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Aldehyde dehydrogenase activity as a functional marker for lung cancer

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Abstract

Aldehyde dehydrogenase (ALDH) activity has been implicated in multiple biological and biochemical pathways and has been used to identify potential cancer stem cells. Our main hypothesis is that ALDH activity may be a lung cancer stem cell marker. Using flow cytometry, we sorted cells with bright ($ALDH^{br}$) and dim ($ALDH^{lo}$) ALDH activity found in H522 lung cancer cell line. We used in vitro proliferation and colony assays as well as a xenograft animal model to test our hypothesis. Cytogenetic analysis demonstrated that the $ALDH^{br}$ cells are indeed a different clone, but when left in normal culture conditions will give rise to $ALDH^{lo}$ cells. Furthermore, the $ALDH^{br}$ cells grow slower, have low clonal efficiency, and give rise to morphologically distinct colonies. The ability to form primary xenografts in NOD/SCID mice by $ALDH^{br}$ and $ALDH^{lo}$ cells was tested by injecting single cell suspension under the skin in each flank of same animal. Tumor size was calculated weekly. ALDH1A1 and ALDH3A1 immunohistochemistry (IHC) was performed on excised tumors. These tumors were also used to re-establish cell suspension, measure ALDH activity, and re-injection for secondary and tertiary transplants. The results indicate that both cell types can form tumors but the ones from $ALDH^{br}$ cells grew much slower in primary recipient mice. Histologically, there was no significant difference in the expression of ALDH in primary tumors originating from $ALDH^{br}$ or $ALDH^{lo}$ cells. Secondary and tertiary xenografts originating from $ALDH^{br}$ grew faster and bigger than those formed by $ALDH^{lo}$ cells. In conclusion, $ALDH^{br}$ cells may have some of the traditional features of stem cells in terms of being mostly dormant and slow to divide, but require support of other cells ($ALDH^{lo}$) to sustain tumor growth. These observations and the known role of ALDH in drug resistance may have significant therapeutic implications in the treatment of lung cancer.

Keywords

ALDH; lung cancer; stem cell; xenografts

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Conflict of Interest
The authors declare that there are no conflicts of interest

1. Introduction

Aldehyde dehydrogenases (ALDH) are a group of $NAD(P)^+$-dependent enzymes involved in oxidizing a wide variety of aldehydes into their corresponding carboxylic acids [1,2]. The role of some of these ALDHs in endobiotic and xenobiotic metabolism has been reviewed extensively before [1-10] and the specific metabolic pathways affected have been revealed in detail [1]. ALDHs have broad substrate specificity. Their role in alcohol metabolism, vitamin A metabolism, and resistance against oxazaphosphorines are some of the most studied aspects of their activities. Multiple tools have been used to study the role of the ALDH enzymes in cellular metabolism and organ development including antisense [11], siRNA [12], inhibitors such as diethylaminobenzaldehyde (DEAB) [13] and disulfiram [14] and knock out mice models [15-19].

Expression of ALDH isozymes has been shown to be increased in many cancer types including liver, pancreas, breast and colon cancers [20-25]. Previously, we have profiled the ALDH expression levels of 12 different human lung cancer cell lines [26]. Specifically, ALDH1A1 and ALDH3A1 cytosolic forms of the enzymes were highly expressed in some non-small cell lung cancer cell lines as well as in patient lung cancer samples [27]. We also observed that expression of both enzymes gradually increases during the transition from normal to atypical pneumocyte, carcinoma in situ and then adenocarcinoma. Moreover, our results have shown that cigarette smoking alone seems sufficient to elevate ALDH expression in normal pneumocytes [27]. These observations are the basis for our hypothesis that the elevated expression of these isozymes may be related to the malignant transformation.

Major recent new hypotheses such as stem cell plasticity which means that somatic stem cells can regenerate and repair different types of tissues, and that cancer behaves like an organ with its own sustaining cancer stem cells (CSC), have intensified the search for a more practical way of defining stem cell. Stemness markers or genes are badly sought after. ALDH has been known to be highly expressed in hematopoietic stem cells (HSC) for years [28,29] and it provides protection against alkylating agents in the oxazaphosphorines family, such as cyclophosphamide and its derivatives [30-34]. The use of ALDH activity as the basis of flow cytometry-based method to sort hematopoietic progenitors has opened the way to study high ALDH activity as a marker for stem cells in different tissues. This method (Aldefluor, Stem Cell Technologies, Inc., Vancouver, BC, Canada) [26] has allowed the isolation of viable progenitors that can now be studied for their functional characteristics in vitro and in vivo [35].

Recent publications and conference presentations have shown the existence of ALDH positive cells in several cancers including multiple myeloma, leukemia, head and neck, and breast [36-39] which possess some stem cell characteristics and ability to initiate tumors in immunodeficient mice.

In this study we investigate the functional role of ALDH in proliferation and tumor formation using H522 human lung cancer cell line. We demonstrate that cells with high ALDH activity isolated from H522 cell line have distinct phenotypic and functional characteristics compatible with cancer initiating stem or early progenitor cells. On the other hand, low level ALDH expressing cells endowed with characteristics similar to progenitor cells with limited proliferation and tumor formation. Most of the ALDH activity in this cell line is accounted for by ALDH1A1 and ALDH3A1 expression. These findings raise the question of which ALDH gene is the stemness marker and why. Future studies will need to address these important questions.

2. Materials and methods

2.1. Cell lines

Lung cancer cell line H522 was originally obtained from ATCC and used in the described experiments. The cell line was cultured in RPMI-1640 medium (Gibco Invitrogen) with 10% FBS (Gibco Invitrogen) in 5% CO_2 cell culture incubator at 37°C, and used within 2–4 passages when in the log phase of growth. Aliquots of the cell line were kept frozen in -80°C freezer unti l use.

2.2. Cell proliferation and colony formation

Cells were plated at 1×10^5 /well in 6-well plates in order to assess proliferation rate. After 48 hr, the cells were harvested, counted. Furthermore, triplicates of 200 cells/ml/well in a six-well plate were cultured in RPMI-1640 + 10% FBS. The cells were allowed to grow for 5-7 days. The number of colonies adhered to the bottom of the plate was then counted using inverted microscope. The mean number of colonies from the triplicates was calculated and compared.

2.3. ALDH activity measurement

ALDH enzyme activity using the spectrophotometric assay was performed as described before [11,26]. Briefly, aliquots of 600 μl lysing buffer were incubated at 37°C in Beckman DLC 64 spectrophotometer cuvettes with the addition of 30 μl cell lysate, 5 mM NAD^+ and 5 mM propionaldehyde as a substrate. The rate of change in absorbance at 340 nm was measured in 3 replicates over 5 min. A control reaction in which the substrate was not added monitored the endogenous rate of NAD^+ reduction. The ALDH activity was expressed in nmoles/10^7 cells.min.

2.4. Aldefluor assay and cell sorting

Based on our published data using Aldefluor assay, H522 cell line contains a proportionate numbers of $ALDH^{br}$ and $ALDH^{lo}$ cells while A549 was made of mostly $ALDH^{br}$ cells. Because of that, we chose H522 cell line sorting cells into two cell populations in order to compare the two in terms of cell proliferation, colony formation, stemness markers and the ability to form tumors in a xenograft animal model.

The cells were first labeled using the Aldefluor kit as described before [26], and then used FACS Vantage (BD Biosciences, San Jose, CA) to sort cells with the gates set to collect the brightest 2% as the $ALDH^{br}$ population and the dimmest 2% as $ALDH^{lo}$ cells. After sorting, the cells were washed and cultured for expansion. ALDH activity was measured after one week both by Aldefluor assay and by spectrophotometry. With establishing two different cell populations in terms of ALDH activity, the cells were then frozen in aliquots and thawed for experiments as needed.

2.5. In Vitro studies comparing $ALDH^{br}$ versus $ALDH^{lo}$ cells

Several in vitro studies, described below, were performed to characterize and compare the stem cell features of $ALDH^{br}$ cells versus the $ALDH^{lo}$ in H522 cell line.

Cytogenetic analysis: Chromosome analysis was performed to detect clonality of the $ALDH^{br}$ cells and differences from $ALDH^{lo}$ cells. Metaphase chromosome spreads were prepared from phytohemagglutinin-stimulated cell cultures in accordance with standard cytogenetic procedures. Chromosome preparations were banded utilizing a standard GTG-banding procedure [40]. Metaphase imaging and karyotype production were facilitated by computer-assisted methods (CytoVision software; Applied Imaging, Foster City, CA). At least

twenty metaphase cells were analyzed for each of 3 cell populations: Parent H522 cell line, $ALDH^{br}$ cells, and $ALDH^{lo}$ cells.

Cell proliferation and maintenance, colony formation, and replating efficiency: After sorted cells are established in culture, repeat Aldeflour assay and ALDH activity were done over time to study the stability of the difference in ALDH activity between $ALDH^{br}$ and $ALDH^{lo}$ cells. Furthermore, equal number of cells from each cell type were cultured for 72 hr and then counted using 1:1 dilution with trypan blue to allow measurements of viable cells only. In addition, triplicates of 200 single cells per 1 ml well were plated for colony formation. Colonies were counted on days 5-7 of culture, unless otherwise specified, using inverted microscope. Finally, after adding trypsin to colonies and under direct vision using inverted microscope, single colonies that just began to detach were collected using volume of 30 μl by pipette and transferred to 500 μl culture media. The cell number of each colony was counted using hematocytometer and then replated for colony formation. Daughter colonies were similarly counted and replating efficiency calculated by dividing the number of colonies by the cell number plated and multiplying by 100 for % plating efficiency.

Stemness markers expression: Multiple efforts to determine stemness markers have been published. We analyzed the expression of several stem cell genes to study differences between $ALDH^{br}$ and $ALDH^{lo}$ cells. We used semiquantitative RT-PCR as described before [26] to measure differences in expression of NANOG and OCT4 genes, both are known markers of human embryonic stem cells [41,42]. The primers used were previously published [42]. In addition, we used standard flow cytometry assays to compare the expression of several surface markers that were used to identify stem and progenitor cells such as CD34, CD133, CD24, and CD44 variant (BD Biosciences).

2.6. Xenoraft mouse model

Six to eight week old Non-Obese Diabetic/Severe Combined Immunodeficient (NOD/SCID) mice were obtained from Charles River Laboratories (Wilmington, Massachusetts). Animals were used and maintained under specific pathogen-free conditions according to defined conditions approved by the Institutional Animal Care and Use Committee of the University of Florida. Prior to cell injections, animals were sublethally irradiated with 200 cGy of gamma irradiation. As a prophylactic treatment, animals were given a regiment of antibiotics (2.5 μl of 100 mg/μl antibiotic solution in 500 μl water bottle) including enrofloxacin (Bayer HealthCare; Shawnee Mission, Kansas), for two weeks. To calculate the cell concentration for injection doses, viability and number of cells were determined by mixing 50μl of cells with 50 μl of 0.4% trypan blue stain (Gibco Invitrogen) and counting the bright cells using a hemacytometer under an inverted microscope. After determining cell density, the cells were injected in 100 μl 1x PBS mixed with 0.4 μl of Matrigel HC under the skin near the scapula. Tumor growth was measured weekly with calipers. The longest (L) and shortest (W) tumor diameters (mm) were obtained and formula for an ellipsoid sphere ($0.52 \times L \times W^2$) was used to calculate the tumor volume.

2.7. ALDH immunohistochemical staining of xenografts

For IHC, the primary antibodies, polyclonal chicken anti-human ALDH1A1 and ALDH3A1, were provided by Dr Sreerama (St Cloud State University, St Cloud, MN, USA). Tumors excised from the animals were fixed overnight in 4% para formaldehyde at 4°C. Samples were sent to the Department of Pathology Core Laboratory for preparation. Paraffin embedded blocks were sectioned for 5 microns. Slides underwent deparaffinization and hydration. Followed by microwave antigen retrieval in 10 mmol citrate buffer at a pH of 6.0. Slides were then rinsed with TBS-T buffer and blocked for 20 minutes at room temperature with 1.5% goat serum (Vector Laboratories) diluted in TBS-T containing avidin (4 drops/μl; Avidin/Biotin

Blocking kit, Vector Laboratories). Primary antibodies of chicken anti-ALDH1A1 IgY (1:200) and chicken anti- ALDH3A1 IgY (1:300) were diluted in Zymed diluent containing 4 drops/μl biotin block (Vector Labs). The diluted anti human ALDH antibodies were incubated for 2 hr at room temperature, followed by a 5-minute washing step with 1X TBS-T. The secondary antibody, biotinylated goat anti-chicken IgG (1:400) (Vector Laboratories) was incubated at room temperature for 30 minutes followed by a 5 minute washing step. Detection of the secondary antibody was performed with the ABC Elite Standard kit (Vector Laboratories) and subsequently visualized with the substrate, Diaminobenzidene (Vector Laboratories). Tissue samples were lightly counterstained by dipping the slides 15-20 times in Gill's 2 hematoxylin (Richard-Allan Scientific). Following the dehydration step in ethanol and xylene, the slides were mounted in Xylseal (Richard-Allan Scientific). Representative tissue sections were stained with a hematoxylin-eosin to assess the morphology of the cells within the tumors.

2.8. Statistical analysis

The results are expressed as mean ± SD. A paired Student's *t* test was used to assess the significance of differences between two means. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. ALDH^{br} cells represent a separate clone and give rise to ALDH^{lo} cells

The detection of H522 cells expressing high ALDH activity by Aldefluor flow cytometry assay is shown in Figure 1. The first set of sorted H522 cells was used in transplantation experiments using a cell dose of 10^5 cells. The enzyme activity of sorted ALDH^{br} cells was 168 versus 67 nmol/10^7 cells. min for ALDH^{lo} cells. The second attempt at sorting the H522 cells resulted in very low yields, which required the sorted cells to be cultured for a longer period of time. During the expansion of the cells from the second set, enzyme activity was measured and determined to be 58 for the ALDH^{br} cells versus 23 nmol/10^7 cells. min for the ALDH^{lo} cells.

Cytogenetic analysis revealed multiple karyotypes in the parent H522 cell line with a dominant one shown in Figure 2. This was similar in the ALDH^{lo}. On the other hand, a different karyotype was dominant in the ALDH^{br} cells and was detectable in the parent cell line but rare. These studies indicate that the ALDH^{br} cells represent a separate clone.

Growing the ALDH^{br} and ALDH^{lo} cells in culture over time and monitoring the ALDH activity by Aldefluor assay and spectrophotometric enzyme activity showed that the ALDH^{br} cells gradually return similar cytogenetics of the parent cell line with concomitant increase in the proportion of the ALDH^{lo} cell population over 4-5 weeks.

3.2. ALDH^{br} cells grow slower in vitro and give rise to different type of colonies

The proliferation of ALDH^{br} in cell culture was noticed to be much slower (2-5 folds) than the ALDH^{lo} cells (data not shown).Colony forming assay in which 200 cells/ml/well of each of ALDH^{br} and ALDH^{lo} cells were cultured for ≥ 7 days revealed a different type of colonies for the ALDH^{br} as compared to both parent and ALDH^{lo} cells (Figure 3A & B). In addition the plating efficiency was 10% and 54% for ALDH^{br} and ALDH^{lo}, respectively, while for the parent cell line it was 63%. The colonies for the ALDH^{br} cells were counted at day 14 versus day 5-7 for the other cell types. The type of colonies was also different (shown in Figure 3A & B). Three different types of colonies were identified in the parent H522 cell colony assay (Fig 3A), based on previously published data [43, 44]. On the other hand, only one type of colony that is completely different in morphology was cultured from the ALDH^{br} cells (Fig 3B). However, these results varied from one sorting session to another depending on the purity of the collected ALDH^{br} cells. Since one of the claimed features of stem cells is reflected by

the ability to give rise to many different types of colonies, single colonies from primary cultures were picked and counted for cell number and then recultured in a similar colony forming assay for ≥ 7 days. The results showed very low replating efficiency of 0-14% for the $ALDH^{br}$ colonies and the resulting colony types were all the same type as the parent colony seen in Figure 3B.

However, when these $ALDH^{br}$ cells are left to grow in cell culture and colony assay is performed at different time intervals, the results show steady increase in plating efficiency with the reappearing of the type of colonies formed by the parent and the $ALDH^{lo}$ cells. Indeed, the mean clonal efficiency from 200 cells plated on day 14 after cell culture of $ALDH^{br}$ cells started was 10%, and then increased to 13% by day 21 and 22% by day 30. At this time, up to 74% of the colonies were holoclones (Fig 3A). These experiments and the change in ALDH activity over time described in the above section suggest that the $ALDH^{lo}$ cells originate from the $ALDH^{br}$ cells. On the other hand, $ALDH^{lo}$ cells remain pure and do not give rise to $ALDH^{br}$ cells within the same culture system and time frame as above.

3.3. Expression of stem cell markers in $ALDH^{br}$ cells

In order to try and establish the stemness of the $ALDH^{br}$ H522 cells, we used known stem cell genes and markers in order to compare their expression in $ALDH^{br}$ versus the $ALDH^{lo}$ cells. Using a traditional flow cytometry approach, we were not able to detect CD34 and CD133 expression on H522 cells. On the other hand, both CD24 and CD44v, adhesion molecules, were detected in $ALDH^{br}$ and $ALDH^{lo}$ cells. We sorted cells into $ALDH^{br}CD24^{+}$ to $ALDH^{br}CD24^{-}$ and studied differences in cell proliferation and colony formation as described above and found no significant differences between the two cell populations (data not shown).

Using semi quantitative RT-PCR, we showed that NANOG and OCT4 are expressed at equal or higher levels in the $ALDH^{lo}$ cells in comparison to $ALDH^{br}$ cells (Figure 4). This was unexpected as we expected these stem cell genes to be expressed in $ALDH^{br}$ lung cancer cells.

3.4. Both $ALDH^{br}$ and $ALDH^{lo}$ cells can form primary xengrafts but with different growth rate

To investigate possible differences in tumor formation potential between the $ALDH^{br}$ and $ALDH^{lo}$ sorted cells, varying cell doses (from 500 to 1×10^5 cells) were injected under the skin of NOD/SCID mice, 3 animals for each cell dose, and monitored for tumor development. As shown in Table 1, both cell groups yielded tumors at every cell dose tested. As expected from the results of in vitro experiments, $ALDH^{lo}$ cells rapidly formed tumors, whereas the tumor growth rate of $ALDH^{br}$ cells was significantly slower. After 5 weeks post-transplantation $ALDH^{lo}$ tumors from 1×10^5 cell dose grew to a mean volume of 3904 ± 360 mm^3, which was 33 fold larger than the $ALDH^{br}$ tumor (117 ± 12 mm^3) at the same time point (Table 1). Interestingly, an additional three weeks was required for the $ALDH^{br}$ cell-generated tumors to reach an equivalent size (3493 ± 310 mm^3). Indeed the results at the level of 10^5 cells show that eventually these $ALDH^{br}$ tumors achieve similar size to that seen at 5 weeks by the comparable $ALDH^{lo}$ group (Figure 5). Representative tumors were excised, and re-established into cell suspension in culture. In order to examine the ability of $ALDH^{br}$ and $ALDH^{lo}$ cells to form secondary and tertiary tumors in new recipients, we used cells obtained from the primary tumors to inject into secondary recipients and then repeated the same experiments with cells obtained from these secondary tumors. The results show that the $ALDH^{br}$ cells grow faster than $ALDH^{lo}$ cells in these secondary and tertiary transplants and thus produce bigger xenografts. Moreover, the $ALDH^{lo}$ tumor growth rates were subsequently decreased with each serial transplant (Table 2).

3.5. ALDH Enzyme Activity Decreased In vivo

To detect ALDH enzymatic activity changes that occur in vivo during the growth of the tumor, tumor samples were assayed for ALDH activity using both the Aldefluor flow cytometry and ALDH enzyme activity assays. Both assays showed decrease in ALDH activity in tumors formed from either $ALDH^{br}$ or $ALDH^{lo}$ cells. The tumor cells originating from $ALDH^{br}$ cells had three-fold reduction in enzymatic activity compared to the initial sorted cell population (from about 168 to 41 nmole/10^7 cells.min), while the decrease in activity was more modest in tumors originating from the $ALDH^{lo}$ cells (from 58 to 44 nmole/10^7 cells.min).

Such change in activity was also demonstrated by IHC staining tumor sections using antibodies against ALDH1A1 and ALDH3A1 which demonstrated similar distribution pattern of ALDH positive cells in both types of tumors. Furthermore, the ALDH positive cells were mainly localized and clustered in a close proximity to blood vessels as shown in Figure 6.

4. Discussion

According to the cancer stem cell (CSC) hypothesis, only a subpopulation of the heterogeneous tumor cells is capable of initiating and re-initiating tumors. This CSC population is defined by their ‘stemness’ properties, more specifically self-renewal and asymmetrical cell division, in addition to their ability to invade. ALDH activity has been used a functional stem cell marker to isolate CSCs in different types of cancers. In this study, a candidate CSC of the H522 human lung cancer cell line was sorted out based on ALDH activity and examined using in vitro assays as well as in vivo animal model.

The in vitro studies using the $ALDH^{br}$ and $ALDH^{lo}$ H522 cells indicate that the $ALDH^{br}$ cells are a separate clone of cells that exist within the parent cell line with its own specific karyotype, typical morphology of slow growing colonies, and low proliferative rate of growth. These $ALDH^{br}$ cells give rise to the $ALDH^{lo}$ cells in long-term culture and re-establish the parent cell line. On the other hand, known established stem cell surface proteins and genes were not exclusively expressed in these $ALDH^{br}$ cells. There are many possible explanations for these findings, including the inherent problem that has hindered stem cell research which is the ability to reproducibly isolate pure population of stem cells. In addition, when isolating cells according to ALDH activity, often it is not known which ALDH isozymes contribute to the activity and therefore which isozymes are specific for the stem cell activity. In separate set of experiments, using knock-down approach by expressing siRNA against ALDH1A1 and ALDH3A1 in H522 and A549 lung cancer cell lines or by the use of known ALDH inhibitors such as DEAB and disulfiram, we have shown that decrease in ALDH activity can affect cell growth, colony formation, and cell migration (manuscript submitted for publication).

Our results provide evidence that as low as 500 ALDH expressing H522 cells were able to give rise to tumors. More importantly, the ALDH levels of the cells were found to be a determinant of the tumor propagation. Although, both $ALDH^{br}$ and $ALDH^{lo}$ sorted cells could develop tumors, in vitro and in vivo the doubling rate of the primary xenografts were inversely correlated to the ALDH levels of the cells. However with the transfer of tumor cells into secondary and tertiary recipient animals, the tumors originating from the $ALDH^{br}$ cells showed faster growth, thus indicating a higher proliferative potential for the $ALDH^{br}$ cells as opposed to the $ALDH^{lo}$ cells. In other words, tumor generating ability of $ALDH^{lo}$ tumors was reduced by each subsequent engraftment, whereas tumor progression of $ALDH^{br}$ cells was increasing. These results are supportive of our main hypothesis that $ALDH^{br}$ are the CSC in lung cancer, but further studies to confirm these results are still needed.

The trend for the characterization of human CSC is evaluating the ‘minimal cell dose’ capable of initiating tumors in animals and their ability to re-establish tumor upon serial transplants

into secondary and tertiary recipients. Indeed, if we were to follow these criteria; the in vivo study results may appear to suggest that the $ALDH^{lo}$ population contains the CSCs due to its fast tumorigenicity and serial engraftment ability. During the first set of animal experiments, by the time the $ALDH^{lo}$ tumors grew to their maximum allowed size, the $ALDH^{br}$ tumors were almost undetectable. If we were to terminate the $ALDH^{br}$ animal study at the same time with the maximized $ALDH^{lo}$ tumors, we would not be able to observe the very slow growing $ALDH^{br}$ tumors and we would have concluded that only the $ALDH^{lo}$ cells are capable of initiating tumors. Thus, our results may indicate the existence of two fractions of CSCs: $ALDH^{br}$ with the long-term repopulating cells, and the $ALDH^{lo}$ CSCs with short term proliferative potential. Such possibility can be explained based on the long established observation in the hematopoietic system in which the stem cells were further fractionated into long-term hematopoietic stem cells (LT-HSC) and short-term HSC (ST-HSC) that acquire limited proliferative capacity [45]. Although the LT-HSCs are considered to be the most primitive HSCs that can regenerate a whole hematopoietic system from a single cell transplantation, the reconstitution progress takes longer period of time due to the steps in generating first the progenitor cells and then the maturation period of these progenitor cells [45]. In this study, what we observed in terms of slow progression of $ALDH^{br}$ tumors might be similar coincidence to the LT-HSC engraftment which is very slow and takes longer time than if both LT and ST-HSC were combined.

In addition, further evidence to the stem cell properties of the high ALDH expressing CSCs may be based on the results of the IHC staining for ALDH1A1 and ALDH3A1 performed on xenografted tumors. The IHC staining displayed a localization of ALDH positive cells in a close proximity to the perivascular niches, which are proposed to be the CSCs niche for brain tumor initiating cells [46]. For primitive cells, niche is a home from where they receive the necessary signaling to maintain their ‘stemness’, and therefore our IHC analysis results perfectly fit into such scenario. However, considering the detoxification functions of these enzymes, it is possible that the high ALDH expression in perivasvular cells reflect the up-regulation of ALDH isozymes as a result of external stimulants diffusing from the blood vessels. Therefore, the underlying mechanism and significance of this perivascular localization must be further investigated before firm conclusions are reached regarding its significance.

In summary, the overall results of this study indicate that the Aldefluor based sorting of lung cancer cells has the potential to enrich for the CSCs. Our results also show that ALDH activity can be a functional marker for lung cancer in addition to its known contribution to drug resistance.

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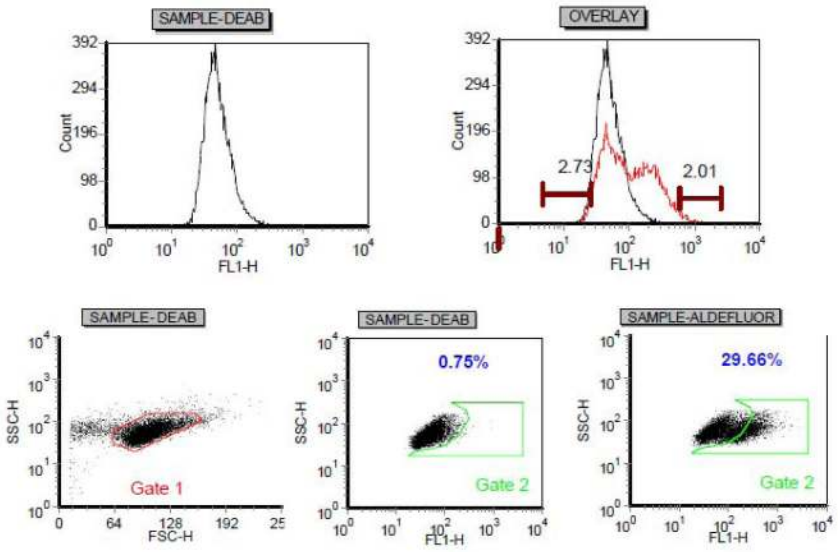

Figure 1.

Aldefluor flow cytometry based assay to identify cells with high ALDH activity (ALDH^{br}) among the H522 lung cancer cells. The top panels show the histograms of Aldefluor fluorescence with and without the addition of DEAB, an ALDH activity inhibitor, which demonstrate shifting of cells with high ALDH activity to the right (right top panel). The same information is shown in the bottom panels, where the 1st panel on the left shows the side scatter of the viable H522 cells (gate 1), and the other two panels show gate 2 that is defined by the addition of DEAB (middle panel) and within which the ALDH^{br} cells fall. In this analysis, the ALDH^{br} cells constitute about 29% of the parent cell line.

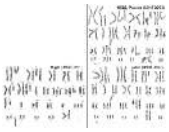

Figure 2.
Comparison of the dominant cytogenetic karyotypes identified in H522 parent cell line as well as H522 cells sorted by flow cytometry with either high or low ALDH activity. Similar karyotypes were dominant in both the parent cell line and the cells with low ALDH activity, while the karyotype of the cells with high ALDH activity was clearly different. Interestingly, other less frequent karyotypes were identified in the parent cell line, including one similar to that found to be dominant among the cells exhibiting high ALDH activity.

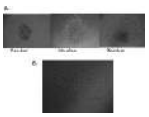

Figure 3.

The morphology of colony types identified during colony forming assay of H522 cells. **A.** Three different known types of colonies were identified when culturing the parent H522 cell lines and the sorted cells with low ALDH activity ($ALDH^{lo}$). **B.** Only one, completely different, type of colony was identified in a similar colony forming assay from sorted H522 cells with high ALDH activity ($ALDH^{br}$).

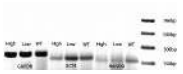

Figure 4.
Semiquantitative RT-PCR for NANOG and OCT4, human embryonal stem cell genes, was performed on RNA obtained from 3 different groups of cells. GAPDH, a house keeping gene, was used as a control. The 3 experimental groups include: the parent H522 cell line (WT), and flow cytometry sorted H522 cells with either high (High) or low (Low) ALDH activity.

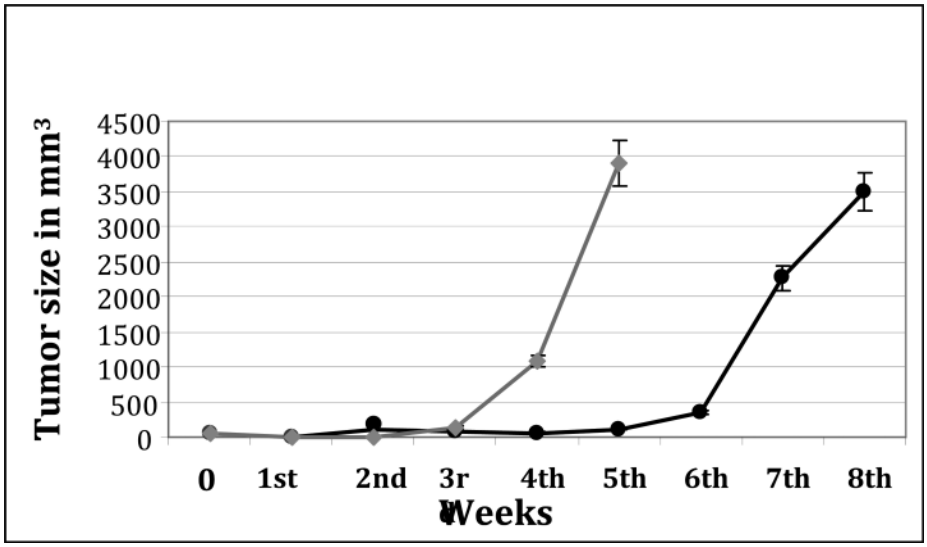

Figure 5.

Comparison of primary xenograft formation in NOD/SCID mice by sorted H522 cells using Aldefluor flow cytometry assay. The curves reflect the increase in size of tumors growing under the skin from 10^5 sorted H522 cells with either low ($ALDH^{lo}$, grey line) or high ALDH activity ($ALDH^{br}$, black line). Each time point represents the mean tumor size (mm^3) calculated weekly from 3 similar animals in each experimental group.

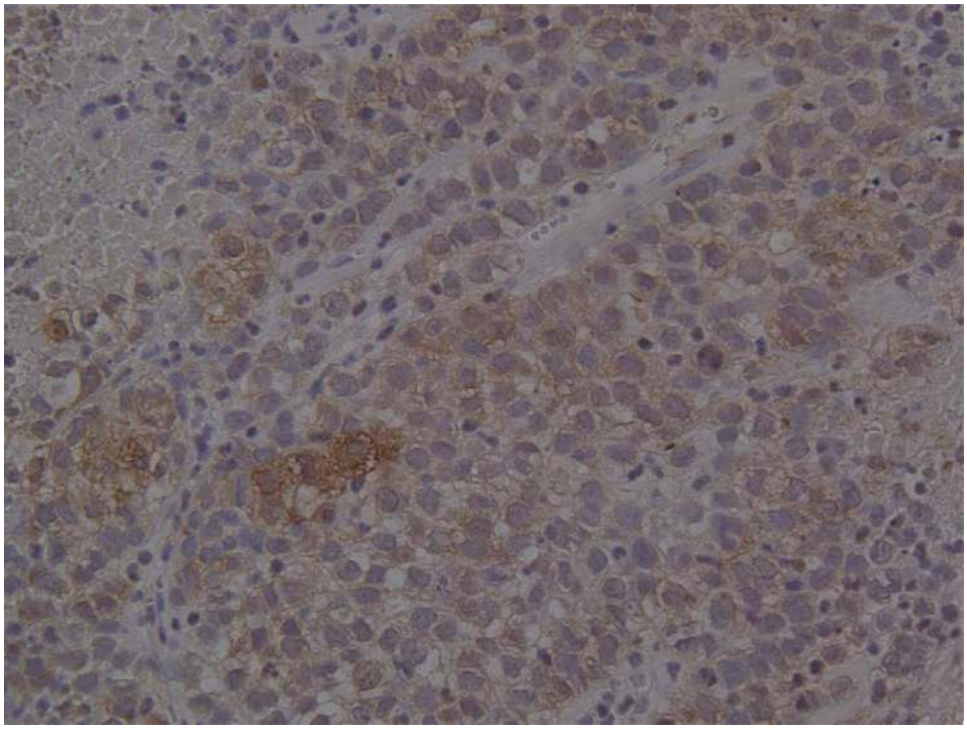

Figure 6.
Immunhistochemistry (IHC) staining for ALDH3A1 of a representative primary xenograft originating from ALDH^{br} H522 cells. The results demonstrate the clustering of cells containing high levels of ALDH3A1 around blood vessels (40X magnification). Similar results were obtained from IHC staining for ALDH1A1 of the same tumor sample (data not shown).

Table 1

Limiting dilution assay for xenografts in NOD/SCID mice: The data represent mean (n=3) of tumor size in mm^3 for each cell dose at weekly intervals after the day of cell injection (Inj Day).

Dose Group	Inj Day	1st wk	2nd wk	3rd wk	4th wk	5th wk	6th wk	7th wk	8th wk
$ALDH^{br}$ 10^5	52	0	106	93	63	117	339	2268	3493
$ALDH^{lo}$ 10^5	52	0	0	140	1081	3904			
$ALDH^{br}$ 10^4	52	0	95	148	438	1423			
$ALDH^{lo}$ 10^4	52	0	107	377	1194	4235			
$ALDH^{br}$ 10^3	52	0	0	38	68	1133			
$ALDH^{lo}$ 10^3	52	0	0	107	528	2984			
$ALDH^{br}$ 500	52	0	0	45	68	371			
$ALDH^{lo}$ 500	52	0	0	59	363	1524			

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Table 2

Comparison of the mean tumor size (mm^3) formed by ALDHbr and ALDHlo cells in primary (1^{st}), secondary (2^{nd}) and Tertiary (3^{rd}) transplants in NOD/SCID mice.

	Inj‡ Day	1st wk	2nd wk	3rd wk	4th wk	5th wk	6th wk	7th wk	8th wk
1st ALDHbr 10^5 (n=3)*	52	0	107	93	63	117	339	2268	3493
1st ALDHlo 10^5 (n=2)	52	0	0	140	1081	3904			
2nd ALDHbr 10^6 (n=2)	52	47	326	1423	3373				
2nd ALDHlo 10^6 (n=1)	52	68	175	568	1582				
3rd ALDHbr 10^6 (n=2)	52	68	123	435	2392	4405			
3rd ALDHlo 10^6 (n=2)	52	31	60	211	762	2512			

*The numbers reflect cell dose injected into each mouse. The n represent the number of animals injected for each cell dose.

‡Inj Day= the first day cells were injected under the skin of animals.