



In Situ Detection of Cancer Stem Cells in Breast Cancer through Multiplexing for ALDH1 and CD44 on Tissue Microarrays



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Abstract

Background: Cancer Stem Cells (CSC) have been characterized by CD44 positivity and CD24 negativity in flow cytometric studies. In a recent study ALDH1 was identified as a putative marker for breast cancer stem cells showing overlap with CD44 positivity in a small percentage of the cells (1.6%) and defining cells capable of tumorigenesis in immunodeficient mice. Here we attempt to move this multiplexed, flow-based method of characterization to an in situ method in order to define CSCs in formalin fixed, paraffin embedded breast cancer tissue. **Material and Methods:** Our breast cancer cohort is a random retrospective collection of 319 node negative and 319 node positive patients with a mean follow up time of 12.6 years. The TMA's were assessed using the AQUA technology, a recently commercialized quantitative microscopy platform that allows reproducible, objective analysis of protein expression levels within molecularly defined architectural compartments. To identify breast CSCs we assessed both ALDH1 in the cytokeratin compartment and also ALDH1 in the CD44 compartment within a cytokeratin mask. Using a CD44 compartment decreases the number of pixels in the denominator of the AQUA score resulting in higher assay sensitivity. Increased sensitivity is achieved by increasing the signal to noise ratio by decreasing the number of pixels in which ALDH1 can be measured. **Results:** Measurement of ALDH1 expression within a keratin mask was performed on TMAs at 4-fold redundancy and also on a cell line array to define the minimal threshold for ALDH1 expression over background noise. The highest score from each patient's set of 4 spots was used because of tumor heterogeneity in order to identify as many positive patients as possible. This method identified 27 cases (of 546) that were above the AQUA threshold for ALDH1. These cases had significantly worse outcome by Kaplan Meier analysis (log rank p=0.027). Using AQUA to multiplex ALDH1 with CD44 within a cytokeratin mask identified 45 cases (of 397) over the threshold score using only a single fold redundancy. This subset shows significantly worse outcome (log rank p=0.001). Furthermore after performing multivariate analysis (Cox proportional hazard model) the marker combination remains significant independent of tumor size, histological grade, nodal status, as well as ER-, PR- and HER2-status. The appearance of the cells and their intra-epithelial location was confirmed by examination of positive spots with convolution/deconvolution microscopy. **Discussion:** These observations suggest a subset of cells can be identified using an in situ technique that is similar to that used to identify CSCs by flow cytometry. The putative CSCs, when present, appear in variable sized clusters. Whether or not they represent true stem cells, they are prognostic for poor outcome independent of the standard prognostic factors used for breast cancer. Identification of these cells may be valuable in identifying high risk patients and potentially lead to a subset of patients that should be targeted for more aggressive therapy.

Results

Figure 1: AQUA Method for protein expression measurement

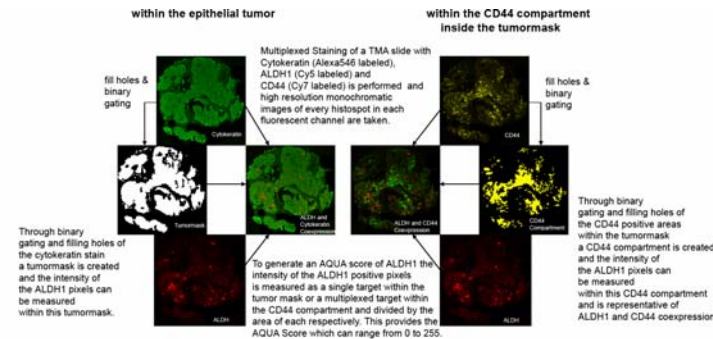


Figure 2: Analysis of CD44 Expression on the Yale Breast Cancer Cohort YTMA 49

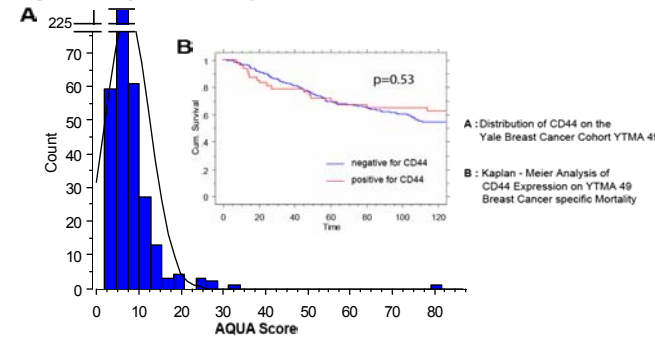
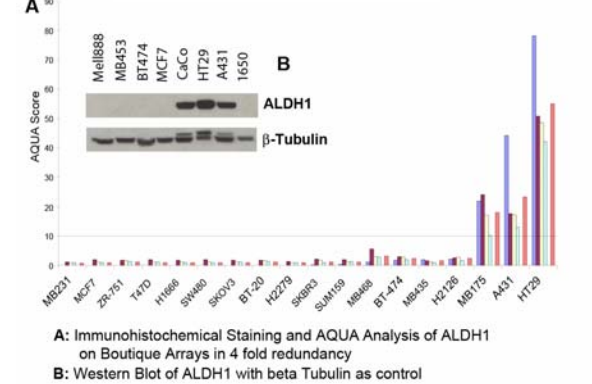


Figure 3: Cell Line Expression of ALDH1



Results

Figure 4: Distribution of maximal ALDH1 Scores 4 fold Redundancy on YTMA 49

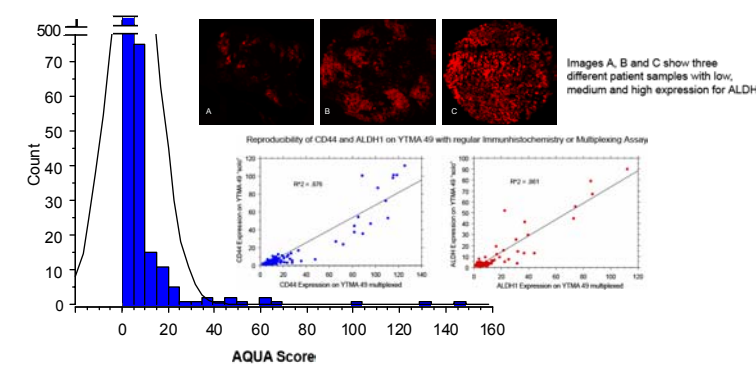


Figure 5: Kaplan-Meier Analysis for ALDH1 Expression within the Tumormask and within the CD44 Compartment

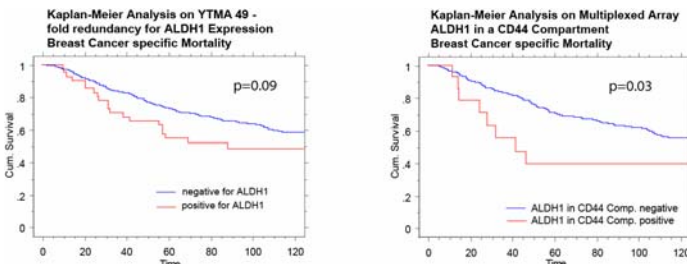
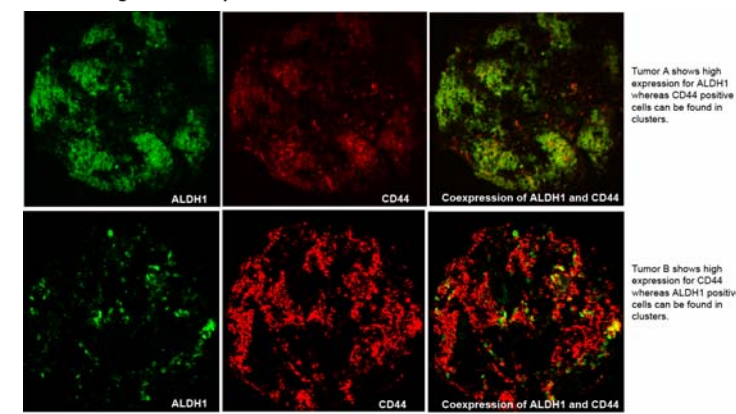


Figure 6: Coexpression of ALDH1 and CD44



Results

Table 3 : Multivariate Analysis of ALDH1 in the CD44 Compartment Cox proportional Hazard Model

Parameter	HR	95% CI	p-value
Nuclear Grade			
low	1.00		
high	1.392	0.991 - 1.954	0.0562
Nodal Status			
negative	1.00		
positive	2.463	1.695 - 3.584	<0.0001
ER			
low	1.00		
high	0.795	0.551 - 1.145	0.217
PR			
low	1.00		
high	0.759	0.526 - 1.097	0.1426
HER2			
low	1.00		
high	1.598	1.048 - 2.437	0.0294
ALDH1 in CD44 Compartment			
negative	1.00		
positive	2.433	1.179 - 5.025	0.0161

Background/ Materials & Methods

Within the last few years research has been focusing on identification of cancer stem cells (CSCs) in various tissues to support the "cancer stem cell model", which says that tumors may originate in stem cells, or progenitor cells, that undergo deregulation of their normally tightly regulated process of proliferation and differentiation. Stem cells are characterized by their unique ability of self renewal through symmetric cell division as well as their ability of differentiation through asymmetric cell division. Recently in breast cancer research low cytometry was performed and cells were isolated that were capable of growing mammospheres as well as creating new tumors in nude mice. These cells are characterized through CD44 positivity and CD24 negativity. In a recent study ALDH1 was also described to be a cell surface marker for breast cancer stem cells supported by flow cytometry that showed some overlap with CD44 positivity in a small percentage of the cells (1.6%). These cells were also capable of creating new mammary tumors in immunodeficient mice. In order to define an assay to identify CSCs in situ, we performed multiplexed immunohistochemical staining for ALDH1, CD44 and Cytokeratin on the Yale Breast Cancer Cohort described above and assessed the level of protein expression with AQUA. To validate the antibodies, we first performed regular immunohistochemical staining for ALDH1 and CD44 separately on "Boutique Arrays". These tissue micro arrays contain tumors samples of breast cancer patients in addition to breast cancer cell lines and are used in order to define the protein's cell line expression, to define the protein's minimal threshold for positivity above background noise, as well as to prove reproducibility of the performed assay. For ALDH1, the cell line expression was confirmed through Western Blot as well. Following antibody validation, we performed analysis of ALDH1 expression alone in our breast cancer cohort of 638 patients represented on a TMA containing cell lines and controls. This assessment was performed in 4 fold redundancy in order to identify as many positive patients as possible due to tumor heterogeneity. Analysis of CD44 expression alone was performed on this breast cancer cohort as well. Following these steps we developed a multiplexed assay to perform immunohistochemical staining for Cytokeratin, ALDH1 and CD44 at the same time. This assay enabled us to measure ALDH1 expression not only within the epithelial tumor area (defined through Cytokeratin positivity) but also to measure ALDH1 and CD44 coexpression and therefore to identify breast cancer stem cells in situ. To perform AQUA we use a recently commercialized quantitative microscopy platform that allows reproducible, quantitative and objective analysis of protein expression levels as well as localization of these proteins in a nuclear, cytoplasmic or membranous compartment. Furthermore we created a "CD44 compartment" defined through CD44 staining above a certain threshold within the epithelial tumor. This allowed us to measure ALDH1 expression within this CD44 compartment and thus to show coexpression of ALDH1 and CD44 for those patients showing an AQUA score for ALDH1 above background within this CD44 compartment.

Table 2: Yale Breast Cancer Cohort - Patient Characteristics

	n(%)	median (range)
Follow-up (y)		6.9 (0.13 - 43)
Age (y)		
>50	170 (26)	
>50	465 (53)	
not specified	7 (1)	
Histology		
Infiltrating duct	520 (81)	
Infiltrating lobular	14 (2)	
Carcinoma (not other spec.)	83 (13)	
Other	25 (4)	
Tumorsize (cm)		2.5 (0.13-14.5)
<2	212 (33)	
2-5	279 (44)	
>5	99 (15)	
Not Specified	52 (8)	
Nodal Status		
Positive	317 (49)	
Negative	320 (51)	
ER		
Positive	320 (50)	
Negative	287 (45)	
Not available	35 (5)	
PR		
Positive	298 (47)	
Negative	290 (46)	
Not available	49 (7)	
HER2		
Positive	487 (77)	
Negative	109 (16)	
Not specified	46 (7)	

Table 1: Primary Antibodies and Dilutions used in Multiplexed Immunohistochemistry

Target	Antibody	Dilution
ALDH1	BD Biosciences mouse monoclonal	1:1000 overnight
CD44	Abcam rabbit monoclonal	1:1000 overnight
Cytokeratin	Sigma guinea pig	1:200 overnight

Conclusions

- Through AQUA analysis and multiplexing for the breast cancer stem cell markers ALDH1 and CD44 we are able to identify in situ a subset of cells with properties seen in stem cells.
- Separate assessment of either CD44 or ALDH1 identifies subset cell populations, but neither alone significantly predicts poor outcome.
- Coexpression of ALDH1 and CD44 in breast cancer is significant in Kaplan-Meier survival analysis with a p-value <0.05. Furthermore after performing multivariate analysis (Cox proportional hazard model) the marker combination remains significant, independent of nuclear grade, nodal status, as well as ER-, PR- and HER2-status.
- Identification of a stem-like population of breast cancer cells may be valuable in identifying high risk patients independent of their nodal status and conventional protein marker expression.

References

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Ginestier, C., Wicha, M.S., and Dontou, G. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcomes. *Cell Stem Cell*. 2007