Aldehyde Dehydrogenase Discriminates the CD133 Liver Cancer Stem Cell Populations

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Abstract

Recent efforts in our study of cancer stem cells (CSC) in hepatocellular carcinoma (HCC) have led to the identification of CD133 as a prominent HCC CSC marker. Findings were based on experiments done on cell lines and xenograft tumors where expression of CD133 was detected at levels as high as 65%. Based on the CSC theory, CSCs are believed to represent only a minority number of the tumor mass. This is indicative that our previously characterized CD133$^+$ HCC CSC population is still heterogeneous, consisting of perhaps subsets of cells with differing tumorigenic potential. We hypothesized that it is possible to further enrich the CSC population by means of additional differentially expressed markers. Using a two-dimensional PAGE approach, we compared protein profiles between CD133$^+$ and CD133$^-$ subpopulations isolated from Huh7 and PLC8024 and identified aldehyde dehydrogenase 1A1 as one of the proteins that are preferentially expressed in the CD133$^+$ subfraction. Analysis of the expression of several different ALDH isoforms and ALDH enzymatic activity in liver cell lines found ALDH to be positively correlated with CD133 expression. Dual-color flow cytometry analysis found the majority of ALDH$^+$ to be CD133$^+$, yet not all CD133$^+$ HCC cells were ALDH$^+$. Subsequent studies on purified subpopulations found CD133$^+$ALDH$^+$ cells to be significantly more tumorigenic than their CD133$^-$ALDH$^+$ or CD133$^-$ALDH$^-$ counterparts, both *in vitro* and *in vivo*. These data, combined with those from our previous work, reveal the existence of a hierarchical organization in HCC bearing tumorigenic potential in the order of CD133$^+$ALDH$^+$ > CD133$^+$ALDH$^-$ > CD133$^-$ALDH$^-$. ALDH, expressed along CD133, can more specifically characterize the tumorigenic liver CSC population.

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Introduction

In the last several years, accumulating evidence has been lending support to the idea that human cancer can be considered a stem cell disorder. According to the hierarchical "stem cell model of carcinogenesis," tumors are not to be viewed as simple monoclonal expansions of transformed cells, but rather as complex tissues where abnormal growth is driven by a rare cancer stem cell (CSC) pool that has not only acquired tumor-like features such as uncontrolled growth but also maintains its innate capacity to self-renew and differentiate into a phenotypically heterogeneous progeny (1). CSCs, like normal stem cells, are also generally believed to possess unique survival mechanisms. And in view of their stem like properties, these cells are believed to be responsible for the maintenance and propagation of the entire tumor. Therefore, to completely eradicate a tumor and prevent recurrence, it is imperative that CSCs be specifically targeted. To date, CSCs have been identified as the driving force behind tumor formation in several tumor types including those of leukemia (2), cancers of the breast (3), brain (4), and prostate (5), and more recently, cancers of the colon (6) and liver (7-10).

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide, affecting 1 million individuals annually. Despite extensive research efforts, there is little understanding of the characterization of the specific group of cells that are the target of tumorigenic transformation. Recently, we and others have identified, characterized, and isolated a CD133$^+$ CSC-like subpopulation from human HCC cell lines and xenograft tumors. Studies in our laboratory found that CD133$^+$ HCC cells not only possess a greater colony-forming efficiency, higher proliferative output, and greater ability to form tumor *in vivo* but are also endowed with characteristics similar to those of stem/progenitor cells including the preferential expression of "stemness" genes, the ability to self-renew, and the ability to differentiate into non–hepatocyte-like, angiomyogenic-like lineages. CD133 expression is found to represent only a minority of the tumor cell population in human HCC specimens (8-10). In addition, CD133$^+$ cells are also more resistant to conventional chemotherapeutic drugs such as doxorubicin and 5-fluorouracil, the underlying mechanism of which required the preferential activation of the Akt/protein kinase B and Bcl-2 survival pathways (11).

Our recent findings were based on experiments done primarily on HCC cell lines Huh7 and PLC8024 or xenograft

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tumors derived from $CD133^+$ (isolated from Huh7) cells, where expression of CD133 was detected at levels as high as 65%. Yet, based on the CSC theory, it is believed that CSCs only represent a minority number of the entire tumor mass (<5% of the total tumor mass), and if true, may be indicative that the $CD133^+$ HCC CSC population is still heterogeneous, consisting of perhaps subsets of cells with differing tumorigenic potential. In fact, CSCs in other cancer types have also been characterized by more than one marker [e.g., $CD44^+CD24^{-/low}$ in breast (3), $CD44^+\alpha_2\beta_1^{hi}CD133^+$ in prostate (5), and $CD133^+CD166^+$ in colon (6)]. For this reason, identification of other markers, expressed along with CD133, with the goal of better characterizing the CSC population in HCC, is much needed and was our chosen focus in this present study. Using a proteomic approach, differential profiles of $CD133^+$ and $CD133^-$ cells isolated from HCC cell line Huh7 were analyzed by two-dimensional PAGE. Candidate protein spots were selected for identification by mass spectrometry (MS) analysis followed by the study of their potential correlation to $CD133^+$ cells and their prospective role as liver CSC markers. Our results reveal, for the first time, that aldehyde dehydrogenase (ALDH), expressed along CD133, can more specifically characterize the liver CSC population.

Results

Identification of Differentially Expressed Proteins in $CD133^+$ and $CD133^-$ Subpopulations Sorted from the Huh7 HCC Cell Line by Two-Dimensional PAGE

To identify candidate genes differentially expressed in $CD133^+$ and $CD133^-$ subpopulations, two-dimensional PAGE analysis was done to compare protein expression profiles between the two groups of cells sorted from the HCC cell line Huh7. Nearly 300 protein spots were resolved in each two-dimensional gel, with the majority of the spots matching between the two gels of $CD133^+$ and its corresponding $CD133^-$ counterparts (data not shown). Those spots that were significantly differentially expressed were selected for further identification by MS analysis. A total of 10 spots, all found to be up-regulated in the $CD133^+$ subpopulation, were selected (Fig. 1; Table 1). Following MS/MS analysis, the matching score of all 10 selected proteins were found to be statistically significant, indicating identity or extensive homology ($P < 0.05$) to their proposed protein identity. Of these, aldehyde dehydrogenase 1A1 (ALDH1A1) was chosen for further study.

Identification of ALDH1A1 as Being Preferentially Expressed in the $CD133^+$ Subpopulation

To confirm the preferential expression of ALDH1A1 found predominantly in the $CD133^+$ subpopulation as detected by two-dimensional PAGE analysis, both quantitative PCR (Fig. 2A, *first two column sets*) and Western blot analyses (Fig. 2B) were carried out to confirm the results at both the genomic and proteomic levels, respectively. In accordance with our two-dimensional PAGE results, ALDH1A1 was found predominantly in the $CD133^+$ subpopulation in cells isolated from both PLC8024 and Huh7 HCC cell lines.

ALDH1A1 Expression and Activity Positively Correlated with CD133 Expression in Liver Cell Lines

To determine whether ALDH1A1 is implicated in hepatocarcinogenesis or whether it is correlated with CD133 expression, we proceeded with examining the expression of ALDH1A1 in a panel of liver cell lines, including hepatoblastoma cell line HepG2 and HCC cell lines Huh7 (12), H2P, H2M (13), PLC8024, Hep3B, 7701, 7703, and 7402, by quantitative PCR analysis. The trend of ALDH1A1 expression correlated with expression of CD133 in that those cell lines that expressed little CD133, like HepG2 and H2P, correspondingly expressed fewer ALDH1A1, and those cell lines that expressed high levels of CD133, such as Huh7, PLC8024, and Hep3B, also correspondingly expressed high levels of ALDH1A1 (Fig. 2C). Subsequent studies for ALDH activity in liver cell lines by the novel ALDEFLUOR technique were consistent with our quantitative PCR data, showing a direct correlation between ALDH activity and CD133 expression (Fig. 2D; Table 2). Because the substrate used for the ALDEFLUOR assay is known to be processed by ALDH1A1, as well as by other ALDH genes in the superfamily, we also went on to examine by quantitative PCR whether other ALDH isoforms (ALDH1A2, ALDH1L1, ALDH2, ALDH3A1, and ALDH9A1) were also differentially expressed in $CD133^+$ and $CD133^-$ cells sorted from HCC cell lines Huh7 and PLC8024 (Fig. 2A). Selection of which ALDH isoforms to test was based on its relevance to cancer, drug resistance, or its expression in human or in the liver. Results indicate that isoforms ALDH1A2 and ALDH3A1 also seem to be preferentially expressed in the $CD133^+$ subfractions. Because we cannot rule out that cells sorted by labeling with the ALDEFLUOR assay exclusively contain $ALDH1A1^{+/-}$ subpopulations only, we chose to address this population as the more general ALDH, rather than ALDH1A1.

The Majority of $ALDH^+$ Cells Were Found to Express CD133, but on the Contrary, Not All $CD133^+$ Cells Are $ALDH^+$

To determine the immunophenotype of $ALDH^+$ HCC cells in liver cell lines, we conducted dual-color flow cytometry analysis for both CD133 expression and ALDH activity. As evident in Fig. 3, the majority of $ALDH^+$ cells gated in R2 (the entire $ALDH^+$ population) were $CD133^+$. It is also interesting to note that a subfraction of $CD133^+$ cells were indeed $ALDH^-$, which indicated that ALDH activity may possibly be used to further enrich the $CD133^+$ subpopulation and can potentially be used to better characterize the representative liver CSC population. Table 3 summarizes the expression of $CD133^+ALDH^+$, $CD133^+ALDH^-$, $CD133^-ALDH^+$, and $CD133^-ALDH^-$ in liver cell lines that were found to express ALDH, including HepG2, Huh7, PLC8024, Hep3B, and H2M, when dually stained with CD133-phycoerythrin and for ALDH activity.

$CD133^+ALDH^+$ HCC Cells Possess Higher Tumorigenic Ability Both In vitro *and* In vivo *When Compared with $CD133^+ALDH^-$ or $CD133^-ALDH^-$ HCC Cells*

To determine the tumorigenic potential of different subpopulations of cells dually stained for CD133 expression

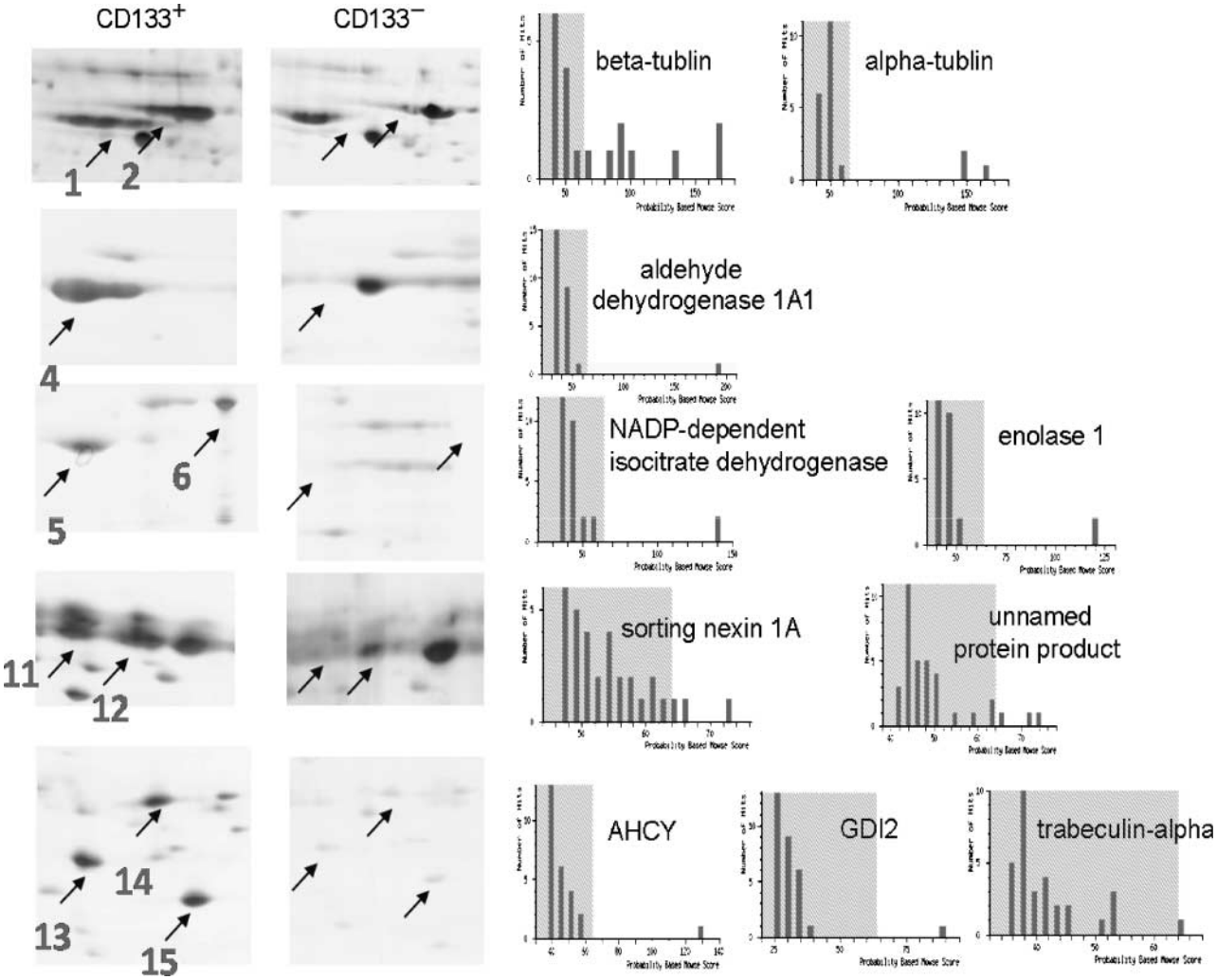

FIGURE 1. Two-dimensional PAGE protein expression profiles of $CD133^+$ and $CD133^-$ cells sorted from the Huh7 HCC cell line. The 10 individual spots selected for protein identification by MS are enlarged. Right, output of database search by the Mascot program using MS/MS data. The scores for each individual protein spot were significant, indicating identity or extensive homology ($P < 0.05$) to their characterized protein as listed in Table 1.

and ALDH activity, $CD133^+ALDH^+$, $CD133^+ALDH^-$, or $CD133^-ALDH^-$ cells were isolated from HCC cell line PLC8024 (Fig. 4A) and subsequently subjected to both clonogenic and proliferative assays *in vitro* (Fig. 4B and C) and tumorigenic experiments *in vivo* (Fig. 4D; Table 4). Unsorted PLC8024 and $CD133^+$ cells isolated from PLC8024 were incorporated as controls. $CD133^+ALDH^+$ cells were able to proliferate significantly faster, induce bigger and greater

Table 1. Characterization of Protein Spots Identified by Two-Dimensional PAGE Proteomic Profiling

Spot No.	Identification (Protein Name, Accession No.)*	Mass †	p*I* value
1	β-Tubulin (CAA26203)	50255	4.79
2	α-Tubulin (AAH21564)	37707	4.87
4	aldehyde dehydrogenase 1A1 (NP000680)	55454	6.3
5	NADP-dependent isocitrate dehydrogenase (AAD02918)	46944	6.34
6	Enolase I (CAC42425)	47481	7.01
11	Sorting nexin 1A (AAC17183)	51885	5.5
12	Unnamed protein product (CAA24758)	70458	5.48
13	*S*-Adenosylhomocysteine hydrolase (AAA51681)	48254	6.03
14	Human rab GD1, GDP dissociation inhibitor 2 (BAA03095)	51088	5.94
15	Trabeculin-α (AAF06360)	617073	5.26

Abbreviation: p*I*, isoelectric point.
*Identification was done by MS study followed by data base search.
† Mass in daltons.

number of tumor colonies, and induce tumor formation with fewer numbers of cells and in a significantly lesser time than their respective $CD133^+ALDH^-$ and $CD133^-ALDH^-$ counterparts or $CD133^+$ cells alone. As few as 500 $CD133^+ALDH^+$ cells were sufficient for consistent tumor development in severe combined immunodeficient (SCID) mice, whereas at least 1,000 $CD133^+$ cells or 100,000 $CD133^+ALDH^-$ cells were necessary to consistently generate a tumor in the same model, and not to mention, requiring a significantly longer period of time. Tumor nodules that formed subcutaneously in the mice were confirmed by pathologic study (data not shown).

Discussion

The growing understanding of stem cell and cancer biology has led to the clear concept that stem cells play a critical role in the development of cancer. According to the CSC hypothesis, not all cells in a tumor are equal and that tumors are hierarchically organized and are composed of a heterogeneous

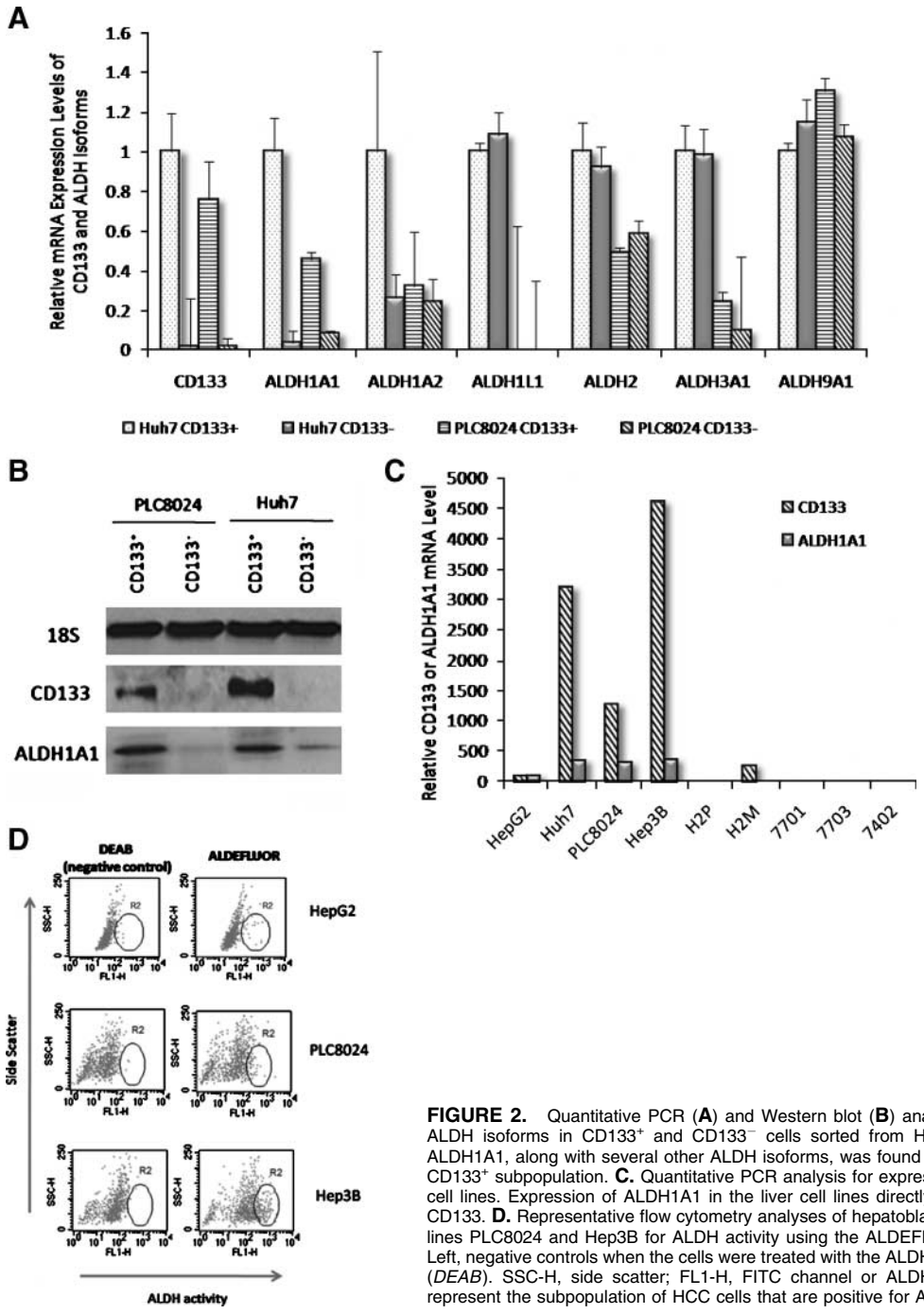

FIGURE 2. Quantitative PCR (**A**) and Western blot (**B**) analyses for expression of CD133 and ALDH isoforms in $CD133^+$ and $CD133^-$ cells sorted from HCC cell lines PLC8024 and Huh7. ALDH1A1, along with several other ALDH isoforms, was found to be preferentially expressed in the $CD133^+$ subpopulation. **C.** Quantitative PCR analysis for expression of ALDH1A1 in a panel of liver cell lines. Expression of ALDH1A1 in the liver cell lines directly correlated with their expression of CD133. **D.** Representative flow cytometry analyses of hepatoblastoma cell line HepG2 and HCC cell lines PLC8024 and Hep3B for ALDH activity using the ALDEFLUOR Kit (Stem Cell Technologies). Left, negative controls when the cells were treated with the ALDH inhibitor diethylaminobenzaldehyde (*DEAB*). SSC-H, side scatter; FL1-H, FITC channel or ALDH activity. Those cells gated in R2 represent the subpopulation of HCC cells that are positive for ALDH activity.

Table 2. CD133 Expression and ALDH Activity in a Panel of Liver Cell Lines

Cell Line	$CD133^+$ (%)*	$ALDH^+$ (%)*
HepG2	8	1.8
Huh7	65	8
PLC8024	60	6
Hep3B	90	55
H2P	4	0
H2M	6	1
7701	0	0
7703	0	0
7402	0	0

*The numbers represent the mean value of at least three independent flow cytometry analyses.

population of cells where a rare population called CSCs sit at the top. These cells possess unique survival mechanisms and distinctive stem cell properties, including the ability to self-renew and differentiate, and also exhibit a marked ability to proliferate following a prolonged period of quiescence.

Our work (10, 11), along with the work of other research groups (8, 9), suggests that human HCC is very likely to be also hierarchically organized and originates from a primitive stem/progenitor group of cells for which $CD133^+$ precursors constitute one of the most immature stage. Results from our previous studies were mainly derived from experiments done on HCC cell lines or xenograft tumors where CD133 expression was as high as 48% to 65% (It was recently observed that HCC cell lines Huh7 and PLC8024 express varying levels of CD133 expression, ranging from 48% to 60% for PLC8024 and from 49% to 65% for Huh7. The authors speculate that the cause for this variance in CD133 expression may partially be dependent on the confluence of cell culture conditions; but the reason remains elusive). Based on the CSC theory, it is believed that CSCs should only represent a minority number of the entire tumor mass; if this is true, it is unlikely that all 65% of the $CD133^+$ cells in PLC8024 and Huh7 cell lines are CSCs and is also indicative that the $CD133^+$ HCC CSC population is still heterogeneous, consisting of perhaps subsets of cells with differing tumorigenic potential. In fact, CSCs in other cancer types have also been characterized by more than one marker. Keeping in mind that the $CD133^+$ HCC CSC population is heterogeneous, we seek to further dissect tumorigenic HCC subsets in the current study. Here, we extended our study to identify the differentially expressed protein profiles of $CD133^+$ and $CD133^-$ cells sorted from the HCC cell line Huh7 by two-dimensional PAGE technique. From the list of identified proteins, ALDH1A1 was chosen for further study. The potential correlations of other identified differentially expressed proteins with CD133 expression and their possible role as liver CSC markers are also currently being investigated in our laboratory.

ALDH is a cytosolic enzyme that is responsible for the oxidation of intracellular aldehydes. To date, more than 17 human ALDH isoforms have been identified to belong in the ALDH superfamily, of which most are highly conserved across a variety of species (14). Recent studies have shown that ALDH is also highly expressed in human and murine hematopoietic stem/progenitor cells (HSC; refs. 15, 16). In particular, a

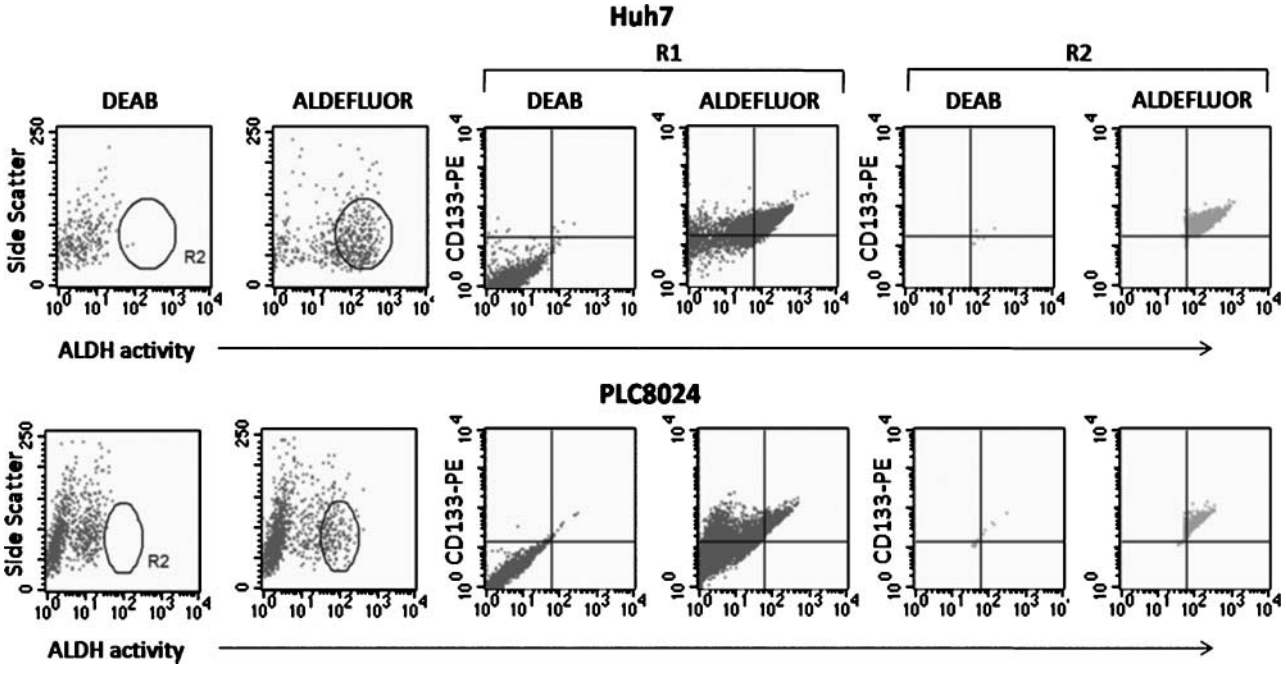

FIGURE 3. Representative flow cytometry analyses of expression and activity of $CD133^+ALDH^+$, $CD133^+ALDH^-$, $CD133^-ALDH^+$, and $CD133^-ALDH^-$ subpopulations in HCC cell lines Huh7 and PLC8024 when dually stained with CD133-phycoerythrin and for ALDH activity. The first two columns on the left represent negative controls when cells were treated with the ALDH inhibitor diethylaminobenzaldehyde and test samples when cells were treated with ALDEFLUOR substrate alone, respectively. Those cells gated in R2 represent the subpopulation of HCC cells that are positive for ALDH activity. The two columns in the middle represent the same samples when gated on R1, which is the entire cellular population, and the last two columns on the right represent the same samples when gated on R2, the $ALDH^+$ population only.

Table 3. Percentages of Sorted Subpopulations in Liver Cell Lines

Cell Line	$CD133^+$ $ALDH^+$ (%)*	$CD133^+$ $ALDH^-$ (%)*	$CD133^-$ $ALDH^+$ (%)*	$CD133^-$ $ALDH^-$ (%)*
HepG2	1.95	6.05	0	92
Huh7	9.43	39.16	0	51.41
PLC8024	7.95	38.61	0	54.44
Hep3B	55.71	33.16	0.11	11.02
H2M	0.94	4.16	0.13	94.9

NOTE: The table summarizes the percentages of different cell subpopulations when dually stained for CD133 expression and ALDH activity.
*The numbers represent the mean value of at least three independent flow cytometry analyses.

functional role of ALDH was recently shown, in which diethylaminobenzaldehyde, a specific inhibitor of ALDH, deregulated human HSC self-renewal by interfering with endogenous retinoic acid biosynthesis (17). These data suggested that ALDH may regulate HSC function and is a potential therapeutic target. More importantly, ALDH has also recently been shown to play a pivotal role in mediating chemoresistance in CSCs where it acts to detoxify harmful substances like aldehydes that would otherwise insult these crucial stem/progenitor cells. This is based on the theory that a number of chemotherapy drugs work through conversion in the body to an aldehyde that attacks cancer cells. These therapies lose potency over time because the relevant ALDH increases in concentration in cancer, deactivating the aldehyde more

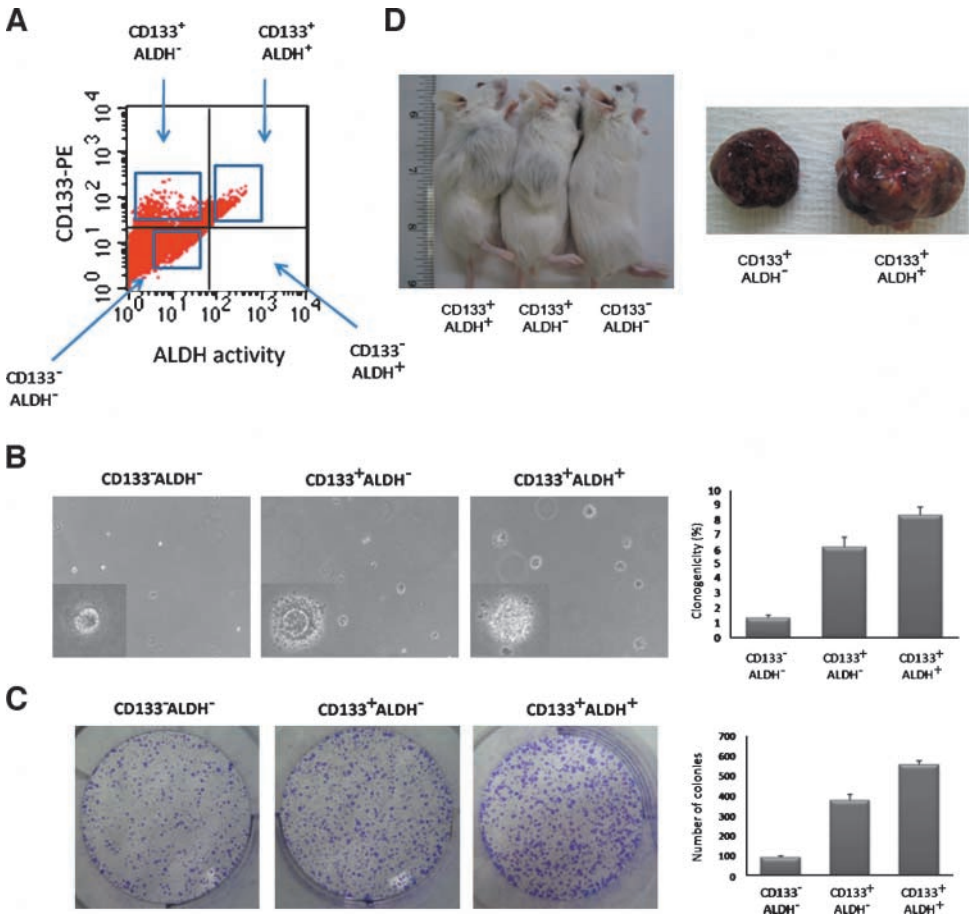

FIGURE 4. A. Flow cytometry cell sorting dot plot of HCC cell line PLC8024 dually stained for CD133 expression and ALDH activity. With reference to the negative diethylaminobenzaldehyde control as shown in Fig. 3A, the dot plot is divided into four quadrants for $CD133^+ALDH^+$, $CD133^+ALDH^-$, $CD133^-ALDH^+$, and $CD133^-ALDH^-$. Cells were collected for each quadrant as gated in the blue boxes. Note that the $CD133^-ALDH^+$ subpopulation does not exist because most, if not all, $ALDH^+$ cells are also $CD133^+$. Freshly isolated $CD133^+ALDH^+$, $CD133^+ALDH^-$, and $CD133^-ALDH^-$ PLC8024 cells were subjected to both soft agar colony formation (**B**) and proliferation (**C**) assays *in vitro*. **B.** Insets, representative examples of clonogenic assays magnified at high power. Each experiment was done thrice, and representative examples are shown. **D.** Representative examples of SCID mice injected subcutaneously with $CD133^+ALDH^+$, $CD133^+ALDH^-$, and $CD133^-ALDH^-$ cells isolated from the PLC8024 HCC cell line. Tumor nodules were only observed in mice injected with $CD133^+ALDH^+$ and $CD133^+ALDH^-$ cells. Injection site of $CD133^-ALDH^-$ cells revealed no tumor.

quickly, thus rendering these therapies ineffective. In particular, ALDH bright cells from human umbilical cord blood and peripheral blood leukemic stem cells have been shown to contain all the nonobese diabetic/SCID engrafting cells in $CD34^+$ cells (18, 19). In addition, recent research efforts have shown that ALDH is highly expressed in non–small-cell lung, colon, and pancreatic cancers (20-22), and that higher ALDH activity levels can be used to better identify the colon CSC population with $EpCAM^{high}CD44^+$ phenotype (6), as well as the glioblastoma (23), retinoblastoma (24), and breast (25) CSC populations. In view of these recent advances, it was both reasonable and logical to speculate that ALDH1A1 or the more general ALDH may possibly play an important role in the better characterization of $CD133^+$ HCC CSCs.

Subsequent studies in the present work found ALDH to be differentially expressed in the $CD133^+$ subfraction of both HCC cells lines Huh7 and PLC8024. Analysis of ALDH expression and enzymatic activity in a panel of liver cell lines found ALDH to be positively correlated with CD133 expression. Further analysis with dual-color flow cytometry for both CD133 expression and ALDH activity found the majority of $ALDH^+$ cells in cultured HCC cell lines to be $CD133^+$. Most importantly, not all $CD133^+$ HCC cells were $ALDH^+$, indicating that ALDH can possibly be used to further enrich the $CD133^+$ subpopulation and thus, in turn, better characterize the representative liver CSC population. In addition, purified $CD133^+ALDH^+$ cells were found to be significantly more tumorigenic than their respective $CD133^+ALDH^-$ or $CD133^-ALDH^-$ counterparts, both *in vitro* and *in vivo*, in that they were able not only to proliferate significantly faster but also to induce bigger and greater number of tumor colonies or nodules with less number of cells in a significantly shorter period of time.

Table 4. *In vivo* Tumor Development Experiments in SCID Mice

Cell Type	Cell Numbers Injected	Tumor Incidence*	Latency (d) †
PLC8024 unsorted	10,000	0/3	—
	100,000	5/5	70
	300,000	8/8	58
$CD133^+$ only	500	0/3	—
	1,000	2/3	85
	10,000	3/3	68
	100,000	4/4	49
	300,000	3/3	35
$CD133^+$ $ALDH^+$	500	3/3	82
	1,000	3/3	56
	10,000	3/3	45
	100,000	4/4	36
	300,000	6/6	22
$CD133^+$ $ALDH^-$	1,000	0/3	—
	10,000	1/3	80
	100,000	2/4	60
	300,000	4/4	43
$CD133^-$ $ALDH^-$	10,000	0/3	—
	100,000	0/5	—
	300,000	1/5	85

NOTE: The table provides a summary of the *in vivo* tumor development experiments of different subpopulations sorted from the PLC8024 cell line in SCID mice.
*The number of tumors detected divided by the number of injections.
†Approximate number of days from tumor cell injection to appearance of a tumor.

Thus, in summary, our work provides concrete experimental evidence showing that ALDH, when coexpressed with CD133, can more specifically characterize the liver CSC cell population. The work presented is novel in that not only is it the first study showing the use of ALDH in the identification of HCC CSCs but it is also the first study showing the preferential ALDH enzymatic activity in the $CD133^+$ subpopulation. The more specific identification of the tumorigenic liver CSC population will shed new light not only on the cellular origin of HCC but also on the underlying biological mechanism of this specific CSC population and, thus, will ultimately lead to the development of more specific therapeutic agents for the treatment of this deadly cancer.

Materials and Methods

Cell Culture

Hep3B was obtained from the American Type Culture Collection. Huh7 was provided by Dr. H. Nakabayashi (Hokkaido University School of Medicine, Sapporo, Japan; ref. 12). QGY-7701, QGY-7703, BEL7402, HepG2, and PLC8024 were obtained from the Institute of Virology, Chinese Academy of Medical Sciences (Beijing, China). H2P and H2M were previously established in our laboratory (13).

ALDEFLUOR Assay, Flow Cytometry, and Cell Sorting

ALDEFLUOR reagent (Stem Cell Technologies, Inc.) was used for the immunofluorescent detection of intracellular ALDH enzyme activity. In some experiments, cells were costained with phycoerythrin-conjugated antihuman CD133/1 antibody (Miltenyi Biotec). In the latter case, isotype-matched mouse immunoglobulins were incorporated as controls. All samples were analyzed and sorted with BD FACSVantage SE cell sorter and CellQuest software (BD Biosciences). Aliquots of $CD133^+ALDH^+$, $CD133^+ALDH^-$, and $CD133^-ALDH^-$ sorted cells were evaluated for purity with a FACSCalibur (BD Biosciences) using the ALDEFLUOR Kit (Stem Cell Technologies) and phycoerythrin-conjugated antihuman CD133/2 antibody (Miltenyi Biotec).

Two-Dimensional PAGE

For the first dimension, 150-μg proteins were separated on a precast immobilized pH gradient strip (pH 4-10, 18 cm; Amersham Biosciences). Separation in the second dimension was done on 12.5% polyacrylamide/bis gel. Gels were fixed overnight and stained with PlusOne Silver Staining Kit (Amersham Biosciences) according to the manufacturer's protocol. Stained gels were scanned with an ImageScanner and analyzed and quantified with ImageMaster 2D Platinum software (Amersham Biosciences). For comparison of protein levels among gels, normalization was done against the total intensity of all spots present in the gel. Only those significantly different spots (2-fold increase or decrease) were selected for analysis by MS.

Protein Identification by MS Analysis

Proteins of interest were excised from gels and digested with trypsin. MS analyses were done on an ABI4700 Voyage-DE STR matrix-assisted laser desorption/ionization-time of flight MS (Applied Biosystems). Generated data were searched via

the Mascot search engine to obtain protein identity by undertaking the peptide mass fingerprinting approach.

Western Blotting

Quantified protein lysates were resolved on SDS-PAGE gel, transferred onto a polyvinylidene difluoride membrane (Millipore), and immunoblotted with mouse anti-human CD133/1 (Miltenyi Biotec), goat anti-human ALDH1A1 (Santa Cruz Biotechnology), or goat anti-human β-actin (Santa Cruz Biotechnology), followed by incubation with secondary horseradish peroxidase. Blots were visualized by enhanced chemiluminescence (Amersham Biosciences).

RNA Isolation, cDNA Synthesis, and Quantitative PCR

Total RNA was isolated with TRIzol reagent (Invitrogen), and complementary DNA (cDNA) was synthesized with an Advantage RT-for-PCR Kit (Clontech Laboratories) according to the manufacturer's instructions. cDNA was subjected to quantitative PCR with a SYBR Green PCR Kit (Applied Biosystems) using primers listed in Supplementary Table S1. Amplification protocol consisted of incubations at 95°C for 15s, 60°C for 1 min, and 72°C for 1 min for 40 cycles. Quantification was done using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). All quantitative PCR reactions were done in triplicate. The C_T of each gene of interest [C_T(gene of interest) test] in each sample was normalized with control β-actin [C_T(β-actin) test] for RNA amount variation and calibrator for plate-to-plate variation using the following formula: ΔC_T(test) = C_T(gene of interest) test − C_T(β-actin) test. Relative expression level was presented as the relative fold change and calculated using the following formula: $2^{-\Delta\Delta C_T} = [C_T(\text{gene of interest}) - C_T(\beta\text{-actin})]\text{test} - [C_T(\text{gene of interest}) - C_T(\beta\text{-actin})]\text{calibrator}$.

Proliferation Assay

Experiments were done as previously described (10).

Anchorage-Independent Assay

Experiments were done as previously described (10).

In vivo *Tumorigenicity Experiments*

The study protocol was done in accordance with the Committee of the Use of Live Animals in Teaching and Research at The University of Hong Kong. SCID mice, ages between 4 and 8 wk, were used to test the tumorigenicity potential of sorted cells. Observation for growth of tumors was done as previously described (10).

Statistical Analysis

Statistical analysis was done by applying the independent *t* test using Microsoft Office Excel software. Statistical significance was declared if $P < 0.05$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer and cancer stem cells. Nature 2001;414:105–11.

2. Lapidot T, Sirard C, Vormoor J, et al. A cell initiating human acute myeloid leukemia after transplantation into SCID mice. Nature 1994;367:645–8.

3. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A 2003;1000:3983–8.

4. Singh SK, Hawkins C, Clarke ID, et al. Identification of human brain tumor initiating cells. Nature 2004;432:396–401.

5. Patrawala L, Calhoun-Davis T, Schneider-Broussard R, Tang DG. Hierarchical organization of prostate cancer cells in xenograft tumors: the $CD44^+\alpha_2\beta_1^+$ cell population is enriched in tumor-initiating cells. Cancer Res 2007;67:6796–805.

6. Dalerba P, Dylla SJ, Park IK, et al. Phenotypic characterization of human colorectal cancer stem cells. Proc Natl Acad Sci U S A 2007;104:10158–63.

7. Chiba T, Kita K, Zheng YW, et al. Side population purified from hepatocellular carcinoma cells harbors cancer stem cell-like properties. Hepatology 2006;44:240–51.

8. Suetsugu A, Nagaki M, Aoki H, Motohashi T, Kunisada T, Moriwaki H. Characterization of $CD133^+$ hepatocellular carcinoma cells as cancer stem/progenitor cells. Biochem Biophys Res Commun 2006;351:820–4.

9. Yin S, Li J, Hu C, et al. CD133 positive hepatocellular carcinoma cells possess high capacity for tumorigenicity. Int J Cancer 2007;120:1436–42.

10. Ma S, Chan KW, Hu L, et al. Identification and characterization of tumorigenic liver cancer stem/progenitor cells. Gastroenterology 2007;132:2542–56.

11. Ma S, Lee TK, Zheng BJ, Chan KW, Guan XY. $CD133^+$ HCC cancer stem cells confer chemoresistance by preferential expression of the Akt/PKB survival pathway. Oncogene 2008;27:1749–58.

12. Nakabayashi H, Taketa K, Miyano K, Yamane T, Sato J. Growth of human hepatoma cell lines with differentiated functions in chemically defined medium. Cancer Res 1982;42:3858–63.

13. Hu L, Wen JM, Sham JS, et al. Establishment of cell lines from a primary hepatocellular carcinoma and its metastasis. Cancer Genet Cytogenet 2004;148:80–4.

14. Sophos NA, Vasilious V. Aldehyde dehydrogenase gene superfamily: the 2002 update. Chem Biol Interact 2003;143:5–22.

15. Povsic TJ, Zavodni KL, Kelly FL, et al. Circulating progenitor cells can be reliably identified on the basis of aldehyde dehydrogenase activity. J Am Coll Cardiol 2007;50:2243–8.

16. Hess DA, Wirthlin L, Craft TP, et al. Selection based on CD133 and high aldehyde dehydrogenase activity isolates long-term reconstituting human hematopoietic stem cells. Blood 2006;107:2162–9.

17. Chute JP, Muramoto GG, Whitesides J, et al. Inhibition of aldehyde dehydrogenase and retinoid signaling induces the expansion of human hematopoietic stem cells. Proc Natl Acad Sci U S A 2006;103:11707–12.

18. Pearce DJ, Taussig D, Simpson C, et al. Characterization of cells with a high aldehyde dehydrogenase activity from cord blood and acute myeloid leukemia samples. Stem Cells 2005;23:752–60.

19. Cheung AMS, Wan TSK, Leung JCK, et al. Aldehyde dehydrogenase activity in leukemic blasts defines a subgroup of acute myeloid leukemia with adverse prognosis and superior NOD/SCID engrafting potential. Leukemia 2007;21:1423–30.

20. Moreb JS, Zucali JR, Ostmark B, Benson NA. Heterogeneity of aldehyde dehydrogenase expression in lung cancer cell lines is revealed by Aldefluor flow cytometry-based assay. Cytometry B Clin Cytom 2007;72:281–9.

21. Sreerama L, Sladek NE. Class 1 and class 3 aldehyde dehydrogenase levels in the human tumor cell lines currently used by the National Cancer Institute to screen for potentially useful antitumor agents. Adv Exp Med Biol 1997;414:81–94.

22. Jelski W, Zalewski B, Chrostek L, Szmitkowski M. Alcohol dehydrogenase (ADH) isoenzymes and aldehyde dehydrogenase (ALDH) activity in the sera of patients with colorectal cancer. Clin Exp Med 2007;7:154–7.

23. Bar EE, Chaudhry A, Lin A, et al. Cyclopamine-mediated hedgehog pathway inhibition depletes stem-like cancer cells in glioblastoma. Stem Cells 2007;25:2524–33.

24. Seigel GM, Campbell LM, Narayan M, Gonzalez-Fernandez F. Cancer stem cell characteristics in retinoblastoma. Mol Vis 2005;11:729–37.

25. Ginestier C, Hur MH, Charafe-Jauffret E, et al. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell Stem Cell 2007;1:555–67.