Aldehyde Dehydrogenase in Combination with CD133 Defines Angiogenic Ovarian Cancer Stem Cells That Portend Poor Patient Survival

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Abstract

Markers that reliably identify cancer stem cells (CSC) in ovarian cancer could assist prognosis and improve strategies for therapy. CD133 is a reported marker of ovarian CSC. Aldehyde dehydrogenase (ALDH) activity is a reported CSC marker in several solid tumors, but it has not been studied in ovarian CSC. Here we report that dual positivity of CD133 and ALDH defines a compelling marker set in ovarian CSC. All human ovarian tumors and cell lines displayed ALDH activity. ALDH+ cells isolated from ovarian cancer cell lines were chemoresistant and preferentially grew tumors, compared with ALDH- cells, validating ALDH as a marker of ovarian CSC in cell lines. Notably, as few as 1,000 ALDH+ cells isolated directly from CD133+ human ovarian tumors were sufficient to generate tumors in immunocompromised mice, whereas 50,000 ALDH- cells were unable to initiate tumors. Using ALDH in combination with CD133 to analyze ovarian cancer cell lines, we observed even greater growth in the ALDH+/CD133+ cells compared with ALDH+ CD133- cells, suggesting a further enrichment of ovarian CSC in ALDH+/CD133+ cells. Strikingly, as few as 11 ALDH+/CD133+ cells isolated directly from human tumors were sufficient to initiate tumors in mice. Like other CSC, ovarian CSC exhibited increased angiogenic capacity compared with bulk tumor cells. Finally, the presence of ALDH+/CD133+ cells in debulked primary tumor specimens correlated with reduced disease-free and overall survival in ovarian cancer patients. Taken together, our findings define ALDH and CD133 as a functionally significant set of markers to identify ovarian CSCs. Cancer Res; 71(11); 3991–4001. ©2011 AACR.

Introduction

Some tumors follow a cancer stem cell (CSC) model, whereas others do not. In tumors that follow a CSC model, CSCs are believed to make up a limited percentage of the tumor cells, yet be the driving force behind cancer growth (1, 2). The presence of such cells could explain why cancer often relapses despite a complete clinical remission with initial therapy; with time a few residual treatment-resistant stem cells could repopulate the tumor.

Ovarian cancer is a tumor that commonly shows a complete remission in response to chemotherapy, yet the majority of patients relapse. This suggests that a CSC model may be relevant in ovarian cancer. Supporting the idea of ovarian CSCs, Zhang and colleagues used a primary human ovarian tumor specimen to generate in vitro tumor "spheroids" (3). One hundred isolated spheroid cells were capable of generating tumors in mice reminiscent of the primary tumor. Tumor spheroids had enriched expression of the stem cell markers CD117 and CD44, suggesting these were possible markers of ovarian CSC. CD133 has also been reported as a marker of ovarian CSC. Limited numbers of CD133+ cells from ovarian cancer cell lines generated large tumors more rapidly than CD133- cells, and CD133+ cells produced tumors in mice with CD133+ and CD133- cells (4). Furthermore, a second group reported that the CD133+ cell population in the primary human tumor xenografts in mice was primarily responsible for serial tumor passage (5). Unfortunately, CD133 is only expressed in ~40% of ovarian cancer cell lines and ~30% of primary ovarian tumors; thus, CD133 may not be a useful CSC marker for a majority of ovarian cancers (4). Finally, CD24 alone, and in combination with CD44 and EpCAM, was reported to mark cells with ovarian CSC activity in various cancer cell lines (6, 7). Interestingly, a proportion of these cells also expressed CD133.

Images
Despite these studies of ovarian CSC, several issues remain. To date, no study has isolated an ovarian CSC population directly from human tumors to initiate tumors in mice; all used cell lines, or stem cells isolated from primary cells following in vitro or in vivo passage. This limits the ability to isolate and characterize ovarian CSCs, without concerns for genetic and phenotypic changes associated with the passage of cells.

Recent studies in other solid tumors identified aldehyde dehydrogenase (ALDH) enzymatic activity as a potential marker for CSC. In a study of breast cancer, ALDH+ cells were present in a majority of tumors and capable of directly generating tumors in vivo (8). Independent studies in colon cancer suggest ALDH identifies colon CSCs; as few as 25 ALDH+ cells could generate tumors whereas ALDH− cells could not (9, 10). Similarly, ALDH has been proposed as a marker of CSC in leukemia, head and neck, lung, and pancreatic cancers (11–14). Interestingly, ALDH has been proposed, together with CD133, to identify CSC population in hepato- and pancreatic cancers (11–14). Interestingly, ALDH has been proposed, together with CD133, to identify CSC population in hepatocellular carcinoma (15). Based on the evidence for ALDH as a stem cell marker in solid tumors, we performed an extensive analysis of ALDH activity alone, and in combination with CD133, as a marker of ovarian CSC.

Materials and Methods

Tumor processing

Informed consent was obtained from all patients before tissue procurement. All studies were performed with the approval of the Institutional Review Board of the University of Michigan. All tumors were stage III or IV epithelial ovarian or primary peritoneal cancer. Tumors were mechanically dissected into single-cell suspensions and isolated on a ficoll gradient as previously described (16). For ascites, studies of cell pellets were collected by centrifugation, and red cells were lysed using ACK buffer, washed, passed through a 40-μm filter, then passed 4 times through a Standard Hub Pipetting needle to isolate single cells.

Sphere formation

Sphere culture was performed as previously described (8, 17). Briefly, FACS-isolated ALDH+/−/CD133+/− cell populations were plated in triplicate in ultra-low attachment plates in serum-free MEBM-2 (Lonza). Cells were plated at the indicated density and from 1,000 to 10,000 cells/mL in subsequent passage. Sphere formation was assessed 2 weeks after seeding the cells.

Flow cytometric analysis and fluorescence-activated cell sorting

Primary ovarian tumor/ascites or cell line single-cell suspensions were counted and incubated with primary antibodies (antibody information for all experiments provided in Supplementary Table S1). ALDH+ enzymatic activity was defined using the ALDEFLUOR kit per protocol (Stem Cell Technologies). For each sample, half of cell/substrate mixture was treated with 50 mmol/L diethylaminobenzaldehyde (DEAB). Cells were incubated for 45 minutes. Gating was established using propidium iodide (PI) exclusion for viability and ALDEFLUOR/DEAB treated cells were used to define negative gates. FACS was performed with ≥1 × 10⁶ cells using the BD FACS Canto II (Becton Dickinson) or FACSAria (Becton Dickinson) under low pressure in the absence of UV light.

Chemotherapy resistance of ALDH+ cells

1 × 10⁶ SKOV3 cells were plated in triplicate in DMEM-10% FBS overnight. Cells were treated with cisplatin (0.1–3 μg/mL; SICOR Pharmaceuticals, Inc.). After 72 hours cells were harvested and FACS analyzed with PI/ALDEFLUOR assay as described. Alternatively, ALDH+ and ALDH− SKOV3 cells were FACS isolated, allowed to recover for ~48 hours, counted, and then ~200,000 cells were plated in replicate. Cells were treated with 1.5 μg/mL of cisplatin or media alone for 72 hours. The number of viable cells was then determined using the Countess Automated Cell Counter (Invitrogen) 3, 7, and 14 days after cisplatin therapy and plotted as a percentage of initial cell input.

Human tumor xenotransplants

Animals were housed in the University of Michigan Unit for Laboratory Animal Medicine and protocols were approved under the University Committee on the Use and Care of Animals. Tumor cells were FACS isolated, resuspended (1:2) in PBS:Matrigel (BD Biosciences) and implanted s.c. into the axillas of nonobese diabetic/severe combined immunodeficient (NOD/SCID)/interleukin (IL)-2Rγ-null (NOG) mice (18–20). Animals were euthanized when the tumors were approximately 0.5 to 1.0 cm in the largest diameter unless otherwise indicated. Tumors were resected and weighed, and a portion of each tumor was snap frozen for histologic analysis. The remaining portion was processed into a single-cell suspension as described. Tumor volumes were calculated using the L × W × W/2 formula. Tumor weights were compared using a Student’s t test. Tumor growth curves were compared using ANOVA and a Student t test.

For in vivo serial passaging, single-cell suspensions of ALDH+ and ALDH− cell initiated tumors (SKOV3 1,000 cells and Hey-1 100 cells) were sorted into ALDH+ and ALDH− fractions and reinjected into mice (n = 4 for each sorted cell subtype). Four passages were performed. A2780, Ovcar8, and PEO4 cell lines were provided by Susan Murphy (Duke University, Durham, NC). All others were provided by Rebecca Liu (University of Michigan).

For passaging of human tumor xenografts, tumors were resected and processed as above. Five thousand ALDH+ or ALDH− cells were FACS isolated and then reimplanted into NOG mice as described. For ALDH+/−/CD133+ cell–derived tumors, ~1,000 to 2,000 cells for each ALDH+/−/CD133− population were reimplanted into NOG mice.

Immunohistochemistry and microvascular density assessment

Fresh tumors were harvested in linear growth phase (~500 mm²) embedded in OCT-Compound (Tissue-Tek) and snap frozen. Seven micrometers of cryosections were processed as previously described (16). Primary anti-mouse CD31 (1:600) and anti-mouse CD105 (1:200; Supplementary Table S1) were
incubated for 2 hours at 20°C. Slides were processed using the Envision system (Dako) per protocol. CD31 and CD105 immunohistochemistry (IHC) were performed on 7 to 11 independent sections of 4 independent ALDH+ or ALDH– cell–derived tumors (Hey-1, SKOV3), or ALDH ‘CD133–’, ALDH ‘CD133+’, ALDH ‘CD133’, or ALDH ‘CD133’ cell–derived tumors (A2780-DK). Microvascular density assessment was then performed as previously described (16). Images were captured on an Olympus BX41 fluorescent microscope with a 12 MB digital camera at 16-bit depth/300 dpi. Total stain area/low power field (∗100), as defined by pixel area (X Y: 1:1) and hue, was assessed using Olympus Microsuite Biological Suite software and compared between groups using a 2-sided Student t test.

**ALDH and CD133 expression in the ovarian tissue microarray**

Confirmed, formalin-fixed, paraffin-embedded ovarian epithelial tumors were obtained from the Department of Pathology, University of Michigan. Cores were obtained from the most viable/non-necrotic areas of the tumor. Each tumor had 3 to 7 independent cores. A tissue microarray (TMA) was constructed from 56 ovarian cancer patients, with staging surgery between 1995 and 2002 [7 (12.5%), 6 (10.7%), 37 (66.1%), and 3 to 7 independent cores. A tissue microarray (TMA) was constructed from 56 ovarian cancer patients, with staging surgery between 1995 and 2002 [7 (12.5%), 6 (10.7%), 37 (66.1%), and 6 (10.7%) patients with stage I–IV disease, respectively], median age 58 years (minimum: 30; maximum: 84).

TMA sections were subjected to microwave epitope retrieval in 1 mmol/L EDTA, pH8, washed, and blocked with Background-Stopper (Biocare Medical) for 30 minutes. Primary antibody [anti-Aldehyde dehydrogenase (1:100) and anti-CD133 (1:100)] was incubated overnight at 4°C. Slides were then washed and incubated with goat anti-rabbit IgG (1:200) and goat anti-mouse IgG (1:200) in background-sniper for 60 minutes at 20°C. The slides were washed, stained with 4',6-diamino-2-phenylindole, and mounted (ProLong Gold, Molecular probes). Monochrome images of each TMA core were captured with an Olympus BX51 microscope at 3 different extinction/emission wavelengths (DAPI, AF488, and AF648), artificially colored and combined to form images.

Tumors were scored as ALDH+ or ALDH–, ALDH ‘CD133–’, ALDH ‘CD133+’, or ALDH ‘CD133’. Only cells associated with tumor islets were scored; stromal and vascular staining was not evaluated. The product limit method of Kaplan and Meier was used to estimate overall and recurrence-free survival. Follow-up time was calculated from the date of diagnosis (staging surgery) until the date of death or first disease recurrence for the end points, respectively. Patients not reaching end points were censored on their last known follow-up date. Comparisons in the estimated overall or recurrence-free survival by CD133 and ALDH expression groups were conducted using the log-rank test statistic. P values ≤ 5% suggest meaningful differences between groups.

**Results**

**Characterization of ALDH activity in human ovarian tumors and ovarian tumor cell lines**

Previous studies reported that CD117/CD44, CD133, CD90, CD24, CD44, and ALDH may identify CSCs in ovarian or other solid tumors (3–5, 8, 21–28). We analyzed more than 13 primary human ovarian tumors and 5 ascites specimens for these tumor markers. ALDH was the only marker detectable in all primary tumor and ascites (Table 1; representative gating provided in Supplementary Fig. S1). CD133 expression was detected in 9 of 13 primary tumors. Higher levels of CD133+ cells were present in ovarian tumor ascites. CD117 was present in only 5 of 11 tumors tested. In contrast, CD44 was expressed at very high levels in all of the tumors tested. CD24 and CD90 showed variable expression.

We also characterized the expression of these CSC markers in human ovarian cancer cell lines. Once again ALDH was the only marker present in all cell lines in a limited subpopulation of cells (Table 1). At least one cell line expressed very high levels (≥40%) of each of the other putative stem cell markers. This may be due to the fact that cell lines may have increased "stemness" associated with in vitro growth selection and passage necessary for the generation of cell lines.

**ALDH activity identifies CSC like cells in ovarian tumor cell lines**

From our studies, ALDH was the only potential stem cell marker expressed in all primary tumor specimens and detected in limited cellular subpopulations of human primary tumor cells (0.25%–7.78%, Table 1). This suggests that ALDH may be a useful CSC marker in ovarian cancer. One of the proposed characteristics of CSCs is chemoresistance. We therefore assayed the percentage of ALDH+ cells after treatment with increasing doses of cisplatin. Although there was a clear, dose-dependent decrease in the total number of viable cells, we observed a significant increase in the percentage of ALDH+ cells (Fig. 1Aii), suggesting chemoresistance of ALDH+ cells and/or the induction of ALDH by cisplatin. We next treated FACS-isolated ALDH+ and ALDH– SKOV3 cells with PBS or cisplatin. We observed no differences in the growth of PBS-treated ALDH+ or ALDH– cells (data not shown). Cisplatin treatment induced cell death in both ALDH+ and ALDH– cell populations. However, ALDH+ cells showed greater viability and recovered from cisplatin treatment more rapidly than ALDH– cells, suggesting relative chemoresistance in ALDH+ versus ALDH– cells (Fig. 1Aii).

A primary characteristic of CSC is the ability to initiate tumors with limited numbers of cells. We next tested the cancer-initiating capability of ALDH+ cells in mice. In 3 cell lines tested [SKOV3, HEY1 (Fig. 1B), and OVCA8 (data not shown)], ALDH+ cells generated larger tumors at a significantly faster rate than equal numbers of ALDH– cells. As few as 100 ALDH+ SKOV3 or HEY1 cells formed tumors, whereas similar numbers of ALDH– cells did not, or rarely formed tumors, respectively (Table 2). FACS analysis of 1,000 ALDH+ versus ALDH– cell–derived tumors showed that tumors derived from ALDH+ cells harbored ALDH+ and ALDH– cells in the same ratios as observed in the original tumor cell line. Tumors derived from 1,000 ALDH+ cells had 10– to 20-fold reduction in the number of ALDH+ cells (Fig. 1C). Both SKOV3 and HEY1 ALDH+ cells successfully generated tumors over 4 consecutive serial passages, whereas ALDH– tumors were only able to passage twice.

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Finally, CSCs are reported to have increased angiogenic capacity (29, 30). Immunohistochemical analysis of the ALDH⁺-derived tumors, compared to ALDH⁻/CD0-derived tumors, showed significantly (2-fold) increased microvascular density. This was true for either the pan-endothelial marker CD31 or the angiogenic endothelial marker CD105 (31) (Fig 1D). FACS analysis of ALDH⁺ and ALDH⁻/CD0 cell-derived tumors revealed a similar increase in the numbers of CD31⁺ and CD105⁺ cells (data not shown). Taken together our data in ovarian cancer cell lines suggest ALDH identifies a population of cells enriched for a CSC phenotype; ALDH⁺ cells are chemoresistant, angiogenic cells with significant tumor-initiating capacity.

ALDH⁺ cells from human ovarian cancers initiate tumors in mice

Given our findings in cell lines, we tested the ability of ALDH⁺ cells isolated from human tumors to generate tumors in mice; the ultimate proof of a putative human CSC. Although 50,000 ALDH⁺ cells did not grow tumors, 1,000 ALDH⁺ cells generated tumors from 2 of 9 primary tumors (Table 2). Tumor generation required approximately ~9 months. Histological analysis of the xenograft tumors revealed well-differentiated papillary-serous tumors identical to that seen in the primary tumor (Fig 2A). FACS analysis of tumor xenografts revealed robust ALDH⁺ and ALDH⁻ cell populations (Fig 2C). Similar to cell line studies ALDH⁺ cell–derived tumors were highly vascular (Fig 2B). We have successfully passaged ALDH⁺ cells from the Pt118 tumor 3 times. These data suggest ALDH activity identifies a population of ovarian cancer cells enriched for CSC activity.

Tumorigenicity of cell lines coexpressing ALDH and CD133

In the hematopoietic system, ALDH used in combination with CD133 improves the identification of stem cells. We next examined the role of ALDH in combination with CD133 in ovarian tumors. FACS confirmed the presence of rare ALDH⁺CD133⁺ cells in several cell lines (Supplementary Table S2). Using limiting dilution studies of A2780 cells we observed similar tumor initiation capacity of both ALDH⁺CD133⁺ and ALDH⁻CD133⁺ cells. In contrast, ALDH⁺CD133⁻ and ALDH⁻CD133⁻ cells had limited tumor initiation capacity at the lowest cell numbers (Table 3). Interestingly, the ALDH⁺CD133⁻ cells generated larger tumors faster than ALDH⁻CD133⁻ cells, and ALDH⁺CD133⁻ cell derived tumors were significantly larger than ALDH⁺CD133⁺ and ALDH⁻CD133⁻ cell tumors (Fig 3A). ALDH⁺CD133⁺ and ALDH⁻CD133⁻ cell tumors both showed increased microvascular density.

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Abbreviation: ND, not done.
Figure 1. *In vitro* and *in vivo* outgrowth of ALDH⁺ cells from human ovarian cancer cell lines. Ai, absolute cell number and %ALDH⁺ SKOV3 cells following treatment with indicated concentrations of cisplatin. Aii, percent viable FACS sorted ALDH⁻/C₀ and ALDH⁺ cells prior to and 3, 7, or 14 days following treatment with 1.5 µg/mL cisplatin. Bi and Bii, tumor growth curves of 100 FACS-isolated ALDH⁺ and ALDH⁻/C₀ SKOV3 and Hey1 cells. Ci and Cii, ALDEFLUOR staining of ALDH⁺ and ALDH⁻/C₀ SKOV3 and Hey1 tumor xenografts with DEAB controls. D, CD105 IHC from Hey-1 ALDH⁻ and ALDH⁺ xenografts tumors. E, quantification of CD31⁺ and CD105⁺ microvascular density in the indicated tumor xenografts. Scale bar indicates 100 µm. All data are representative of at least 2 independent experiments (n = 5 tumors/experiment). *, P < 0.01 versus controls.
density compared to ALDH\(^+\)CD133\(^-\) and ALDH\(^-\)CD133\(^+\) tumors (Figs. 3B and S2). FACS analysis of both ALDH\(^-\)CD133\(^+\) and ALDH\(^+\)CD133\(^+\) cell–derived tumors recapitulated the cellular populations observed in the primary cell line giving rise to all 4 CD133\(^+/\)ALDH\(^+/\) cell populations. In contrast, ALDH\(^+\)CD133\(^-\) or ALDH\(^-\)CD133\(^+\) cell xenografts had little or no capacity to generate CD133\(^+\) cells (Fig. 3C). This observation indicates that ALDH\(^+\)CD133\(^-\) cells and ALDH\(^-\)CD133\(^+\) cells must be downstream of CD133\(^+\) expressing cells in a differentiation pathway. Similar results were obtained with the OVCAR-8 cell line (data not shown). These studies suggest a potential hierarchy of angiogenic ovarian cancer stem/progenitor cells based on ALDH activity and CD133 expression.

ALDH\(^+\)CD133\(^+\) cells from human ovarian cancers have a CSC phenotype and have an increased ability to initiate tumors in mice

Given these findings, we assessed if the combination of ALDH activity and CD133 expression in primary human tumors could improve the isolation of ovarian CSC. Analysis of CD133\(^+\) primary human ovarian tumors showed a small percentage of cells that coexpressed ALDH activity and CD133 (0.1%–0.01%, Supplementary Table S3). We used the tumor sphere assay to analyze the phenotype of the primary ALDH\(^+\)/CD133\(^+\) cell populations from 8 patients. When 5,000 cells were analyzed from 6 of 8 patients we observed significantly greater numbers of spheres in the ALDH\(^+\)CD133\(^+\) cell population (Figs. 3D and S3). In the other 2 patient samples we observed preferential sphere formation in the ALDH\(^+\)CD133\(^-\) cells and the CD133\(^+\)ALDH\(^+\) cells respectively (Supplementary Fig. S3). ALDH-CD133\(^-\) negative cells were only able to generate spheres when 100,000 cells were assayed. These spheres were unable to passage suggesting these were adhesive cell clusters rather than true spheres.

We next tested the in vivo cancer-initiating ability of ALDH\(^+\)/CD133\(^+/\) cells from primary human ovarian tumors. From 9 primary tumors tested, we were able to successfully generate tumors from 11, 500, 500, and 500 ALDH\(^+\)CD133\(^+\) cells from 4 patients (Pt 32, Pt55, Pt94, and Pt171, respectively; Table 3). All 4 of these patient samples showed the greatest tumor sphere generation in the ALDH\(^+\)CD133\(^+\) cell populations (Supplementary Fig. S3). Tumor growth required 4 to 7 months. Tumors were not obtained from similar numbers of ALDH\(^-\)CD133\(^+\) cells from primary human ovarian tumors have a CSC phenotype and have an increased ability to initiate tumors in mice

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ALDH and CD133 Define Ovarian Cancer Stem Cells

Table 3. Tumor-initiating capacity of limiting dilutions of ALDH+/−/CD133+/− cells from the A2780 ovarian cancer cell line and 9 primary human ovarian tumors

<table>
<thead>
<tr>
<th>Cell type</th>
<th>A2780 cell line</th>
<th>No. of cells injected</th>
<th>Tumors formed</th>
<th>Primary human tumor samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>4/6</td>
<td>0/5</td>
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<tr>
<td></td>
<td></td>
<td>100</td>
<td>5/5</td>
<td>3/6</td>
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<tr>
<td></td>
<td></td>
<td>1,000</td>
<td>10/10</td>
<td>10/10</td>
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<tr>
<td></td>
<td></td>
<td>10−500</td>
<td>0/9</td>
<td>4/9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5,000</td>
<td>ND</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50,000</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not done.

Consistent with the CSC hypothesis, (i) ALDH activity is present in a small percentage of ovarian tumor cells; (ii) small numbers of ALDH+ cells and ALDH+CD133+ cells are capable of tumor initiation and propagation; and (iii) these cells generate tumors that recapitulate the original tumor cell composition. In addition, these cells show resistance to chemotherapy and increased angiogenic capacity. Importantly, ALDH+ cells were identified in all tumor cell lines and primary human tumor samples analyzed. Although CD133 appears to be in an important ovarian CSC marker, it is undetectable in approximately half of ovarian cancers. ALDH offers a means to isolate ovarian CSC in CD133− tumors.

This study is the first in ovarian cancer to successfully generate in vivo tumors from a putative stem cell population directly isolated from human tumors. Other studies of ovarian CSC investigated cell lines, used in vitro culture prior to isolation and growth in vivo, or injected total tumor cell suspensions for growth in vivo followed by isolation of stem cells from primary xenografts (4, 5, 32, 33). Although representing important tools, these manipulations could lead to genetic/phenotypic changes in the CSC population.

Using ALDH and CD133 to define a hierarchy of ovarian CSC

In CD133− tumors, the combination of ALDH and CD133 appears to enhance ovarian CSC isolation. As few as 11 primary human ALDH+CD133+ cells generated tumors in vivo. In addition, we observed an increased tumor engraftment rate of ALDH+CD133− compared with ALDH+CD133− cells. ALDH+CD133+ cell–derived tumors, in both patient samples and cell lines, were able to generate all ALDH+/−/CD133+/− cellular populations. In contrast, ALDH+CD133− cell–derived tumors from both patients and cell lines did not generate CD133+ cells. This implies that CD133+ cells are “upstream” of CD133− cells in a potential differentiation pathway.

Taking all of our observations together we hypothesize a hierarchical model of ovarian CSC differentiation (Fig. 4C). In this model, ALDH+CD133+ cells with self-renewal capacity give rise to ALDH+CD133− cells with limited self-renewal capacity.

Discussion

ALDH as a marker of ovarian CSC

We present here a thorough analysis of ALDH activity, alone and in combination with CD133, as an ovarian CSC marker. The presence of ALDH+CD133+ cells predicts clinical outcome.

Because ALDH+CD133+ cells showed greater sphere forming potential, greater engraftment potential, and more rapid tumor generation than ALDH−CD133− tumors, we speculated that ALDH+CD133+ cells may identify a more aggressive tumor phenotype. We therefore scored a panel of 56 ovarian tumors for the presence of ALDH and CD133 expressing cells using immunofluorescence. Tumors were scored as (i) ALDH−CD133− (no ALDH or CD133 expression detectable in tumor islets); (ii) ALDH+CD133− (ALDH expression but no CD133 expression in tumor islets); (iii) ALDH+CD133+ (any tumor with more than 1 ALDH+CD133+ cell/section); or (iv) ALDH−CD133+ (any tumor without ALDH+CD133+ cells in the tumor islet, and at least 1 ALDH−CD133− tumor cell; Fig. 4A). Stromal and vascular staining were excluded. Although we did not observe any correlation with tumor stage, grade, or platinum resistance, Kaplan–Meier analysis showed that patients with ALDH+CD133+ tumor cells have worse progression-free and overall survival compared to the other groups individually or combined (Fig. 4B).

cells, 5,000 ALDH+CD133−, or 50,000 ALDH−CD133− cells isolated simultaneously from the same tumor samples.

Histological analysis of the xenografts revealed highly vascular, high-grade papillary-serous carcinomas which mirrored that of the primary tumor (Fig. 3E and data not shown). FACS analysis of these tumors revealed that ALDH+CD133− cells were able to generate tumors with ALDH+/−/CD133+/− cells (Fig. 3F). ALDH+CD133− cell–derived tumors from 2 patients’ samples have successfully passed 4 times. A third is currently in first passage. These studies indicate that in CD133+ human ovarian tumors, CSCs may be identified by the dual expression of ALDH and CD133.
Figure 3. Characterization of ALDH+CD133+ cells as CSC in ovarian tumor cell lines and primary human epithelial ovarian tumors. A, tumor weights from 1,000 cells of the indicated FACS-isolated A2780 tumor cell populations (n = 5/group). B, quantification of CD31+ and CD105+ microvascular density (vessels/low power field) of the indicated A2780 cell line populations. C, FACS analysis of indicated A2780 xenografts for expression ALDH, and CD133 in ALDH+ gate. Di, Spheres generated from FACS-isolated ALDH+CD133+ cells from a primary ovarian tumor sample. Cell numbers plated at the initiation of the sphere assay are indicated. Scale bar (lower left) indicates 100 μm. Dii, average sphere formation in the indicated cell population from 8 different patients. E, histology of Pt94 primary tumor and tumor xenograft generated from ALDH+CD133+ isolated from Pt94. F, FACS analysis of Pt 94 tumor; left shows DEAB control, middle shows ALDEFLUOR activity in Pt 94 xenograft, and right shows histogram for CD133 with isotype control. *, P < 0.05 compared to ALDH+CD133+ and ALDH-/CD133- groups. **, P < 0.05 versus all other groups.
and ultimately give rise to the differentiated ALDH⁺/CD133⁻ cells. We propose that ovarian CSC can arise from any of the cellular populations with self-renewal capacity. Given that we observed some tumor generation in the CD133⁻/ALDH⁺ cellular compartment of cell lines, we postulate that there may be an ovarian CSC/progenitor, which lacks appreciable expression of either CD133 or ALDH. Alternatively, this may be an artifact of tumor cell lines, or due to trace ALDH⁺/CD133⁻ cellular contamination of the ALDH⁺/CD133⁻ cells.

The combination of ALDH and CD133 to enhance stem cell identification is parallel to the hematopoietic system (34–39). Our model is consistent with hematologic malignancies where partially differentiated progenitor cells with self-renewal capacity can manifest stem cell properties (40–42). Such a model accounts for the variability observed in patients and ovarian cancer cell lines. The expression of other reported ovarian CSC markers, including CD24, CD44, and CD117 in relation to ALDH and CD133 remains to be determined. It seems likely, given the heterogeneity of ovarian tumors, that there may be other distinct stem cell populations.

Based on our model we would predict that tumors derived from early stem cells (i.e., ALDH⁺/CD133⁺ cells) would portend a poorer prognosis, whereas tumors driven by stem cells derived from more differentiated cells (ALDH⁻/CD133⁻) would have a better outcome. In our study, although the presence of ALDH⁺/CD133⁺ cells did not correlate with tumor grade, the presence of ALDH⁻/CD133⁻ cells strongly correlated with poor outcome. Consistent with our model, increased ALDH expression in ovarian tumor islets has been associated with improved prognosis (43). However, the role of ALDH alone...
as a prognostic factor is controversial with a new report suggesting increased ALDH expression indicates poor prognosis in ovarian cancer (44). It is possible that stratification of ALDH expression based on CD133 coexpression could explain this discrepancy.

**ALDH CD133** in ovarian cancer

The role of ALDH CD133 cells remains uncertain. In primary specimens, ALDH CD133 cells did not generate significant numbers of spheres and did not generate tumors. In cell lines ALDH CD133 cells grew more rapidly than ALDH CD133 cells, and had similar tumor-initiating capacity as the ALDH CD133 cells at low cellular concentrations. ALDH CD133 cell–derived tumors generated all ALDH CD133–/– CD133 +/− cellular populations. Insensitivity of the ALDEFLUOR assay and conservative ALDH + gating may allow for ALDH “dim” cells within the ALDH CD133 + population of cells and may explain these observations. However, ALDH CD133 + cells were clearly observed with immunofluorescent analysis. It is possible that ALDH CD133 cells isolated from human tumors may not tolerate the stressful conditions associated with FACS isolation. Finally, ALDH CD133 + cells isolated from whole tumor cellular suspensions may be predominantly nontumor cells such as endothelial progenitors, diluting the number of ALDH CD133 + tumor cells. Cell line studies would suggest a close relationship between ALDH CD133 + and ALDH CD133− cells. Further studies are necessary to clarify the role of ALDH CD133− cells.

**Improving ovarian CSC growth in vivo**

Our study is the first to show the generation of tumor xenografts from CSC directly isolated from primary ovarian carcinoma. However, the tumor generation rate for cells isolated from primary human ovarian tumors was low (20–40%). This could be secondary to FACS induced cellular toxicity. Alternatively, the s.c. tumor inoculation may be an inferior location for tumor growth; only 50% of glioblastoma stem cells engrafted when implanted s.c., whereas 100% engraftment was obtained when these cells were orthotopically implanted into the cranium (45). Similarly, increased tumorigenesis was obtained when pancreatic CSCs were injected into the pancreas as opposed to an s.c. site (46). Finally, use of a humanized microenvironment significantly enhanced engraftment of breast CSC (27). Thus, future studies using orthotopic CSC injection or a humanized microenvironment for ovarian CSC may increase engraftment rates.

**Conclusions**

Collectively, our data indicate that ALDH is a marker of CSC in some ovarian tumors that lack CD133 expression. In tumors that express CD133, the combination of ALDH and CD133 can be used to identify a more aggressive CSC suggesting a potential hierarchy of cells with distinct cancer growth potential. Based on this we propose a model of ovarian CSC differentiation similar to that observed for hematopoietic cells.

**Disclosure of Potential Conflict of Interest**

The authors have no conflicts of interest to report.

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**References**

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Aldehyde Dehydrogenase in Combination with CD133 Defines Angiogenic Ovarian Cancer Stem Cells That Portend Poor Patient Survival

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