Aldehyde Dehydrogenase 1, a Potential Marker for Cancer Stem Cells in Human Sarcoma

Birgit Lohberger1*, Beate Rinner2, Nicole Stuendl1, Markus Absenger2, Bernadette Liegl-Atzwanger3, Sonja M. Walzer4, Reinhard Windhager4, Andreas Leithner1

* E-mail: birgit.lohberger@medunigraz.at

1 Department of Orthopaedic Surgery, Medical University of Graz, Graz, Austria, 2 Center for Medical Research, Medical University of Graz, Graz, Austria, 3 Institute of Pathology, Medical University of Graz, Graz, Austria, 4 Department of Orthopaedic Surgery, Medical University of Vienna, Vienna, Austria

Abstract

Tumors contain a small population of cancer stem cells (CSC) proposed to be responsible for tumor maintenance and relapse. Aldehyde dehydrogenase 1 (ALDH1) activity has been used as a functional stem cell marker to isolate CSCs in different cancer types. This study used the Aldefluor® assay and fluorescence-activated cell sorting (FACS) analysis to isolate ALDH1high cells from five human sarcoma cell lines and one primary chordoma cell line. ALDH1high cells range from 0.3% (MUG-Chor1) to 4.1% (SW-1353) of gated cells. Immunohistochemical staining, analysis of the clone formation efficiency, and xCELLigence microelectronic sensor technology revealed that ALDH1high cells from all sarcoma cell lines have an increased proliferation rate compared to ALDH1low cells. By investigating of important regulators of stem cell biology, real-time RT-PCR data showed an increased expression of c-Myc, β-catenin, and SOX-2 in the ALDH1high population and a significant higher level of ABCG2. Statistical analysis of data demonstrated that ALDH1high cells of SW-982 and SW-1353 showed higher resistance to commonly used chemotherapeutic agents like doxorubicin, epirubicin, and cisplatin than ALDH1low cells. This study demonstrates that in different sarcoma cell lines, high ALDH1 activity can be used to identify a subpopulation of cells characterized by a significantly higher proliferation rate, increased colony forming, increased expression of ABC transporter genes and stemness markers compared to control cells. In addition, enhanced drug resistance was demonstrated.

Citation: Lohberger B, Rinner B, Stuendl N, Absenger M, Liegl-Atzwanger B, et al. (2012) Aldehyde Dehydrogenase 1, a Potential Marker for Cancer Stem Cells in Human Sarcoma. PLoS ONE 7(8): e43664. doi:10.1371/journal.pone.0043664

Editor: Javier S. Castresana, University of Navarra, Spain

Received April 19, 2012; Accepted July 23, 2012; Published August 23, 2012

Copyright: © 2012 Lohberger et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: Financial support by the Medical University of Graz (START grant), OENB grant (s.i.14356) and “EccoCell” grant is gratefully acknowledged. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: birgit.lohberger@medunigraz.at

Introduction

The cell population of most tumors is heterogeneous with regard to its proliferation capacity and the ability to initiate tumor formation in immune-deficient mice. A cancer stem cell (CSC) is defined as a cell within a tumor that possesses the capacity to self-renew and to generate the heterogeneous lineages of cancer cells that comprise the tumor [1,2]. Numerous investigations have provided evidence that CSCs exist in a variety of human tumors such as hematopoietic malignancies, brain tumors, breast cancer, and gastroenterological cancer [3,4,5,6].

Cytosolic aldehyde dehydrogenases (ALDHs) are a group of enzymes involved in oxidizing a wide variety of intracellular aldehydes into their corresponding carboxylic acids [7]. Among these enzymes, ALDH1 is thought to have an important role in oxidation of alcohol and vitamin A and in cyclophosphamide chemotherapy. Gimestier et al. [8] showed that ALDH1 was a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome of breast cancer patients. High ALDH1 activity has been used to define stem cell populations in many cancer types including human multiple myeloma, acute myeloid leukemia [8], pancreatic cancer [9], and breast cancer [10]. Therefore, ALDH1 activity might be used as a common marker for malignant stem cell populations [11].

Failure of cancer chemotherapy can occur through increased efflux of chemotherapeutic agents, leading to the reduction of intracellular drug levels and consequent drug insensitivity. ABC transporters have the capacity to export many cytotoxic drugs and recent evidence suggests that the cancer stem cell phenotype is associated with high-level expression of the ABCG2 transporter [12,13,14].

In this study, we used the Aldefluor® assay and fluorescence-activated cell sorting (FACS) analysis to isolate ALDH1high cells from five human sarcoma cell lines and one recently established chordoma cell line. We analyzed ALDH1high cells in vitro for their repopulation capacity, clonogenicity, cell proliferation properties, the expression of stem cell markers and ABC transporters, and their multidrug resistance capacities.

Materials and Methods

Cell Culture

All human sarcoma cell lines (SW-684, SW-872, SW-982, SW-1353, and TE-671) were obtained from CLS (Eppelheim, Germany) and cultured in Dulbecco’s-modified Eagle’s medium (DMEM-F12) containing 10% foetal bovine serum (FBS), 1% L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 0.25 μg amphotericin B. MUG-Chor1 cells were cultured in...
IMDM/RPMI 1649 (4:1) (PAA, Pasching, Austria) supplemented
with 1% L-glutamine and 1% ITS (PAA). All cell incubation was
carried out at 37°C in a humidified atmosphere of 5% CO₂ and
cultures are periodically checked for mycoplasma. Culture
medium and supplements were purchased from GIBCO®,
Invitrogen (Darmstadt, Germany).

Aldefluor® Assay and Separation of the ALDH1+ Cell
Population by FACS Analysis

Aldehyde dehydrogenase (ALDH) enzyme activity in viable cells
was determined using a fluorogenic dye based Aldefluor® assay
(Stem Cell Technologies, Grenoble, France) according to the
manufacturer’s instructions. 1×10^6/ml cells were suspended in
Aldefluor® assay buffer containing ALDH substrate (Bodipy-
Aminoacetaldehyde) and incubated for 45 min at 37°C. As a
reference control, the cells were suspended in buffer containing
Aldefluor® substrate in the presence of diethylaminobenzaldehyde
(DEAB), a specific ALDH1 enzyme inhibitor. The brightly
fluorescent ALDH1-expressing cells (ALDH1high) were detected
in the green fluorescence channel (520–540 nm) of FACSAria (BD
Biosciences, San Diego, CA) and the data was analyzed using
FACS DIVA software (BD Biosciences). To exclude nonviable
cells propidium iodide (PI; Sigma Aldrich, Vienna, Austria) was
added at a final concentration of 2 μg/ml.

Repopulation Assay

To compare the repopulation ability of sarcoma ALDH1high
 cells with ALDH1low cells in vitro, freshly sorted cells were cultured
separately under the same culture condition. After 2 weeks, cells
were re-stained with the Aldefluor® assay and reanalyzed via
FACSAria (BD Biosciences).

Western Blot Analysis

For total protein analysis, cells were re-suspended in lysis buffer
(50 mM Tris-HCl pH 7.4, 150 mM NaCl, 50 mM NaF, 1 mM
EDTA, 10% NP-40, 1% Triton-X and protease inhibitors),
incubated on ice for 10 min and centrifuged at 15,000 rpm for
15 min. Aliquots of protein extracts (20 μg) were separated on
12% SDS-PAGE and electro-blotted onto 0.45
μm Hybond ECL
nitrocellulose membrane (Amersham Biosciences, Little Chalfont,
UK). The membrane was blocked with 3% milk blocking buffer
for 1 h and then incubated with the primary antibodies for 2 h at
room temperature. As the primary antibody, rabbit polyclonal
ALDH1/2 antibody (#sc50385; Santa Cruz Biotechnology, Santa
Cruz, CA) was used. The major liver isoform ALDH1 localized to
cytosolic space, while ALDH2 localized to the mitochondria. The
blots were developed using horseradish peroxidase-conjugated
secondary antibodies (Dako, Vienna, Austria) at room temperature
for 1 h and the SuperSignal® West Pico Chemoluminescent
Substrate (Thermo Scientific, Rockford, IL), in accordance with
the manufacturers’ protocol.

Immunohistochemistry

Each 1×10^4 ALDH1high and ALDH1low cells were seeded in
polystyrene culture slides (BD Biosciences), fixed with 4%
formalin/PBS solution, and dehydrated in an ascending series of
alcohol. Immunohistochemical (IHC) studies using the streptavi-
din-biotin peroxidase complex method were carried out employing
antibody against the anti-
Ki-67 (clone 30-9) rabbit monoclonal
primary antibody (Ventana Medical Systems, Tucson, AZ) using
the BenchMark Ultra instrument (Ventana Medical Systems).
Cells were imaged using an Olympus BX51 microscope with
Olympus DP71 microscope digital camera. The stained slides
were digitally scanned and positive and negative cells were
quantified using the ImageScope software (ImageScope Virtual
Slide, version 6.25, Aperio Technol.,Vista, CA). The positivity
= N positive cells/N total cells.

| Table 1. Primer Sequences used for real-time RT-PCR. |
| --- | --- | --- | --- |
| target gene | name | primers | oligonucleotide sequence 5’-3’ |
| ABCG2/BCRP1 | breast cancer resistance protein | forward | ACC TGA AGG CAT TTA CTG AA |
| | | reverse | TCT TCC CTT GCA GCT AAG AC |
| ABCA2 | ABCA2 | forward | AGA TGG ACA AGA TGA TCG AG |
| | | reverse | GCT TGT ACT TCA GGA TGA GG |
| ABCB1/MDR1 | multidrug resistance protein | forward | GAG GAA GAC ATG ACC AGG TA |
| | | reverse | CTG TCG CAT TAT AGC ATG AA |
| c-Myc | v-myc myelocytomatosis | forward | GGA AGC AGC TAA AAC GGA GCT |
| | | reverse | GCC CTT TTC ATT GTT TTC CAA CT |
| β-catenin | cadherin-associated protein | forward | CGA GCC GAC ACC AAG AAG |
| | | reverse | CGA ATC AAT CCA ACA GTA GCC |
| SOX-2 | SRY (sex determining region | forward | CGA GTG GAA ACT TTT GTT GGA |
| | | reverse | TGT GCA GCC CTC GCA G |
| GAPD | glyceraldehyde 3-phosphate | forward | AAGGTCGGAGTCAACGGA |
| | | reverse | ACCAGTTAAAAAGGCACCT |
| hprt-n | hypoxanthine | forward | ATGGGAGGCCATACATTT |
| | | reverse | ATGTAATCCAGCGAGCTACGAA |
| ACTB | β-actin | forward | CGTAAAGGTTAGGGTACA |
| | | reverse | AAGGGACTTCCCTGATAACATGCA |
The xCELLigence DP device from Roche Diagnostics (Mannheim, Germany) can be used to quantitatively and dynamically monitor cell proliferation in real-time [15]. Respectively 1 × 10⁴ freshly sorted ALDH1<sup>high</sup> and ALDH1<sup>low</sup> cells were seeded in electronic microtiter plates (E-Plate<sup>TM</sup>; Roche Diagnostic) and measured for 72 h with the xCELLigence system according to the instructions in the user’s manual. Application of a low-voltage (less

Figure 1. Aldehyde dehydrogenase 1 (ALDH1) expression in sarcoma cell lines using the Aldefluor<sup>®</sup> assay. Fluorescence versus forward scatter was shown in a density blot from (A) DEAB control cells and (B) ALDH1-expressing cells (called ALDH1<sup>high</sup>). (C) ALDH1 expression in % of gated cells. The highest proportion of ALDH1<sup>high</sup> cells is represented by SW-684 cells (1.77 ± 0.9%; n = 12), SW-982 cells (2.23 ± 1.0%; n = 11), and SW-1353 cells (2.69 ± 1.3%; n = 8). (D) After two weeks cultured the ALDH1<sup>high</sup> population generated a significant higher account of ALDH1<sup>high</sup> cells. (E) The enhanced ALDH activity was also demonstrated by western blot. doi:10.1371/journal.pone.0043664.g001

xCELLigence System

The xCELLigence DP device from Roche Diagnostics (Mannheim, Germany) can be used to quantitatively and dynamically monitor cell proliferation in real-time [15]. Respectively 1 × 10⁴ freshly sorted ALDH1<sup>high</sup> and ALDH1<sup>low</sup> cells were seeded in electronic microtiter plates (E-Plate<sup>TM</sup>; Roche Diagnostic) and measured for 72 h with the xCELLigence system according to the instructions in the user’s manual. Application of a low-voltage (less
Table 2. Overview of all results including the corresponding significances.

| Method                  | SW-684 ALDH1<sup>high</sup> | SW-684 ALDH1<sup>low</sup> | SW-982 ALDH1<sup>high</sup> | SW-982 ALDH1<sup>low</sup> | SW-1353 ALDH1<sup>high</sup> | SW-1353 ALDH1<sup>low</sup> |
|-------------------------|-------------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|                        | % ALDH1<sup>high</sup> cells  | 1.77±0.9                    | 2.23±1.0                    | 2.69±1.3                    |                             |                             |
|                        | % ALDH1<sup>low</sup> cells   | 41.7±18.6                   | 52.5±8.7                    | 31.7±11.1                   |                             |                             |
|                        | Repopulation assay p-value    | p=0.0039                    | p=4.4E-7                    | p=0.0025                    |                             |                             |
|                        | Colony formation assay p-value |                             |                             |                             |                             |                             |
|                        | Ki-67 positivity (%)          | 67.17%                      | 32.83%                      | 55.34%                      | 44.66%                      | 72.57%                      | 27.43%                      |
|                        | ALDH1<sup>high</sup> western blot |                             |                             |                             |                             |                             |
|                        | ALDH1<sup>high</sup> expression (%) | 67.17%                      | 32.83%                      | 55.34%                      | 44.66%                      | 72.57%                      | 27.43%                      |
|                        | Relative gene expression      |                             |                             |                             |                             |                             |
|                        | β-catenin expression (%)      | 0.98±0.82                   | 0.87±0.87                   | 0.41±0.06                   | 0.81±0.18                   | 1.16±0.13                   | 0.95±0.37                   |
|                        | SOX-2 expression (%)          | 25.2±19.4                   | 9.66±2.77                   | 1.96±1.29                   | 1.02±0.59                   | 4.37±3.83                   | 39.82±28.61                 |
|                        | ABCG2/BCRP1 expression (%)    | 2.42±1.56                   | 1.05±0.15                   | 2.28±0.78                   | 1.15±0.29                   | 4.38±0.73                   | 1.76±0.23                   |
|                        | ABCA2 expression (%)          | 1.04±0.34                   | 0.94±0.32                   | 3.42±1.37                   | 3.12±0.25                   | 1.88±0.41                   | 0.62±0.17                   |
|                        | ABCB1/MDR1 expression (%)     | 1.66±0.61                   | 1.14±0.28                   | 2.25±1.03                   | 1.08±0.27                   | 11.15±2.55                  | 1.52±0.22                   |
|                        | Doxorubicin IC<sub>50</sub> (μM) | 1.5 μM                      | 1.4 μM                      | 1.8 μM                      | 0.9 μM                      | 1.1 μM                      | 0.7 μM                      |
|                        | Epirubicin IC<sub>50</sub> (μM) | 2.2 μM                      | 2.0 μM                      | 1.3 μM                      | 0.5 μM                      | 1.5 μM                      | 0.7 μM                      |
|                        | Cisplatin IC<sub>50</sub> (μM) | 25.7 μM                     | 24.9 μM                     | 23.7 μM                     | 18.8 μM                     | 30.7 μM                     | 26.2 μM                     |

Real-Time RT-PCR

Real-time RT-PCR was performed according to MIQE criteria [16] to determine the relative expression of the ABC transporter genes ABCG2/BCRP1, ABCA2, and ABCB1/MDR1 and the stemness markers c-Myc, β-catenin, and SOX-2. Total RNA was isolated with RNasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers’ recommended protocol. DNA was digested with 1 U DNase (Fermentas, St.Leon-Rot, Germany) per μg RNA. 1 μg RNA was reverse transcribed using RevertAid cDNA Synthesis Kit (Fermentas). Real-time PCR reactions were performed in triplicates using the Platinum SYBR Green Super Mix with ROX (Invitrogen) on AB7900HT (Applied Biosystems, Invitrogen). The housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-actin (ACTB) and hypoxanthine phosphoribosyltransferase (hprt-n) served as an internal control due to their stable expression in different tissues. Table 1 lists the primers used for real-time PCR. The expression levels were calculated based on the 2–DD<sub>CT</sub> method [17].

Drug Sensitivity Assay

Sorted cells were adjusted to a density of 5×10<sup>3</sup> cells/100 μl and incubated in 96-well microplates. The cells were exposed to

than 20 mV) AC signal leads to the generation of an electric field that interacts with the ionic environment inside the wells of the E-Plates and is differentially modulated by the number of cells in the well, the morphology of the cells, and the strength of cell attachment. Cell density measurements were performed in quadruplicate with a programmed signal detection every 20 min and were normalized to the 6 h time point. Data acquisition and analysis was performed with the RTCA software (version 1.2, Roche Diagnostics).

Colony Formation Assay

To determine the clone formation efficiency (CFE) of sorted cells in vitro, ALDH1<sup>high</sup>, ALDH1<sup>low</sup> cells and unstained cells (control) were counted and 200 cells per well were seeded in six well plates. Triplicate wells were used for each group. Cells were cultured in DMEM-F12 with supplements for 14 days, fixed in methanol for 10 min and stained with crystal violet (Sigma Aldrich, Hamburg, Germany). The clone’s number which consisted of more than 50 cells was counted. The CFE was calculated according to the formula: (the clone number/the plated cell number)×100.

doi:10.1371/journal.pone.0043664.t002
various concentrations of chemotherapeutic drugs (doxorubicin hydrochloride, epirubicin hydrochloride, and cis-diammineplatinum(II)chloride (cisplatin); Sigma Aldrich) for 48 h. Chemotherapeutic drug sensitivity was determined by the MTS assay (Promega, Mannheim, Germany) following the manufacturers’ instructions in quadruplicates using a photometer (Spektramax; BMG Labtech., Offenburg, Germany) at the wavelength of 490 nm. IC50 values were determined from the growth inhibition data.

Statistical Analysis
The outcome variables were expressed as mean ± SD. Student’s unpaired t-test and the exact Wilcoxon’s test was used to evaluate differences between groups with the PASW statistics 18 software (IBM Corporation, Somers, NY). Two-sided P-values below 0.05 were considered statistically significant. IC50 curves were fitted according the Hill equation (sigmoid, 3 parameters). Graphic data were prepared with SigmaPlot® (Systat Software Inc., San Jose, CA).

Results

Sarcoma Cell Lines Display a Distinctive Fraction of ALDH1high Cells
The Aldefluor® assay system has been developed to detect the activity of the ALDH1 isoform. We used this assay followed by FACS analysis to assess the presence and quantity of ALDH1high cell populations in five human sarcoma cell lines and one chordoma cell line [18].

To set a marker for ALDH1high cells DEAB control cells was used to ensure the accuracy of the analysis. The representative SW-1353 cells were treated in the presence of the ALDH1 inhibitor DEAB (Figure 1A) or stained with Aldefluor® reagent, which are defined as ALDH1 high cells (Figure 1B). Sorting experiments have been performed a minimum of five times on each cell line. The amount of ALDH1high cells given in average ± SD was 1.77 ± 0.9% for the fibrosarcoma cell line SW-684 (n = 12), 0.77 ± 0.4% for the liposarcoma cell line SW-872 (n = 5), 2.23 ± 1.0% for the synovial sarcoma cell line SW-982 (n = 11), and 2.69 ± 1.3% for the chondrosarcoma cell line SW-1353 (n = 8) respectively. The chordoma cell line MUG-Chor 1 showed only a small proportion of 1.11 ± 0.5% ALDH1high cells (n = 9) (Figure 1C). We therefore focused on the three sarcoma cell lines SW-684, SW-982, and SW-1353 in the following experiments. In the repopulation assay the ALDH1high population generated a statistic significant higher account of 41.73 ± 18.5% (*p = 0.0039) ALDH1high cells in the SW-684 cells, 52.5 ± 8.75% (*p = 4.39E-07) in the SW-982 cell line, and 31.7 ± 11.1% (*p = 0.0025) (n = 5) in SW-1353 chondrosarcoma cells (Figure 1D). Additional our observations of enhanced ALDH1 expression could be further be substantiated by western blot analysis (Figure 1E; Table 2).

ALDH1high Cells Show Higher Proliferation and Clonogenicity
Using the ImageScope software Ki-67 positive and negative cells were quantified after immunohistochemical staining. ALDH1high cells from all three cell lines have an increased proliferation level compared to ALDH1low cells. Representative staining of SW-982 ALDH1high (Figure 2A) and ALDH1low cells (Figure 2B) are shown and summarized in Table 2 (n = 5). Furthermore, ALDH1high and ALDH1low cells differed significantly in logarithmic growth velocity measured with the xCELLigence-System (Figure 2C–E).

Figure 2. Proliferation analysis of ALDH1high and ALDH1low sarcoma cells. The immunohistochemical analysis using anti-Ki-67 proliferation marker revealed a decreased proliferation level of (A) SW-1353 ALDH1low cells and compared to (B) SW-1353 ALDH1high cells. (C–E) Dynamic proliferation curves for ALDH1high and ALDH1low cells seeded at 10,000 cells per well measured with the xCELLigence system. doi:10.1371/journal.pone.0043664.g002
significantly higher in SW-1353 ALDH1<sup>high</sup> cells compared to corresponding ALDH1<sup>low</sup> cells (*p = 0.0005). For the other two cell lines these effect could also be demonstrated, however in a smaller extent. The higher number of colonies in the SW-684, SW-982, and SW-1353 ALDH<sup>high</sup> cells is presented (Figure 3B).

The mRNA Expression of ABCG2, c-Myc, β-catenin, and SOX-2 are Upregulated in ALDH1<sup>high</sup> Cells

We investigated whether ALDH1<sup>high</sup> cells are enriched for expression of genes that have been postulated to play key roles in stem cell biology, such as c-Myc, β-catenin, and SOX-2 [19]. Quantitative RT-PCR showed increased expression of c-Myc in the ALDH1<sup>high</sup> population, while unsorted control cells (ctrl) and ALDH1<sup>low</sup> cells had only minimal expression (Figure 4A). Similarly, a slight but not significant increase in the expression of β-catenin, and SOX-2 in the ALDH1<sup>high</sup> fraction could be observed (n = 6) (Figure 4B–C).

The relative expression of the three major drug transporters ABCG2/BCRP1, ABCA2, and ABCB1/MDR1 was determined by real-time RT-PCR (n = 5). Interestingly the ALDH1<sup>high</sup> population of all sarcoma cell lines demonstrated, with statistic significance, increased expression levels of ABCG2 compared to control or ALDH1<sup>low</sup> cells (Figure 4D), whereas the p value for ABCA2 was not significant (Figure 4E). In addition, in ALDH1<sup>high</sup> SW-1353 cells a statistic significant higher expression of ABCB1 (p = 0.0302) could be observed (Figure 4F). The 2<sup>−ΔΔCt</sup> values and the corresponding p-values are listed in Table 2.

ALDH1<sup>high</sup> Cells Show Enhanced Drug Resistance

The cancer stem cell hypothesis proposes that the discrepancy between treatment response and patient survival noted in most cancer types reflects an inherent resistance of the cancer stem cells to chemotherapy. To investigate possible differences in drug resistance ALDH1<sup>high</sup> and ALDH1<sup>low</sup> sorted SW-982 and SW-1353 cells were treated with increasing doses of three commonly used chemotherapeutic agents after a two weeks recovery phase. ALDH1<sup>high</sup> SW-982 cells treated for 48 h with 1.0 μM (p = 0.016) and 5.0 μM (p = 0.001) doxorubicin were significantly increased compared with ALDH1<sup>low</sup> cells (Figure 5A). Treatment with 1.0 μM epirubicin (p = 0.045) induced an enhanced drug resis-

![Figure 3. Clonogenic activity of ALDH1<sup>high</sup> and ALDH1<sup>low</sup> cells.](image)

![Figure 4. Relative mRNA expression of stemness markers and ABC transporters genes in ALDH1<sup>high</sup> and ALDH1<sup>low</sup> cells.](image)
tance (Figure 5B). SW-1353 ALDH1high cells showed a similar significant effect after the treatment with 5.0 μM doxorubicin (p = 2.77E-05) (Figure 5D) and 0.5 μM epirubicin (p = 0.039) and 1.0 μM epirubicin (p = 0.021) (Figure 5E). The treatment with cisplatin caused only small differences between ALDH1high and ALDH1low SW-982 and SW-1353 cells (Figure 5C and 5F). In the fibrosarcoma cell line SW-684 no significant differences could be detected. Mean value ±SD of all experiments was fitted according the Hill equation. The corresponding calculated IC50 values are listed in Table 2.

**Discussion**

Based on the current cancer stem cell (CSC) hypothesis, only a small subpopulation within the heterogeneous tumor population is capable of initiating and re-initiating tumors. The concept of CSCs was based on the observation that when cancer cells of many different types were assayed for their proliferative potential in various assays *in vitro* and *in vivo*, only a minority of cells showed extensive proliferation [20]. CSCs have been identified in a variety of malignancies [21,22,23]. One widely accepted method for identifying CSCs is based on the enzymatic activity of aldehyde dehydrogenase 1 (ALDH1), a detoxifying enzyme responsible for the oxidation of intracellular aldehydes [8,21]. There are different isoforms of ALDH. The Aldefluor® assay system has been developed to detect the activity of the ALDH1 isoform. ALDH1 activity showed to be increased in CSCs and has been used to isolate CSCs in different cancers [24,25,26]. Therefore, ALDH1high cells display several features typically seen in CSCs, including the ability for self-renewal, generation of differentiated progeny, and increased expression of stem cell marker genes. The study of CSC biology is predicated on the ability to accurately assess CSC representation within cancer cell populations. As suggested by more recent findings CSC representation may be a function of the cell type of origin, stromal microenvironment, accumulated somatic mutations and stage of malignant progression reached by a tumor [27,28].

To date, the existence of such a stem-like cell population in human osteosarcoma and Ewing’s sarcoma cell lines has been based on the expression of stem cell marker genes as well as their ability to form spheroids *in vitro* [29,30,31]. It has been suggested that identification of the CSC cannot solely rely on side population (SP) sorting using efflux of Hoechst 33342 dye. However, the SP phenotype is not presented in all CSCs and there may exist other defensive mechanisms for CSCs to evade drug therapies that cannot be identified by Hoechst dye staining [32]. Therefore, we chose the marker ALDH1. Our results show that all five sarcoma cell lines contained different percentages of ALDH1high cells, with the highest percentage in fibrosarcoma, synovial sarcoma, and chondrosarcoma cell lines. The small ALDH1 expression of the ALDHlow cells in the western blot analysis can be explained by the use of the ALDH1/2 primary antibody. The proliferation rate and clonogenicity of SW-684, SW-982, and SW-1353 ALDH1high cells *in vitro* were significantly higher than that of ALDH1low cells, consistent with the characteristics of the high ALDH1 activity phenotype in other cancer cells [33,34], which may indicate that ALDH1high cells from sarcoma are partially responsible for tumor metastasis and recurrence and should be focused during the cancer therapy. As c-Myc has been recently recognized as an important regulator of stem cell biology, it may serve as a link connecting malignancy and “stemness” [35]. Introduction of c-Myc with other transcription factors (including SOX-2) generates induced
pluripotent stem cells from differentiated cells [36]. Wnt/β-catenin signaling plays an important role not only in cancer, but also in cancer stem cells [37]. Our quantitative RT-PCR data showed increased expression of c-Myc, β-catenin, and SOX-2 in the ALDH1high population, while unsorted control cells (ctrl) and ALDH1low showed minimal expression.

A proposed mechanism of chemotherapeutic resistance of cancer stem cells is based on the enhanced expression of ATP-binding cassette (ABC) transport proteins, which are responsible for drug efflux. Higher expression of ABC transport proteins in stem cells compared to non-stem cells results in relative resistance of the stem cells to the toxic effects of chemotherapeutic drugs [12,13]. We analysed the mRNA expression of three major drug transporters (ABCG2/BCRP1, ABCA2, ABCB1/MDR1) of ABC transporter family. In the present study, ABCG2 was upregulated in ALDH1high cells from all three sarcoma cell lines. Furthermore, another ABC transporter ABCB1/MDR1 was also found with higher mRNA expression level in SW-1353 ALDH1 high cells compared to ALDH1low cells. These genes may be responsible for multi-drug resistance of cancer cells and should be ideal targets for clinical cancer therapy.

Additional, ALDH1high cells showed increased resistance to commonly used chemotherapeutic drugs. ALDH1high cells of SW-982 and SW-1353 showed significantly lower sensitivity to both doxorubicin and epirubicin compared with ALDH1low cells. The cisplatin treatment showed only slight differences. Together, we successfully isolated ALDH1high cells from different sarcoma cell lines using the Aldeflour assay. ALDH1high cells exhibited in vivo a significant higher proliferation rate, increased clone formation efficiency, elevated expression of ABC transporters and stemness marker, as well as increased chemotherapeutic drug resistance compared to ALDH1low cells.

In conclusion, the presence of stem-like cells with increased ALDH1 expression could be one of the possible contributors to the development of drug resistance in sarcomas. Further study will be required to define the sarcoma stem cells and the mechanisms of drug resistance, but ALDH1high population may serve as an in vitro model to search for new therapeutic treatment options.

Acknowledgments

The authors would like to thank Heike Kaltenegger and Alexandra Novak for excellent technical assistance.

Author Contributions

Conceived and designed the experiments: BL BR SMW. Performed the experiments: BL BR NS MA. Analyzed the data: BL BR. Contributed reagents/materials/analysis tools: BLA AL RW. Wrote the paper: BL.
36. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126: 663–676.
37. Teng Y, Wang X, Wang Y, Ma D (2010) Wnt/beta-catenin signaling regulates cancer stem cells in lung cancer A549 cells. Biochem Biophys Res Commun 392(3): 373–379.