ORIGINAL ARTICLE

SINGLE-MARKER IDENTIFICATION OF HEAD AND NECK SQUAMOUS CELL CARCINOMA CANCER STEM CELLS WITH ALDEHYDE DEHYDROGENASE

Matthew R. Clay, BSc,[1] Mark Tabor, MD,[2] John Henry Owen, BSc,[3] Thomas E. Carey, PhD,[3] Carol R. Bradford, MD,[3] Gregory T. Wolf, MD,[3] Max S. Wicha, MD,[4] Mark E. Prince, MD[3]

[1] Department of Molecular and Cell Biology, University of Wisconsin, Madison, Wisconsin
[2] Department of Otolaryngology–Head and Neck Surgery, College of Medicine, University of South Florida, Tampa, Florida
[3] Department of Otolaryngology–Head and Neck Surgery, University of Michigan, Ann Arbor, Michigan. E-mail: mepp@umich.edu
[4] Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan

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Abstract: *Background.* In accord with the cancer stem cell (CSC) theory, only a small subset of cancer cells are capable of forming tumors. We previously reported that CD44 isolates tumorigenic cells from head and neck squamous cell cancer (HNSCC). Recent studies indicate that aldehyde dehydrogenase (ALDH) activity may represent a more specific marker of CSCs.

Methods. Six primary HNSCCs were collected. Cells with high and low ALDH activity ($ALDH^{high}$/$ALDH^{low}$) were isolated. $ALDH^{high}$ and $ALDH^{low}$ populations were implanted into NOD/SCID mice and monitored for tumor development.

Results. $ALDH^{high}$ cells represented a small percentage of the tumor cells (1% to 7.8%). $ALDH^{high}$ cells formed tumors from as few as 500 cells in 24/45 implantations, whereas only 3/37 implantations of $ALDH^{low}$ cells formed tumors.

Conclusions. $ALDH^{high}$ cells comprise a subpopulation cells in HNSCCs that are tumorigenic and capable of producing tumors at very low numbers. This finding indicates that ALDH activity on its own is a highly selective marker for CSCs in HNSCC. © 2010 Wiley Periodicals, Inc. *Head Neck* **32:** 1195–1201, 2010

Keywords: cancer stem cells; aldehyde dehydrogenase; head and neck cancer; squamous cell cancer

Correspondence to: M. E. Prince

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Head and neck cancer is a common malignancy that affects approximately 40,000 new patients in the United States each year.[1] Despite advances in therapy that have improved quality of life, survival rates have remained static for many years. Mortality from this disease remains high because of the development of distant metastasis and the emergence of treatment-resistant local and regional recurrences. To develop more effective therapies for head and neck squamous cell cancer (HNSCC), it is essential that we gain a deeper understanding of the biology

of this disease and the cells that are responsible for recurrent and persistent cancer.

The cancer stem cell (CSC) theory of carcinogenesis postulates that tissue stem cells or progenitor cells are a target for genetic changes that lead to malignant transformation. Based on their similarity to normal stem cells, CSCs are also likely to be more resistant to therapy and may be responsible for tumor persistence and recurrence. Evidence has been accumulating that supports the validity of this theory in a number of malignant diseases.[2–8] Epithelial cancers, including HNSCCs, contain heterogeneous populations of cells, some of which are tumorigenic and many others that are not. Accumulated evidence indicates that CSCs retain phenotypic features of their cell of origin, irrespective of whether that is a stem cell or an early progenitor cell.[9,10] Stem cell markers or molecular processes that are also expressed on CSCs should provide the means to identify and study CSCs. Thus, conserved stem cell molecular pathways may possibly be used in the search for CSCs.

In recent studies in breast and central nervous system cancers, tumorigenic subpopulations of "CSCs" have been isolated based on expression of cell surface markers.[11–16] Using a modification of a method used to identify CSCs in breast cancer we isolated a subpopulation of HNSCC cells marked by the cell surface marker CD44 (CD44-positive cells [CD44+]) that produce tumors in the NOD/SCID mouse model.[12,17] In contrast, even 10-fold higher numbers of CD44-negative (CD44−) tumor cells were unable to form tumors. Although this was the first demonstration of CSCs in HNSCC, the high percentage of HNSCCs found to be CD44+ and the number of such cells necessary (5×10^3) to develop a tumor suggested that, although the CD44+ cell population contained CSCs, it was unlikely to be a pure population of CSCs.

Aldehyde dehydrogenase (ALDH) expression has been suggested as a potential functional marker for stem cells and CSCs. ALDH, which detoxifies intracellular aldehydes through oxidation, may have a role in the differentiation of stem cells through the oxidation of retinoic acid.[18–20] High ALDH activity has been used to isolate normal hematopoietic and central nervous system stem cells.[21–24] ALDH activity is also found in subsets of multiple myeloma and acute myeloid leukemia.[24,25] Wicha et al[26] used ALDH activity to isolate breast CSCs. ALDH 1 expression has recently been reported to be a putative marker for CSCs in HNSCC and colon cancer.[27,28] These findings indicate that ALDH expression may be an important new marker for the isolation of CSCs. In this study we investigated the ALDH active population of HNSCC cells for tumorigenic activity.

MATERIALS AND METHODS

Approvals for the collection of cancer specimens and for use of the animal model were obtained through the appropriate review boards. The University of Michigan's *Guide for the Care and Use of Laboratory Animals* was followed. Samples of HNSCC cancer were obtained from subjects undergoing surgical resection or biopsy of their tumor. Specimens were immediately transported to the laboratory in Dulbecco's modified Eagle's medium (DMEM; GIBCO Media/Invitrogen Corp., Carlsbad, CA) containing 10% fetal bovine serum (FBS; Sigma–Aldrich, St. Louis, MO) on ice. Single-cell suspensions were created.

Specimens were cut into small fragments with sterile scissors and minced with a sterile scalpel, rinsed with Hanks' balanced salt solution (HBSS; Invitrogen) containing 2% heat-inactivated calf serum (HICS; Invitrogen), and centrifuged for 5 minutes at 1000 rpm. The resulting tissue specimen was placed in a solution of DMEM F-12 containing 300 U/mL collagenase and 100 U/mL hyaluronidase (STEMCELL Technologies, Vancouver, BC, Canada). The mixture was incubated at 37°C mixing to dissociate cells. The digestion was arrested with the addition of FBS and the cells were filtered through a 40-μm nylon sieve. The cells were washed twice with HBSS/2% HICS and stained for flow cytometry as described in the following text.

ALDH activity was identified in the cancer cells using the ALDEFLUOR substrate in accord with the manufacturer's protocol (StemCo Biomedical, Durham, NC). Specimens that were analyzed for ALDH activity were counterstained with anti-CD44 (allophycocyanin [APC] conjugated; BD Pharmingen, San Diego, CA) at the appropriate dilution. Cells of other lineages were identified and removed using markers anti-CD2, CD3, CD10, CD16, and CD18 (CyChrome [Cy] conjugated; BD Pharmingen) that are not expressed on the tumor cells. Nonviable cells were eliminated using 4′,6-diamidino-2-phenylindole (DAPI; BD Pharmingen). During flow cytometry analysis, other lineage

Table 1. Patient demographics, tumor classification, and characteristics.

Sample	Age/ Sex	Site	Classification	Differentiation
HN72	74/M	Tonsil	T2N0M0	Well
HN76	71/F	Floor of mouth	T3N2cM0	Poor
HN78	67/M	Oral tongue	T2N2cM0	Moderate
HN79	45/F	Tonsil	T2N1M0	Moderate
HN80	44/M	Larynx	T3N0M0	Moderate
HN84	59/M	Tongue base	T4N0M0	Poor

Abbreviations: M, male; F, female.

cells and the DAPI-stained dead cells were eliminated by gating. The specific flow gates for ALDH-positive cells were set using a control sample of the isolated tumor cells in which ALDH activity was inhibited with diethylaminobenzaldehyde (DEAB). Subsequent flow cytometry runs were used to identify populations of cells with high aldehyde dehydrogenase activity ($ALDH^{high}$) and those that express the surface marker CD44 (CD44+), whereas measurements of cell numbers were used to identify the percentage of each population present: $ALDH^{high}$, $ALDH^{low}$, CD44+, and CD44−. Overlap between these populations was assessed to identify populations of cells with the characteristics CD44+ $ALDH^{high}$, CD44+ $ALDH^{low}$, CD44- $ALDH^{high}$, and CD44− $ALDH^{low}$ in HNSCC.

HNSCC subpopulations of interest were collected based on their ALDH expression. Subpopulations of $ALDH^{high}$ and $ALDH^{low}$ cells were injected subcutaneously into NOD/SCID mice and evaluated for their tumorigenic potential. When sufficient numbers were available, the cells were serially diluted prior to injection. The cells were mixed with Matrigel Basement Membrane Matrix (BD Pharmingen) solution to form a final volume of 200 μL. Injection sites were sealed with a liquid skin adhesive. The animals were assessed for tumor growth.

The tumorgenicity of the injected cell populations was evaluated by evidence of tumor growth in NOD/SCID mice and by histology. When the number of cells allowed for serial dilutions to be injected, we determined the minimum number of cells required for each population to produce a tumor in the NOD/SCID mouse. The resultant tumors were assessed by flow cytometric and histologic analysis for tumor heterogeneity and proportion of cells with ALDH activity.

RESULTS

Six primary tumors were collected from subjects with HNSCC. Two tumors originated in the oral cavity (floor of mouth, tongue), 3 originated in the oropharynx (2 in the tonsil, 1 base of tongue), and 1 originated in the larynx. The subjects ranged in age from 45 to 74 years old. There were 3 moderately differentiated, 2 poorly differentiated, and 1 well-differentiated tumor (Table 1). Although most of the tumor samples were small, when sufficient numbers of cells were available costained flow cytometry sorts and injections of serially diluted cells were performed.

HNSCC can be separated into 2 subpopulations using the ALDEFLUOR substrate to

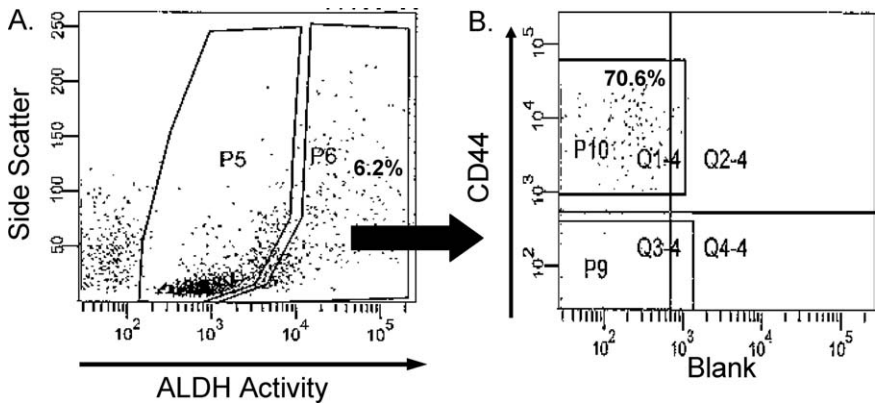

FIGURE 1. Head and neck squamous cell carcinoma (HNSCC) cells that have high levels of aldehyde dehydrogenase (ALDH) expression also express CD44 at high levels. (**A**) HN79 cells sorted for ALDH expression. P6 represents cells with high levels of ALDH expression ($ALDH^{high}$). (**B**) Only HN79 cells with elevated ALDH levels (P6) have been sorted for CD44 expression. The cells in P10 represent the population of cells with high ALDH expression that also express CD44. Approximately 70% of the ALDH high cells are also CD44+.

Table 2. Percentage of ALDH^{high} cells in the HNSCC specimens, percentage of CD44+ cells that are ALDH^{high}, and percentage of ALDH^{high} cells that are also CD44+.

Sample	Percentage ALDH^{high}	Percentage of ALDH^{high} cells that are CD44+	Percentage of CD44+ cells that are ALDH^{high}
HN72	1.0	70.2	23.60
HN76	1.8	74.4	16.49
HN78	1.3	50.6	13.40
HN79	6.2	72.9	N/A
HN80	7.8	69.9	N/A
HN84	2.6	N/A	9.86

Abbreviations: ALDH, aldehyde dehydogenase; HNSCC, head and neck squamous cell cancer, N/A, not applicable.
Note: Not all combinations are reported because of limitations related to cell numbers available for analysis.

determine ALDH activity and flow cytometry to sort and collect the cells. As expected, the majority of HNSCC cells had low ALDH activity. In these tumors, the proportion of ALDH^{high} cells had a mean of 3.5 ± 2.8% (1.0% to 7.8%).

The ALDH^{high} subpopulation overlaps significantly with the CD44+ population of cells. When the ALDH^{high} population was sorted for CD44, the majority of cells also expressed CD44, 50.6% to 74.4% (Figure 1 and Table 2). Conversely, when CD44+ cells were sorted for ALDH activity, only 9.8% to 23.6% of the CD44+ cells had high ALDH activity, indicating that a large proportion of ALDH^{high} cells are CD44+, but a much smaller proportion of CD44+ cells are ALDH^{high}.

ALDH^{high} HNSCC cells are highly tumorigenic compared with ALDH^{low} cells (Table 3). HNSCC cells with high ALDH activity produced tumors in the NOD/SCID mouse model in 24/45 injections (53%), whereas HNSCC cells with low ALDH activity resulted in tumors in only 3/37 injections ($p < .00001$, chi-square test). Tumors developed in 7/15 (47%) mice injected with as few as 50 to 100 ALDH^{high} HNSCC cells, whereas no tumors occurred in 0/14 injections of the same number of ALDH^{low} cells.

ALDH^{high} cells produce tumors that have a histology similar to that of the original tumor (see Figure 2). The ALDH^{high} cells can be passaged in the mouse model and reproduce the original tumor heterogeneity for ALDH activity—that is, the proportion of cells that are ALDH^{high} is similar to the proportion in the original tumor (see Figure 3).

DISCUSSION

The identification of highly tumorigenic subpopulations of cells—the CSCs—in solid tumors has significant implications regarding cancer biology, response to therapy, and the development of new cancer treatments. Therapies based on tumor regression may produce treatments effective against the majority of more differentiated cancer cells while sparing the CSC subpopulation.[8] The development of more effective cancer therapeutics will require the CSCs to be selectively targeted and eliminated. For this strategy to be successful, the CSC cells must be reliably identified and isolated so their characteristics can be studied.

CD44 is a cell surface marker that identifies a subpopulation of cancer cells from HNSCCs that are highly tumorigenic. However, the CD44 subpopulation of HNSCCs likely contains both non-CSCs and CSCs as shown by the need to

Table 3. Number of tumors resulting from implantations of ALSH^{high} and ALDH^{low} cells for each HNSCC specimen.

		No. of cells injected				
Sample	Population	5000+	1001–5000	501–1000	101–500	50–100
HN72	ALDH^{high}		1/2	0/1	1/1	2/3
	ALDH^{low}		0/1	0/2		0/4
HN76	ALDH^{high}	1/3		2/2		0/2
	ALDH^{low}	1/2				
HN78	ALDH^{high}		3/4	2/4		2/4
	ALDH^{low}		0/4	1/4		0/4
HN79	ALDH^{high}		1/1	1/1	1/1	2/4
	ALDH^{low}		0/1	0/2		0/4
HN80	ALDH^{high}		0/2	1/2	2/4	
	ALDH^{low}		0/3		0/2	
HN84	ALDH^{high}			1/2		1/2
	ALDH^{low}			1/2		0/2

Abbreviations: ALDH, aldehyde dehydogenase; HNSCC, head and neck squamous cell cancer.

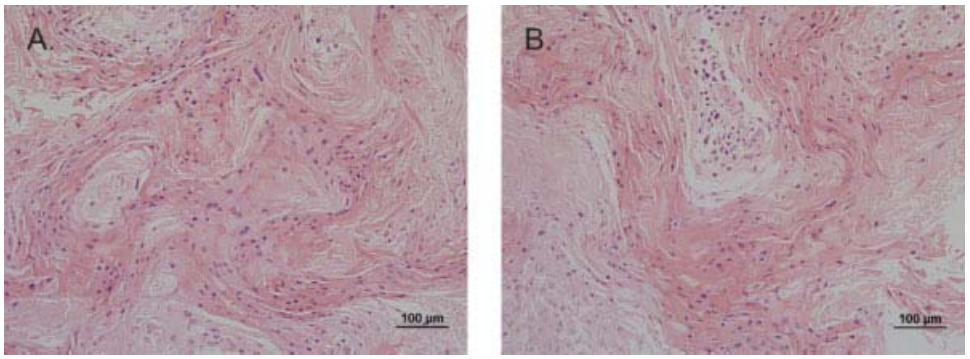

FIGURE 2. ALDH^{high} cells reproduce tumors that are histologically similar to the original tumor. (**A**) HN84 primary tumor (original magnification, ×20). (**B**) HN84 tumor resultant from ALDH^{high} injection (original magnification, ×20). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

inject in the order of 5×10^3 cells to produce a tumor in the animal model.[17] In other cancers, combinations of cell surface markers have been used successfully to isolate CSCs, and smaller numbers of the isolated cells have been shown to produce tumors in an animal model, suggesting this methodology is capable of isolating the CSCs more selectively. These findings indicated

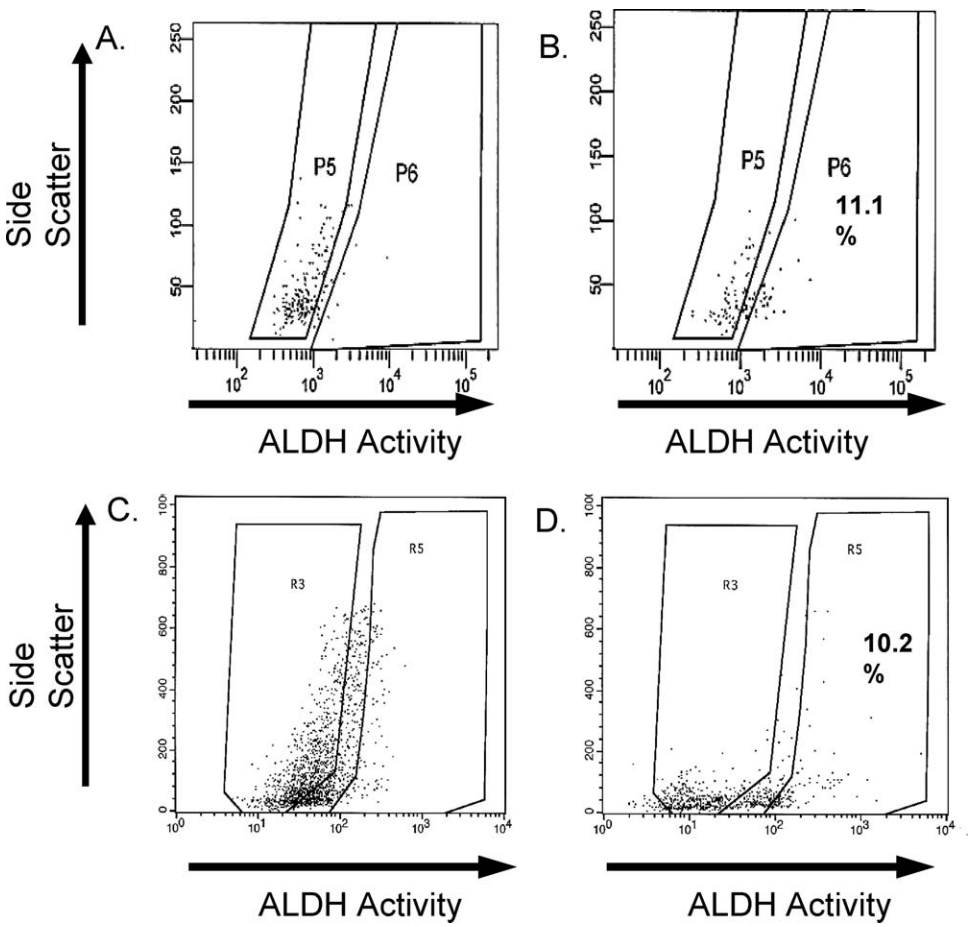

FIGURE 3. ALDH^{high} cells re-create the original tumor heterogeneity for ALDH expression and maintain the distribution of ALDH in cancer cells passaged in the animal model. (**A**) Primary HN84 cancer cells inhibited with diethylamino-benzaldehyde (DEAB). (**B**) Primary HN84 cancer cells sorted for ALDH expression: 11.1% of the cancer cells are ALDH^{high}. (**C**) Cancer cells derived from tumors grown in the animal model (passage 1) inhibited with DEAB. (**D**) Cancer cells sorted for ALDH expression from tumor grown in the animal model from HN84 ALDH^{high} exhibit the same heterogeneity for ALDH expression as the original tumor.

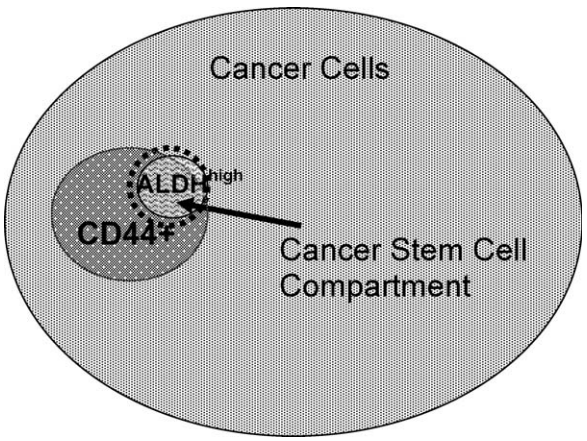

FIGURE 4. Proposed model for the cancer stem cell (CSC) compartment in HNSCC.

a need to identify a more selective single marker or combination of markers for CSCs in HNSCCs.

Wicha et al[26] recently demonstrated that ALDH expression can be used to identify breast CSCs and that injections of small numbers of these cells produce tumors in an animal model. Interestingly, the normal breast stem/progenitor cells also have high expression of ALDH, strongly supporting the concept that stem and progenitor cells are the targets of malignant transformation in breast cancer.[26]

We have shown here that ALDH expression isolates a subpopulation of cancer cells from HNSCCs that are highly tumorigenic. The ALDH-positive cells are able to produce tumors at a 10-fold reduction over that which was possible with CD44+ cells alone.[17] The tumors grown from ALDH^{high} cells re-create the original tumor heterogeneity and histology, and the ALDH^{high} cells can be passaged in the animal model, fulfilling the requirements for CSC phenotype. These data along with the recent report by Chen et al[27] strongly support the use of ALDH expression as a method to select CSCs in HNSCC.

As expected, the ALDH subpopulation of HNSCC cells mainly comprise a small subpopulation of the CD44+ cells, suggesting ALDH expression isolates a subset of CD44+ cells that contain the actual tumorigenic CSCs (see Figure 4). Thus we conclude that the tumorigenic cells have both phenotypic markers. The explanation as to why CD44 and ALDH are expressed by the stem cell population is not known, although one could speculate that the ability to oxidize retinoic acid, a proposed function of ALDH, is a requirement for stem cell activity. It is also not certain that both markers are always expressed on the tumorigenic stem cells because there appears to be a small fraction of ALDH-positive cells that are CD44−. However, the limited number of cells precluded a separate selection of these cells for testing, and our prior experiments had shown that even large numbers of CD44-negative cells are not tumorigenic. No doubt there are other phenotypic markers that are expressed by CSCs. For example, in breast cancer, CD24 is also a stem cell marker. In HNSCC, this does not appear to be the case; thus there seem to be tissue-specific stem cell markers as well as general stem cell markers. ALDH expression may represent a CSC that is generally applicable to all CSCs, although this remains to be proven.

The small numbers of CSCs obtained from HNSCC limit the critical experiments that must be carried out to understand the role of these cells in cancer persistence, recurrence, and resistance to therapy. Repeated recultivation in the NOD/SCID mouse or in vitro culture methods would allow for repeated experiments, and our future work will target these mechanisms.

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