The ALDH1$^+$ subpopulation of the human NMFH-1 cell line exhibits cancer stem-like characteristics

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Abstract. Cancer stem cells (CSCs) have been reported in many tissues. However, CSCs have yet to be identified in a human malignant fibrous histiocytoma (MFH) cell line. Elevated aldehyde dehydrogenase 1 (ALDH1) has been proposed as a stem cell marker for isolating CSCs from cancer. The aim of the present study was to identify a population with elevated ALDH in the human NMFH-1 cell line. ALDH$^+$ and ALDH$^-$ cell populations were isolated and compared for CSC characteristics. ALDH enzymatic activity was used as a marker to identify the cells in the NMFH-1 line. Self-renewal, differentiation capacity, and tumorigenicity of the NMFH-1 ALDH$^+$ cell population were then examined using a spheroid formation assay and xenograft model in nude mice. Chemoresistance levels, ABCG2 drug transport gene expression, and stem cell-associated gene expression were compared in these NMFH-1 populations. The ALDH$^+$ population was better able to form spheres in anchorage-independent serum-starved conditions. Furthermore, the mRNA expression of key stem cell-related genes was enhanced in these cells. Increased expression of the drug transporter gene, *ABCG2*, was detected. Compared with ALDH$^-$, the ALDH$^+$ subpopulation had higher levels of chemoresistance to doxorubicin (DXR) and cisplatin (CDDP). Additionally, the ALDH$^+$ cells more efficiently formed tumors when implanted into BALB/c nude mice. ALDH1 may therefore be used as a marker for the isolation of cells that exhibit several characteristics of CSCs from the NMFH-1 cell line. This finding may lead to the development of novel therapies to specifically kill ALDH1$^+$ subpopulations (CSCs).

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Introduction

Cancer stem cells (CSCs) exist within tumors and are a unique subset of cells with the potential to self-renew, proliferate indefinitely, and differentiate into tumor cells (1,2). Present cancer therapies are known to leave behind some CSCs, explaining why tumor eradication is difficult to achieve (3-5). While the development of CSC-specific drugs may bring new hope to cancer therapy, there are few established models that allow the isolation and the study of CSCs.

Previous findings have shown that aldehyde dehydrogenase 1 (ALDH1) can be used as a CSC marker and is expressed in many stem cell types, including breast, lung, neuronal, and hematopoietic stem cells (HSCs) (6,7). ALDH1 is a member of a family of acetaldehyde dehydrogenase enzymes that oxidize acetaldehyde to acetic acid. In HSCs, ALDH1 can control retinol metabolism, and certain classes of retinoic acid may lead to the differentiation of hematopoietic progenitor cells (8). By contrast, immature HSCs are very rich in retinol, which enhances self-renewal (9). The above-mentioned results suggest that ALDH1 is a necessary factor for the HSC environment. A fluorescent ALDH substrate that passes through the cell membrane by free diffusion (Aldefluor® BAAA-ALDH) can be used to measure ALDH enzyme activity by quantitative flow cytometry (10-12).

Malignant fibrous histiocytoma (MFH) was identified and designated as such in the 1960s. However, the prognostic factors that are predictive of MFH outcome have not been well described (13). The majority of MFH tumors are defined as pleomorphic subtypes, and a few myxoid MFH cell lines have been established (14-16). Kawashima *et al* established the NMFH-1 cell line, which may prove a useful tool for studying CSCs (17).

In the present study, we isolated and characterized the population of NMFH-1 cells that have ALDH enzymatic activity, a trait characteristic of CSCs. The aim was to develop a model for the study of CSCs, which may ultimately provide insights into the clinical treatment of MFH. At present, it remains unknown whether the population of ALDH$^+$ cells may be isolated from the human NMFH-1 cell line. Therefore, we defined the population of NMFH-1 cells with high ALDH enzymatic activity on CSC phenotypes. The findings may impact on the development of more effective MFH therapies.

Materials and methods

Cell lines and culture. The human NMFH-1 cell line used in the present study was obtained from the Niigata University Graduate School of Medical and Dental Sciences (17) and maintained at 37˚C in a 5% CO_2 atmosphere in RPMI-1640 supplemented with 10% fetal bovine serum (FBS). Aldefluor® (Stem Cell Technologies, Durham, NC, USA) was used to isolate the cell populations with high levels of the ALDH enzymatic activity. The cells were labeled with Aldefluor® reagent by collecting cells using 0.25% trypsin according to the manufacturer's instructions, and single-cell suspension was prepared. The cells were washed with PBS and isolated by centrifugation prior to adding the product reagent according to the manufacturer's instructions. The $ALDH^+$ and $ALDH^-$ cells were then analyzed or isolated using a FACSAria.

Sphere formation. A single-cell suspension of $1x10^5$ $ALDH^+$ or $ALDH^-$ cells in serum-free RPMI-1640 medium was seeded in 6-well plates (Corning Inc., Corning, New York, USA). Each well contained 20 μg/l of EGF and bFGF, and wells were supplemented with 2 μg/l of EGF and bFGF every 24 h until spherical cell formations appeared. Following sphere formation, the cells were collected in a common culture bottle with complete media to observe whether the cells could be adhered to the wall. Other spheres were disrupted into a signal-cell solution to assess whether a second sphere could form.

Tumor implantation in nude BALB/c mice. Cells were collected and suspended and the concentration was adjusted with culture medium. The cells were injected into the left side of the front leg of BALB/c nude mice (6 weeks; weight, 18-22 g) obtained from the Animal Research Center, Harbin Medical University, China. The mice were separated into 10 groups of 5 animals and received varying concentrations of the $ALDH^+$ or $ALDH^-$ cells. After 6 weeks, the mice were sacrificed and any tumors were removed for histopathological and immunohistochemical analysis. Tumor samples were also digested using collagenase II (Sigma-Aldrich, St. Louis, MO, USA) and re-injected into mice to generate second-round tumors. Data were collected from three independent experiments.

Histopathological and immunohistochemical analysis of xenografts. The $ALDH^+$ and $ALDH^-$ tumors were placed in flasks with 10% formalin, and then embedded in paraffin. Sections were cut and stained with hematoxylin and eosin (H&E) using a standard protocol to assess tumor type. An ALDH1 antibody (Cell Signaling Technology, Danvers, MA, USA) was used to determine the ALDH1 expression levels in the tumor by immunohistochemistry.

Chemoresistance of cell monolayers and spheres. Cells were transferred to 96-well plates at a density of $2x10^3$ cells/well in RPMI-1640 culture medium with 10% FBS. Increasing concentrations of the drugs doxorubicin (DXR) or cisplatin (CDDP) (Sigma-Aldrich) were added in triplicate and incubated for 40 h (1, 5 and 10 μM). CCK-8 (10 μl) was placed in each well. The OD value was measured at 450 nm. Cell viability was measured using the Cell Titer 96 AQueous One Solution Cell Proliferation Assay kit (Promega), according to the manufacturer's instructions. In addition, we compared drug resistance between the $ALDH^+$ spheroid and adherent cells.

Table I. PCR primers used in this study.

Target gene	Primer sequences	Size (bp)
c-Myc	F: 5'-TCCCTCCACTCGGAAGGAC-3' R: 5'-CTGGTGCATTTTCGGTTGTTG-3'	96
Nanog	F: 5'-TTTGTGGGCCTGAAGAAAACT-3' R: 5'-AGGGCTGTCCTGAATAAGCAG-3'	116
Sox-2	F: 5'-GCCGAGTGGAAACTTTTGTCG-3' R: 5'-GGCAGCGTGTACTTATCCTTCT-3'	155
Bmi-1	F: 5'-CGTGTATTGTTCGTTACCTGGA-3' R: 5'-TTCAGTAGTGGTCTGGTCTTGT-3'	82
ABCG2	F: 5'-ACGAACGGATTAACAGGGTCA-3' R: 5'-CTCCAGACACACCACGGAT-3'	93
STAT3	F: 5'-CAGCAGCTTGACACACGGTA-3' R: 5'-AAACACCAAAGTGGCATGTGA-3'	150
ALDH1	F: 5'-GCACGCCAGACTTACCTGTC-3' R: 5'-CCTCCTCAGTTGCAGGATTAAAG-3'	129
Oct3/4	F: 5'-GTGTTCAGCCAAAAGACCATCT-3' R: 5'-GGCCTGCATGAGGGTTTCT-3'	156
β-actin	F: 5'-CATGTACGTTGCTATCCAGGC-3' R: 5'-CTCCTTAATGTCACGCACGAT-3'	250

F, forward; R, reverse; ALDH1, aldehyde dehydrogenase 1.

Quantitative PCR (qPCR). Total RNA was extracted from both the $ALDH^+$ and $ALDH^-$ cells. We quantified the mRNA transcription levels of highly expressed genes in stem cells, i.e., *c-Myc*, *Bmi-1*, *Sox2*, *Nanog*, *Oct3/4*, and *STAT3*. We also assessed the expression levels of the drug-resistant genes, *ABCG2* and *ALDH1*. β-actin was used as an internal control. The primer sequences used for amplification are listed in Table I.

Western blotting. Western blotting was performed as described previously, with some modifications (18). Briefly, the cells were washed twice with cold PBS and lysed in buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% SDS, 1% Na deoxycholate, 1 mM Na vanadate and protease inhibitors (5 mg/ml pepstatin, 1 mM PMSF, 10 mg/ml leupeptin and 1 mM NaF; Sigma-Aldrich) for 1 h on ice. After centrifugation at 13,000 x g for 10 min at 4˚C, the supernatant protein concentrations were measured using the BCA Protein Assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Membranes were blocked with non-fat milk for 1 h at room temperature and incubated overnight at 4˚C with the corresponding antibodies in Tris-buffered saline (TBS) with 5% BSA (Sigma-Aldrich) and 0.1% Tween-20 (Bio-Rad Laboratories, Hercules, CA, USA). After washing three times in TBS with 0.1% Tween-20, the blots were incubated with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology , Danvers, MA, USA; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Immunoreactive bands were detected with the ECL

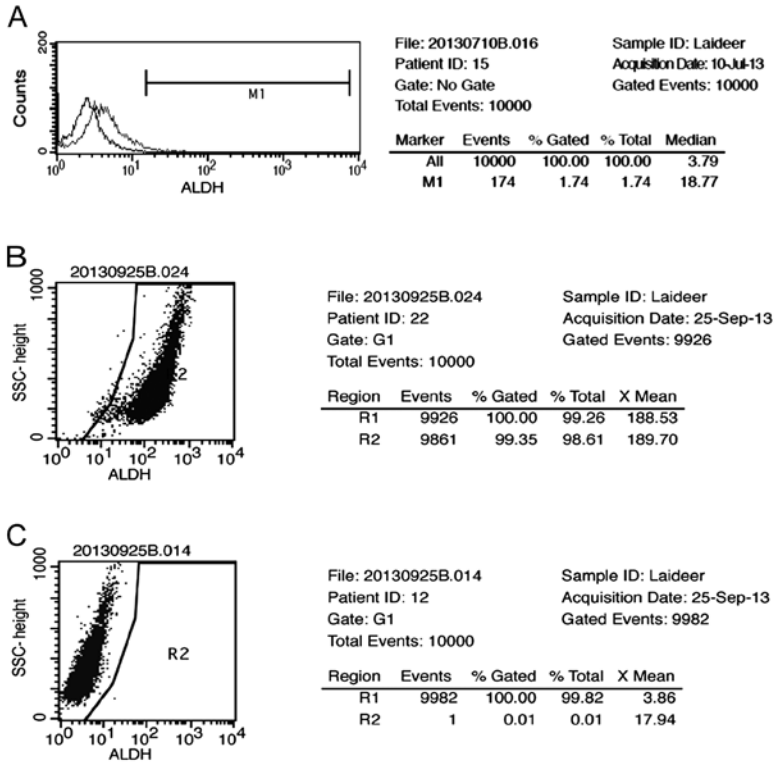

Figure 1. Isolation of an NMFH-1 cell population with increased ALDH activity. (A) FACS analysis of ALDH activity in NMFH-1 cells. Aldefluor assay determined that 1.74% of NMFH-1 cells express high ALDH activity (M1). (B and C) The purity of the ALDH$^+$ (R2, 98.61%) and ALDH$^-$ (R1, 99.82%) cell populations was determined after flow separation. ALDH, aldehyde dehydrogenase.

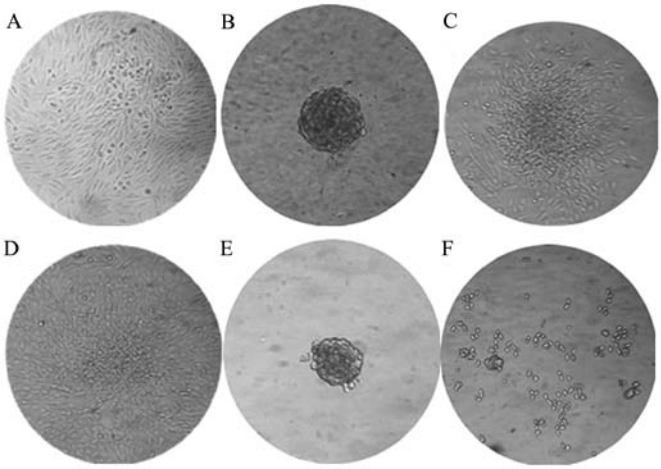

Figure 2. Sphere formation occurs more readily from cell populations with increased ALDH activity. (A) Micrograph of cells with the ALDH enzymatic activity in a monolayer. (B) The formation of a cloned ball appeared after incubating the ALDH$^+$ cells in serum-free suspension medium for 5 days. These spheres were then seeded in serum-containing medium, and adherent cells expanded after (C) 12 h and (D) 48 h. (E) Dissociated ALDH$^+$ sphere cells also had a capacity to form secondary spheres. (F) By contrast, a longer incubation period (10 days) was required to observe smaller spheres from ALDH$^-$ cells. ALDH, aldehyde dehydrogenase.

Plus SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Waltham, MA, USA) for 60 sec. Protein levels were normalized with respect to the band density of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The primary antibodies used were: anti-ABCG2 (1:1,000), anti-Bmi-1 (1:1,000), anti-c-Myc (1:1,000), anti-Nanog (1:1,000), anti-Oct3/4 (1:1,000), anti-Sox2 (1:1,000), anti-STAT3 (1:1,000), and anti-ALDH1 (1:1,000) (all from Cell Signaling Technology), and anti-GAPDH (1:5,000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

Statistical analysis. The statistical software SPSS 20.0 (SPSS Inc., Chicago, IL, USA) was used for data processing and analyzing. Data are presented as the means ± SD, and a comparison was carried between experimental groups of qPCR analysis using one-way ANOVA. The results of the chemosensitivity assay were calculated and compared using one-way ANOVA. $P<0.05$ was considered statistically significant.

Results

Separation and differentiation of the ALDH$^+$ and ALDH$^-$ populations from the human NMFH-1 cell line. We assessed the presence and size of the NMFH-1 cell population with the ALDH enzymatic activity by Aldefluor assay. A small (1.74%) but detectable cell population was identified (Fig. 1A). The ALDH$^+$ and ALDH$^-$ cells were isolated by FACSAria-based cell sorting, and the content of each population was verified after separation. The ALDH$^+$ population reached 98.61% purity (Fig. 1B), and the ALDH$^-$ population reached 99.82% purity (Fig. 1C).

ALDH$^+$ cells exhibit enhanced sphere formation. The ALDH$^+$ cells formed many spheroids when cultured in suspension with serum-free medium. This method was initially developed to select neural stem cells, but has been adapted as a

Table II. Tumor-initiating capacity of ALDH+ and ALDH- populations.

	Cell planting				
Populations	$1x10^3$	$5x10^3$	$5x10^4$	$5x10^5$	$5x10^6$
ALDH+	1/5	2/5	4/5	5/5	5/5
ALDH-	0/5	0/5	1/5	3/5	5/5

ALDH, aldehyde dehydrogenase.

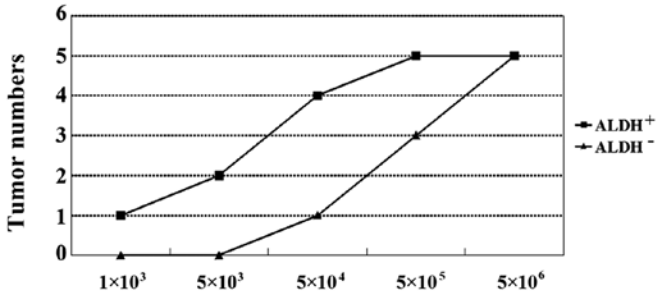

Figure 3. The ALDH+ and ALDH- subpopulations have different tumor initiation capacities. Different concentrations of cells were injected into BALB/c nude mice and tumor formation was determined 6 weeks later. The symbol (■) represents the ALDH+ cells, and the symbol (▲) represents ALDH- cells. ALDH, aldehyde dehydrogenase.

general tumor-initiating cell selection method. The cells grew normally under these conditions (Fig. 2A), but growth in EGF/bFGF-supplemented medium has been shown to allow spherical clones to develop from normal cells and CSCs of epithelial origin. The ALDH+ cells formed a small sphere on the third day, which became clearly visible by the fifth day (Fig. 2B). In comparison, 10 days were required for the ALDH- cells to develop spheres that were smaller than those formed by the ALDH+ populations (Fig. 2F). The ALDH+ spheres could be transferred into complete media containing serum and the cells were able to adhere for continued growth (Fig. 2C and D).

ALDH+ cells exhibit enhanced tumor development in BALB/c nude mice. The implantation of cancer cells into nude mice can lead to tumor development. In order to evaluate the tumor development potential of the ALDH+ and ALDH- cells, we injected them into BALB/c nude mice to generate xenografts. Six weeks following the injection, the mice were euthanized and assayed for tumor development (Table II). As few as $1x10^3$ ALDH+-injected cells were required for tumor growth. By contrast, $5x10^4$ ALDH- cells were required to develop similar tumors (Figs. 3 and 4A and B). H&E staining indicated that the ALDH+ and ALDH--induced tumors have histological differences. Furthermore, ALDH+-induced tumors had a higher ALDH1 expression as determined by immunostaining (Fig. 4C-F). These results suggested that ALDH1 may be the gene responsible for the selected ALDH enzymatic activity.

Drug efficacy on cell monolayers and spheres. The results showed DXR and CDDP have dose-dependent effects on cell survival. The ALDH+ and ALDH- cell survival rate was determined after 48-h drug treatments (Table III and IV, Fig. 5). DXR and CDDP killed the two cell populations; however, the ALDH+ cells were more resistant to the drugs than the ALDH- cells. In order to further validate the resistance of the ALDH+ cells, we compared the drug response of spheroid and adherent cells. The rate of growth inhibition of the ALDH+ spheroids was slightly higher than that of adherent cells in response to the two drugs (Table IV, Fig. 5C and D).

qPCR. Stemness and drug transporter genes are characteristic of CSCs. To determine whether the NMFH-1 subpopulations shared these characteristics, we measured the expression of the *c-Myc*, *Bmi-1*, *Sox2*, *Nanog*, *Oct3/4*, *STAT3*, *ABCG2* and *ALDH1* genes (19-21). The ALDH+ population had increased levels of ALDH1 gene expression. Therefore the CSC population that we isolated can also be referred to as ALDH1+. We found that Bmi-1 expression was not significantly different between the ALDH+ and ALDH- cells. However, the expression of *c-Myc*, *STAT3*, *Sox2*, *Nanog*, *Oct3/4*, and *ABCG2* was significantly increased in the ALDH+ cells as measured by mRNA transcript levels (Fig. 6A). However, the expression of Oct3/4 protein was not higher in the ALDH+ cells compared with the ALDH- cells (Fig. 6B). The transcription and protein expression of the ABCG2 drug transporter was significantly increased in the ALDH+ cells compared with the ALDH- cells. Thus, these data demonstrated that the NMFH-1 cells have an ALDH+ subpopulation that expresses genes highly associated with stem cells.

Discussion

Tumors are difficult to cure due to the existence of CSCs, which have the potential for self-renewal, unlimited proliferation, and differentiation into tumor cells (1,2). Previous findings confirm that CSCs exist in many tumor tissues. However, the isolation of CSCs to develop improved cancer treatments in the future remains to be investigated.

In 1996, the identification of a side population (SP) of cells was proposed as a new method for stem cell separation. The SP, defined by Hoechst 33342 dye exclusion, was identified as a distinct subset of cells. These cells have subsequently been isolated and designated CSCs because they possess stemness characteristics and are responsible for tumorigenesis in several cancer types (22-25).

In 2003, Al-Hajj *et al* (26) isolated CSCs (~2%) from breast cancer specimens with the cell surface markers $CD24^-CD44^+Lin^-$, and verified that these cells were highly tumorigenic. Another series of cell surface markers, such as CD133, CD90, CD44, CD34, and CD38 have also been used to isolate CSCs (27-31). These specific surface markers are mostly present in solid tumors. However, stem cells from different tissues may express unique markers.

ALDH1 has recently been identified as a marker for CSCs in humans, because many types of stem cells express ALDH1 at high levels. This observation has been confirmed in breast stem cells, HSCs, neural stem cells, prostate, colon and lung CSCs (6,7,32-36). As a functional protease, the gene is more common than the cell surface markers. Confirmation that all

Table III. $ALDH^+$ and $ALDH^-$ cell survival rates (%) after 48-h treatment with DXR or CDDP.

	DXR			CDDP		
	1 μM	5 μM	10 μM	1 μM	5 μM	10 μM
$ALDH^+$	94.92±2.16	84.69±3.41	76.34±4.99	98.19±1.67	88.57±1.17	72.40±1.60
$ALDH^-$	86.18±1.39	64.56±3.83	64.56±1.39	85.25±2.26	73.31±3.48	64.08±1.14
F-value	11.86	89.68	49.78	52.44	51.88	11.15
P-value	0.026	0.001	0.002	0.002	0.002	0.029

Values are presented as means ± SD. ANOVA, P<0.05, statistical significance; ALDH, aldehyde dehydrogenase; DXR, doxorubicin; CDDP, cisplatin.

Table IV. Spherical and adherent cell survival rates (%) after 48-h treatment with DXR or CDDP.

	DXR			CDDP		
	1 μM	5 μM	10 μM	1 μM	5 μM	10 μM
Sphere	96.4±0.78	95.95±2.58	85.90±2.22	98.11±1.68	96.80±2.43	85.58±3.77
Adhere	94.92±2.16	84.69±3.41	76.34±4.99	98.19±1.67	88.57±1.17	72.40±1.60
F-value	1.035	20.83	9.019	0.000	28.00	31.11
P-value	0.310	0.010	0.039	0.960	0.006	0.005

Values are presented as means ± SD. ANOVA, P<0.05, statistical significance; ALDH, aldehyde dehydrogenase; DXR, doxorubicin; CDDP, cisplatin.

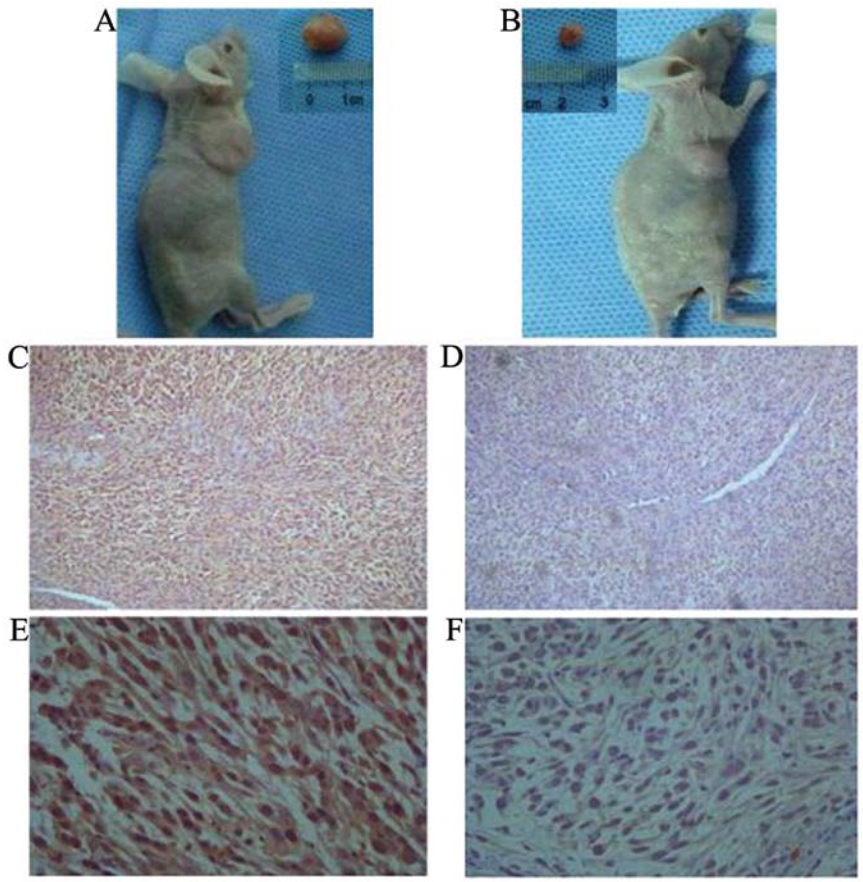

Figure 4. Analysis of tumors grown from the $ALDH^+$ and $ALDH^-$ subpopulations. (A and B) Images show the mice and resected tumors grown from the $ALDH^+$ and $ALDH^-$ cell populations. (C-F) Tumors were resected and prepared for histology. Left panel: H&E staining; right panel: expression of ALDH1. ALDH1, aldehyde dehydrogenase 1.

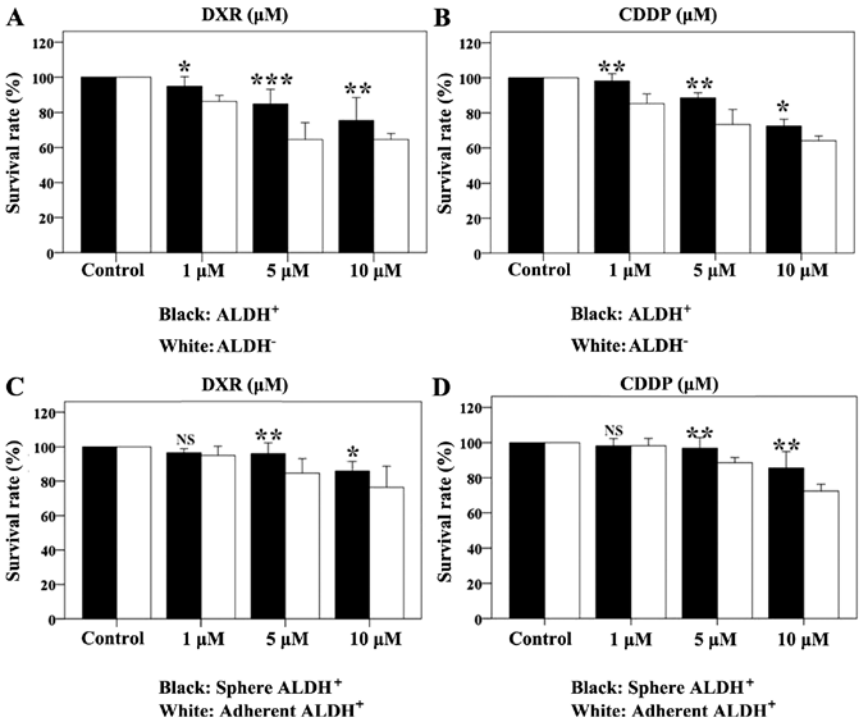

Figure 5. Drug resistance of the ALDH+ and ALDH- cells. The ALDH+ cells were highly resistant to (A) DXR and (B) CDDP in comparison with the ALDH- cells. The spheroids were significantly more resistant to (C) DXR and (D) CDDP for both ALDH+ and ALDH- cells. (means ± SD; NS, no significant statistical difference; *P≤0.05, **P≤0.01, and ***P≤0.00). ALDH, aldehyde dehydrogenase; DXR, doxorubicin; CDDP, cisplatin.

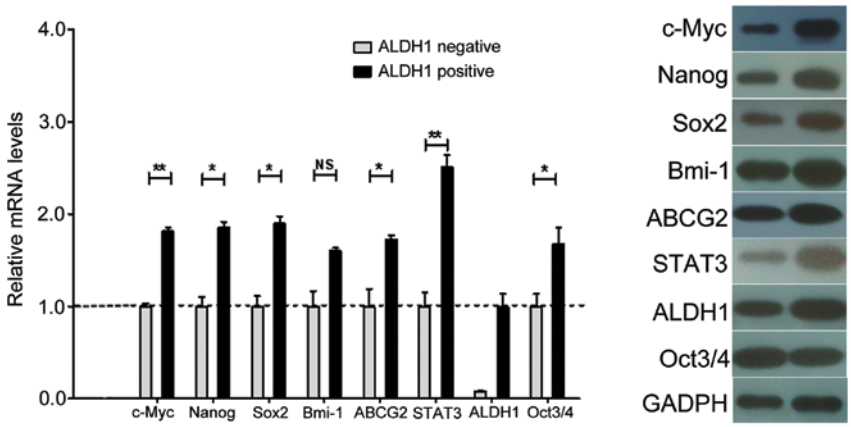

Figure 6. The expression profile was determined for a subset of genes in the ALDH+ and ALDH- populations. Left panel: the qPCR mRNA analysis of genes characteristically expressed in stem cells, such as the drug transporter ABCG2, in the ALDH+ and ALDH- populations. Right panel: western blot analysis confirmed the qPCR results at the level of protein expression. GAPDH was used as a loading control. (means ± SD; NS, no significant statistical difference; *P≤0.0, **P≤0.01, and ***P≤0.001; relative mRNA levels). ALDH, aldehyde dehydrogenase; qPCR, quantitative PCR.

the stem cells express high levels of ALDH1+ may provide a reliable method for the isolation of the CSCs. Our results show that 1.74% of the NMFH-1 cells are ALDH+. We also found that the survival rate following the freeze-thaw of the ALDH+ cells was significantly higher than that in the ALDH- cells. However, subsequent generations of these cells did not have significant growth defects. It is possible that the low temperature had a specific-temporary effect on the growth of the ALDH- cells.

The results showed that the ALDH+ NMFH-1 cells are more capable of forming spheres when cultured in serum-free medium than the ALDH- cells, and these cells can form spheres a second time. The ALDH- cells required longer time periods and formed smaller spheres. These spheres may have been formed by the CSCs of other subpopulations marked by CD133, CD44 or other markers. It is possible that ALDH selection could isolate most of the stem cells but not all of them; however, this remains to be demonstrated. The ability of the ALDH+ cells to form tumors in nude mice is very apparent. Briefly, $1x10^3$ ALDH+ cells grown in nude mice subcutaneously formed a tumor, whereas $5x10^4$ ALDH- cells did not form tumor in nude mice. Furthermore, the ALDH+ cells formed larger tumors than the ALDH- cells during the same time-period. Previous studies have indicated that 500 ALDH+ cells can form tumors

but 5,000 ALDH$^-$ cells are required to form tumors (6). Our experiments required more cells which may be an indication of differences in the laboratory conditions, personnel operation, or experimental design. Immunohistochemistry confirmed that the tumors developed from the ALDH$^+$ cells expressed high levels of the ALDH1 protein in mice. By contrast, low levels of the ALDH1 protein were expressed in tumors developed from the ALDH$^-$ cells. Thus, it can be hypothesized that the ALDH1$^+$ cells contribute to tumor formation, which is consistent with these cells being CSCs. In the present study, ALDH1 expression was maintained even after several rounds of division *in vivo*. However, in ordinary tumor cells, the content of ALDH1 is very limited. Survival competition of the ALDH1$^-$ cells may account for this phenomenon by secreting some factors that limit the ALDH1$^+$ cell proliferation. However, this remains an open area of research.

We compared the expression of genes that play a prominent role in stem cell maintenance, self-renewal, and nuclear reprogramming. These included *c-Myc*, *Bmi-1*, *Sox2*, *Nanog*, *Oct3/4* and *STAT3* in ALDH$^+$ and ALDH$^-$ cells. If these cells expressed these genes at high levels, they could not all be defined as CSCs. We used ALDH enzymatic activity as the marker for isolation and we detected *ALDH1* gene expression in both the ALDH$^+$ and ALDH$^-$ cells. qPCR assays showed that the expression of *c-Myc* and *STAT3* was significantly different between populations ($P<0.01$). c-Myc regulates the G0-G1 cell cycle transition and promotes cell division. It has also been shown to control infinite rounds of proliferation and the development of tumor formation. Many growth factors can stimulate fibroblast cells, which can lead to an enhanced c-Myc expression. Bmi-1 emerged as a Myc-cooperating oncogene (37-39). STAT3 is a signal transduction factor and an important member of a family of activating factors. The STAT signaling pathways are closely associated with cell proliferation, differentiation and apoptosis. Activation of the pathway can lead to abnormal cell proliferation and malignant transformation (35,40). The expression of Sox2, Nanog, and Oct3/4 was significantly different between the ALDH$^+$ and ALDH$^-$ populations ($P<0.05$). Sox2 and Oct3/4 can cooperate to control fibroblast growth factor 4 (FGF4). FGF4 is a signaling molecule that plays an important role in embryonic development, and the *FGF4* gene has a specific enhancer element in the 3'UTR (41-43). Nanog encodes a recently identified divergent homeoprotein that controls cell self-renewal (44).

In the drug resistance experiment, we found that the NMFH-1 ALDH$^+$ cells were more resistant than the ALDH$^-$ cells. Moreover, the ALDH$^+$-derived spheres showed greater resistance than the ALDH$^+$ adherent cells. In clinical practice, MFH is difficult to cure and is not sensitive to these drugs. Comprehensive surgery, radiation, and chemotherapy are known treatment methods used to improve the resection and survival rates, and reduce the local recurrence rate of MFH (45,46). ABCG2 expression is reported to significantly contribute to the CSC phenotype, strongly correlate with drug resistance, and indicate a poor clinical outcome (47,48). The mRNA expression of the *ABCG2* gene between populations had a significant difference, as shown by the RT-PCR results. In the ALDH$^+$ cells, the ABCG2 protein expression was higher than that in the ALDH$^-$ cells, which potentially accounts for ALDH$^+$ cell drug resistance.

In conclusion, our study is the first to successfully isolate the ALDH$^+$ subpopulation from the NMFH-1 cell line. The experiments also show that the ALDH$^+$ subpopulation exhibits several characteristic CSC properties, including high clonogenicity and self-renewal, increased chemotherapeutic drug resistance, elevated expression of stemness and drug transporter genes and high tumorigenic potential. The above results show that ALDH1 can be used as a marker for isolation of the CSCs from the NMFH-1 cell line. We hypothesize that ALDH1 may be used as a biomarker for stem cell specificity and applied to other tumors. ALDH1 expression may also play a future role in the development of clinical treatments.

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