More recently, Liu et al. (2013) demonstrated that $ALDH^{high}/CD44^{high}$ cell subsets showed the highest enhancement of stem cell phenotypic properties compared to $ALDH^{high}/CD44^{low}$, $ALDH^{low}/CD44^{high}$, $ALDH^{low}/CD44^{low}$ and unsorted controls. In a recent study, Cui et al. (2015)revealed that the mRNA levels of Notch1 and β-catenin were significantly higher in $ALDH^{hi}CD44^{+}$ cells compared with those in $ALDH^{low}CD44^{+}$ cells and found a high correlation between $ALDH^{hi}CD44^{+}$ cells and Ki-67 expression; however, they did not find an association between $ALDH^{hi}CD44^{+}$ cells and ER, PR and HER2 expression. Moreover, they suggested that the presence of $ALDH^{hi}CD44^{+}$ cells might serve as a novel diagnostic and prognostic factor in breast cancer. In clinical breast cancers, CD44 and ALDH1 have both been reported as predictors of poor clinical outcomes (Ginestier et al., 2007; Rovira et al., 2010; McFarlane et al., 2015.), but the prevalence of $ALDH^{+}$-$CD44^{+}$ cells and the relationship between the level of expression of these two markers and clinical outcomes have never been clarified.

In our previous study, we showed that out of all the proteins in the ALDH family, ALDH1A3 might be responsible for the poor outcomes of patients with breast cancer (Qiu et al., 2014). In this study, we enrolled 144 patients with invasive ductal breast cancer (IDC) and evaluated the prevalence of ALDH1A3 and CD44 co-expression in tumor cells ($ALDH1A3^{+}CD44^{+}$) and the association of these markers with clinicopathological parameters, distant metastasis, recurrence and survival. Our data demonstrated that patients with $ALDH^{+}/CD44^{+}$ tumor cells were more likely to have distant metastases. Moreover, we showed that disease-free survival and overall survival were decreased in patients with $ALDH^{+}/CD44^{+}$ cells, which suggests that the presence of $ALDH^{+}CD44^{+}$ cells is an independent predictive indicator of poor outcomes in IDC.

2. Materials and methods

2.1. Patients and sample preparation

Specimens from 144 patients with IDC were surgically removed between 2006 and 2009. All specimens were collected and diagnosed in the Department of Pathology of West China Hospital. We prepared tissue microarray (TMA) cores of 1.5 mm from the FPPE samples. Two cores from each individual tumor were arrayed. All patients were female (median age, 51 years; range, 33–77 years), were diagnosed on the basis of histological findings and were staged according to the TNM system. The follow-up time ranged from 2 to 102 months (median, 84.3 months). DFS and OS were defined as the time between the initial surgery and local or distant metastatic relapse, and the time between surgery and death, respectively. Approval for the study was granted by the Ethics Committee of West China Hospital (No. 2013-191).

2.2. Immunohistochemistry (IHC)

Dual IHC with antibodies for the detection of CD44 and ALDH1A3 was performed with the EnVision G|2 Doublestain System Rabbit/Mouse (DAB+/Permanent Red) according to the manufacturer's instructions. Sections with a thickness of 0.4 μm were cut from paraffin-embedded TMA blocks and were mounted on adhesive-coated glass slides. The TMA slides were deparaffinized and rehydrated in dimethylbenzene and ethanol, respectively. Endogenous peroxidase was blocked by 3% H_2O_2, and epitope retrieval was performed in a pressure sterilizer. After blocking with 10% serum for 20 min at room temperature (RT), the slides were further incubated overnight at 4 °C with the following primary antibodies: rabbit anti-CD44 (1:200; 04-1123, Merck Millipore, Darmstadt, Germany) and mouse anti-ALDH1A3 (1:400; Origene, TA502805, Rockville, USA). After 5 washes in phosphate-buffered saline, the slides were incubated with anti-rabbit and anti-mouse secondary antibodies for 30 min at room temperature. CD44 was detected with Permanent Red while ALDH1A3 was detected with diaminobenzidine (DAB). Single IHC with anti-CD44 and anti-ALDH1A3 antibodies was also performed as a control. The hematoxylin and eosin (H&E) as well as the IHC stains were assessed by light microscopy. The staff pathologist at West China Hospital conducted a standard pathological assessment of the tumors from the anonymous patient panel. The status of the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) in the specimens was obtained from the initial pathology reports. HER2 staining was analyzed according to the guidelines of the American Society of Clinical Oncology. CD44 staining was detected primarily in the membrane, and the scoring was performed as follows: 0, 0% positive tumor cells; 1, 1% to 10% positive cells; 2, 11% to 50% positive cells; 3, 51% to 75% positive cells; and 4, 76% to 100% positive cells. ALDH1A3 staining was detected primarily in the cytoplasm, and the scoring was performed as described for CD44 (Honeth et al., 2008).

2.3. Statistical analysis

The associations between the presence of $ALDH1A3^{+}/CD44^{+}$, the clinical variables and the breast cancer subgroups were assessed by Pearson's Chi-square test and Fisher's exact test. Survival curves were plotted according to the Kaplan–Meier method and were compared by log-rank test. The significance of the various parameters for survival was analyzed by the Cox proportional hazards model in a multivariate analysis. Statistical analyses were performed with SPSS version 16.0 software (SPSS Inc. Chicago, USA).

3. Result

3.1. Co-expression of ALDHA3 and CD44 in invasive ductal breast cancer

In order to clearly identify the target tumorigenic cells, we performed both single IHC and dual IHC (Fig. 1A–D) on our TMA samples. In the breast tumor cells, CD44 is predominantly expressed on the membrane, whereas ALDH1A3 is mainly present in the cytoplasm. $ALDH1A3^{+}/CD44^{+}$ cells were present in 39 out of 144 (27.1%, Table 1) patients while $ALDH1A3^{+}/CD44^{+}$ tumor cells ranged between 0% and 80%; the results of the single IHC assay demonstrated that $ALDH1A3^{+}$ and $CD44^{+}$ cells ranged from 0 to 100% (data not shown).

3.2. Baseline clinical characteristics of the tumor tissues

The impact of the clinicopathologic characteristics and prognostic factors was calculated by Kaplan–Meier analysis, and the survival curves were delineated by log-rank test. We observed that larger tumor size ($p_{DFS} = 0.014$, $p_{OS} = 0.003$), increased nodal status ($p_{DFS} < 0.001$, $p_{OS} < 0.001$), clinical stage ($p_{DFS} = 0.013$, $p_{OS} < 0.001$), and the presence of distant metastasis ($p_{DFS} < 0.001$, $p_{OS} < 0.001$) or recurrence ($p_{DFS} < 0.001$, $p_{OS} = 0.015$) all had a close association with poor clinical outcomes. Twelve patients (8.3%) had recurrences, and detailed information in regard to tumor recurrence was available for all patients. Additionally, 59 (40.1%) patients had distant metastases, which were primarily observed in the bone, liver, lungs, and other organs (Table 2).

3.3. Correlation of the prevalence of $ALDH1A3^{+}/CD44^{+}$ cells with tumor characteristics

We compared the expression status of $ALDH1A3^{+}/CD44^{+}$ in different subgroups of invasive breast cancer, which were stratified by clinical characteristics and prognostic factors (i.e., WHO grade, tumor size, nodal status, ER status, PR status, HER2 status, clinical stage, distant metastasis and relapse) by Mann–Whitney U and Pearson Chi-square tests