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Loss of expression of the cancer stem cell marker aldehyde dehydrogenase1 correlates with advanced stage colorectal cancer

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Abstract

Background—Colorectal cancer (CRC) progression is mediated by cancer stem cells (CSCs). We sought to determine if expression of the CSC marker aldehyde dehydrogenase 1 (ALDH1) in CRC tumors varies by AJCC stage or correlates to clinical outcomes.

Methods—Primary and metastatic CRC samples from 96 patients were immunostained with antibodies to ALDH1 and imaged to evaluate marker expression. The percentage of ALDH1⁺ cells was correlated to clinical outcomes.

Results—ALDH1 was overexpressed in CRC tumors compared to non-neoplastic tissue. Marker expression was highest in non-metastatic tumors; loss of expression was associated with advanced stage and metastatic disease. No significant correlation was found between ALDH1 expression and metastasis, recurrence or survival.

Conclusions—ALDH1 was highly expressed in non-metastatic CRC, but expression was lost with advancing stage. ALDH1 could be an effective therapeutic target in early CRC but not late stage disease. No correlation was found between ALDH1 and disease prognosis.

Keywords

Colorectal cancer; cancer stem cells; aldehyde dehydrogenase 1 (ALDH1)

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INTRODUCTION

An increasing amount of evidence has emerged over the last 15 years in support of the hypothesis that tumors derive from a small subpopulation of cancer cells known as tumor-initiating cells or cancer stem cells (CSCs).¹⁻⁴ Similar to endogenous stem cells, CSCs possess the characteristics of self-renewal and pluripotent differentiation making them capable of tumor initiation and maintenance.¹⁻⁴ CSCs have been identified and isolated in several types of solid tumors based on the expression of specific cell surface markers.^{1,5} The phenotype of CSC markers in colorectal cancer (CRC) is still debated though CD166, CD44, EPCAM, and aldehyde dehydrogenase 1 (ALDH1) have been shown to mark highly-tumorigenic CRC cells.⁶⁻⁷ Prior studies have shown that CRC cells positive for one or more of these markers are able to recapitulate the original parent tumor when injected at small numbers into immunodeficient mice.^{4,8-9}

CSCs show resistance to radiation and chemotherapy which can lead to disease recurrence and metastatic spread post-treatment.^{1,10} This resistance is acquired through several mechanisms including high expression of DNA repair enzymes, molecular pumps and detoxifying enzymes such as ALDH1.⁹⁻¹¹ By catalyzing the irreversible oxidization of intracellular aldehydes, ALDH1 confers resistance to alkylating chemotherapeutic agents and protects against oxidative damage.^{9, 11} ALDH1 may also play a role in tumor initiation and growth by converting retinol to retinoic acid, which modulates cell proliferation and motility.⁹ The enzymatic activity of ALDH1 has been used to isolate CSCs in CRC as well as in other malignancies, including breast, prostate, pancreas and lung.¹²⁻¹⁵ Several groups have linked increased ALDH1 expression to poor outcomes, particularly in breast and prostate cancer.¹²⁻¹³ However, others have shown that loss of ALDH1 expression correlates to shortened survival in hepatocellular, pancreatic and lung carcinomas.¹⁴⁻¹⁶ Ultimately, studies are needed to elucidate the significance of ALDH1 in CRC. The aim of our study was to determine if the level of ALDH1 expression in primary and metastatic colorectal tumors from patients with AJCC stage I-IV disease could be correlated to disease stage or used to predict clinical outcomes.

METHODS AND MATERIALS

Archived formalin-fixed, paraffin-embedded (FFPE) primary and metastatic tissue samples from 96 patients who underwent curative resection for primary or metastatic CRC between March 2004 and April 2009 were obtained from Oregon Health & Science University and Kaiser Permanente of the Northwest in Portland, Oregon. Clinical, pathologic and outcome data was obtained and included age, gender, primary tumor location, presence of lymph node metastasis, size and location of distant metastasis, AJCC stage, pre- and post-surgical chemotherapy and radiation treatment, and disease-free and overall survival. Approval from the Institutional Review Board (#2108, 3894, 5169) was obtained from both institutions prior to conducting any review of archived tissue or patient data.

FFPE tissue samples were cut into 5µm-thick sections, deparaffinized and then subjected to antigen retrieval (citrate buffer, 100°C, for 50 minutes followed by a cooling time of 20 minutes). Hepatic tissue sections were incubated for 10 minutes in CytoQ Background

Buster (Innovex Biosciences, Richmond, CA) to reduce native autofluorescence. All slides were incubated in PBS blocking buffer (1% BSA, 0.3% Triton X-100, 1mM CaCl₂) for 10 minutes. Slides were immunostained with a primary antibody to ALDH1 (1:200, Abcam, Cambridge, MA) followed by detection with a species-appropriate fluorescent conjugated secondary antibody. Nuclei were marked by Hoechst staining (50ng/ml). Cytokeratin (1:250, Fitzgerald, Acton, MA) staining was used to identify epithelial tumor cells within the tissue specimen. Tumor samples with no residual tumor or with non-evaluable immunostaining were excluded from the study.

Confocal microscopy was performed on a FluoView FV1000 Confocal Microscope (Olympus, Center Valley, PA) to evaluate the expression of ALDH1 within tumor specimens. Cellular expression of ALDH1 was analyzed using an automated image quantification program (CellProfiler 2.0, Broad Institute Imaging Platform, Cambridge, MA).¹⁷ Marker expression was correlated to patient outcomes (disease recurrence and overall survival) and disease characteristics (presence of nodal and/or distant metastases and AJCC stage).

Immunohistochemical and statistical analysis

Six images were taken at random of each stained tumor specimen using confocal microscopy at 40x magnification. Images were then analyzed using CellProfiler to quantitatively score ALDH1 expression within tumors by identifying the proportion of cells with positive marker expression relative to the total number of tumor cells as denoted by cytokeratin staining. Receiver-operating characteristic (ROC) curves were used to designate tumors as positive or negative for ALDH1 expression. Pearson's χ^2 -tests were used for categorical end points. Paired t-tests were used to compare marker expression in primary tumor and normal colon tissue. Statistical analysis was performed using R version 2.12.1 (R Foundation for Statistical Computing, Vienna, Austria).¹⁸ *P*-values of ≤ 0.05 were considered significant.

RESULTS

A total of 96 patients underwent curative resection for CRC during our study period, which included 14 primary colorectal tumors from patients without nodal or distant metastases (AJCC stage I–II at diagnosis), 34 primary colorectal tumors from patients who presented with or later developed liver metastases (AJCC stage II–IV at diagnosis) and 65 metastatic liver tumors (Table 1). Seventeen patients had matched primary and metastatic tumors available for analysis. All patients who presented with AJCC stage I disease achieved long-term disease-free survival (defined as no disease recurrence within 2 years of curative resection). Among patients who presented with AJCC stage II disease, 69.6 % developed liver metastases within 2 years of resection of the primary colorectal tumor. Only 45.1% of patients with metastatic disease achieved long-term disease-free survival. However, 72.0% of patients with metastatic disease achieved long-term overall survival (defined as survival beyond 2 years of resection).

ALDH1 expression in primary tumors compared to normal colorectal tissue

Using only the primary colorectal tissue samples, ALDH1 expression within tumors was compared to expression within adjacent non-neoplastic colorectal tissue. ALDH1 expression was increased by 18.9% on tumor cells compared to non-neoplastic cells ($p=0.05$). As demonstrated in Figure 1, ALDH1 expression was restricted to the proliferative regions of normal colorectal tissue but was diffusely expressed throughout neoplastic tissue.

ALDH1 expression in early versus late stage CRC

ALDH1 expression was compared between the 14 colorectal tumors from patients without nodal or metastatic disease, the 34 colorectal tumors from patients with metastatic disease, and the 65 metastatic liver tumors. On the basis of ROC analysis, tumors were considered ALDH1⁺ if ≥25% of cells within the colorectal tumor expressed ALDH1 on the cell membrane and if ≥22% of cells within liver tumors demonstrated cell surface marker expression. Interestingly, ALDH1 was highly expressed in all early stage tumors, but expression decreased in late stage and metastatic tumors ($p<0.01$) (Figure 2). ALDH1 expression was lowest among metastatic liver tumors.

ALDH1 as a prognostic marker in CRC

To determine if ALDH1 could be used as a prognostic marker in CRC, expression in primary colorectal tumors was correlated to nodal disease, liver metastasis, disease recurrence and overall survival. Among patients with ALDH1⁺ primary tumors, 52.9% were found to have nodal and liver metastases at diagnosis, 61.6% developed recurrent disease within 2 years of resection, and only 27.6% achieved long-term survival. However, none of these findings reached statistical significance.

DISCUSSION

In this study, we evaluated the relationship of ALDH1 expression in metastatic and non-metastatic colorectal tumors. In contrast to prior studies, we found that early stage, non-metastatic colorectal tumors exhibited increased ALDH1 expression and that expression was lost in more advanced stages of CRC. Interestingly, the lowest marker expression was seen in metastatic tumors to the liver where ALDH1 is normally expressed at very high levels. Furthermore, ALDH1⁺ tumors did not correlate to more aggressive disease behavior (represented by early nodal or liver metastases) or to worse overall survival in our patient cohort.

We found that ALDH1 is overexpressed on tumor cells compared to adjacent non-neoplastic colorectal tissue. This finding is supported by other studies where ALDH1 expression was found to be relatively weak in colonic epithelium except for cells localized to the bottom of normal colonic crypts where stem cells are known to reside.^{9,11} Huang and colleagues have demonstrated that ALDH1⁺ cells increase in number and move farther up the crypt following acquisition of the APC mutation that leads to adenomatous growth.⁹

The functional significance of ALDH1 in tumorigenesis remains unclear. In normal stem cell biology, the enzyme appears to play a role in cell replication as knockdown of ALDH1

using siRNA decreases cell proliferation and motility.¹⁹ Similarly, overexpression of ALDH1 leads to increased cell proliferation, and interestingly, to increased resistance to chemotherapeutic agents.¹⁹ From our data, it appears that overexpression of ALDH1 may indicate increased proliferation in early tumorigenesis but this expression is lost in more advanced disease. Better understanding of the role of ALDH1 in tumor development is crucial for effective therapeutic targeting of this cell population.

Furthermore, the prognostic value of ALDH1 expression has yet to be elucidated. Several studies have reported an association between ALDH1⁺ tumors and poor clinical prognosis in breast, lung, pancreatic, and prostate cancer.^{12–15} In a study by Ohi *et al.*, ALDH1 expression in tumor cells of triple-negative breast cancer was an independent prognostic indicator of shorter disease-free survival on multivariate analysis.¹² Conversely, studies by Deng *et al.*¹¹ and Chang *et al.*²⁰ report a favorable prognosis in serous ovarian cancer with increasing ALDH1 expression. In our study, a larger percentage of patients with ALDH1⁺ tumors had early nodal and distant metastases as well as shortened overall survival. However, these findings did not reach statistical significance. Therefore, we conclude that ALDH1 is not a reliable prognostic marker in CRC.

There were some limitations to our study. First, the data was collected retrospectively in order to obtain survival data for at least two years post-resection. Some patients were lost to follow-up and we were unable to obtain matching primary and metastatic tumor samples for the majority of patients. Second, patients were evaluated and treated at different times, and thus, received different pre- and postoperative chemotherapy and radiation regimens. Lastly, there is inherent subjectivity in immunohistochemical analysis; however, similar immunohistochemical technique and analysis parameters were used for every sample.

In conclusion, this is the first systematic comparison of the CSC marker ALDH1 in metastatic and non-metastatic CRC. Our data shows that ALDH1 is overexpressed in early stage CRC suggesting that it plays an important role in initiation and early proliferation of CRC tumors. However, ALDH1 expression decreases with disease progression, and loss of marker expression can be used a sign of more advanced disease stage. We found no prognostic correlation between ALDH1 expression and early nodal or distant metastases, disease recurrence or overall survival. This suggests that ALDH1 is not a reliable prognostic marker for aggressive behavior or ability to metastasize in CRC. Given that increased expression of ALDH1 may confer resistance to chemotherapy and radiation, ALDH1 could be an effective therapeutic target in early stage CRC. However, our data suggests that it would not be a compelling target in late stage disease.

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Summary

Aldehyde dehydrogenase 1 (ALDH1) has been shown to be a cancer stem cell marker in colorectal cancer (CRC) and may confer resistance to chemotherapy and radiation. The authors found that ALDH1 is expressed at high levels in early stage CRC and may play a role in tumor initiation and early proliferation. ALDH1 expression decreased in late stage and metastatic CRC and showed no correlation to overall prognosis.

Figure 1.

Expression of aldehyde dehydrogenase 1 (ALDH1) in normal colon (A) was restricted to the proliferative region within the colonic crypts. ALDH1 was diffusely expressed on cells throughout AJCC stage I colon tumor (B). ALDH1 was expressed at a lower level in metastatic liver tumors (C). Arrows indicate ALDH1⁺ cells.

Figure 2.

Cut-offs for tumor-positivity were established using receiver-operating characteristic curves. Tumors were considered positive for aldehyde dehydrogenase 1 expression if 25% of cells in the colorectal tumor were marker-positive and if 22% of metastatic liver tumor cells were marker-positive. Early stage refers to non-metastatic primary tumors, while late stage refers to metastatic primary tumors.

Table 1

Patient demographics, n=96

	AJCC Stage at Initial Diagnosis				
	I	II	II*	III	IV
No. patients	7	7	16	15	51
Primary tumor site					
Colon	5	6	10	10	41
Rectum	2	1	6	5	10
Node-positive primary	NA	NA	NA	15	48
No. of liver metastases					
<5	NA	NA	15	13	39
5			1	2	12
Largest liver met 5cm	NA	NA	5	3	16
Neoadjuvant chemotherapy	0	1	6	13	32
Adjuvant chemotherapy	0	1	7	10	32
Negative liver resection margins	NA	NA	12	11	10
Disease-free survival					
Short-term (< 2 years)	0	0	8	9	28
Long-term (2 years)	7	7	8	6	23
Overall survival					
Short-term (< 2 years)	0	0	2	5	16
Long-term (2 years)	7	7	14	10	35

* Patients initially diagnosed with AJCC stage II disease who later developed liver metastases.