European Journal of Cancer (2014) **50**, 137–149

Available at www.sciencedirect.com

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journal homepage: www.ejcancer.com

Identification of brain tumour initiating cells using the stem cell marker aldehyde dehydrogenase

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Available online 5 October 2013

KEYWORDS
Aldehyde dehydrogenase
Brain tumour initiating cell
Primary brain tumours
Stem cell marker

Abstract Aldehyde dehydrogenase (ALDH) has been identified in stem cells from both normal and cancerous tissues. This study aimed to evaluate the potential of ALDH as a universal brain tumour initiating cell (BTIC) marker applicable to primary brain tumours and their biological role in maintaining stem cell status.

Cells from various primary brain tumours (24 paediatric and 6 adult brain tumours) were stained with Aldefluor and sorted by flow cytometry. We investigated the impact of ALDH expression on BTIC characteristics *in vitro* and on tumourigenic potential *in vivo*.

Primary brain tumours showed universal expression of ALDH, with 0.3–28.9% of the cells in various tumours identified as $ALDH^+$. The proportion of $CD133^+$ cells within $ALDH^+$ is higher than ALDH cells. $ALDH^+$ cells generate neurospheres with high proliferative potential, express neural stem cell markers and differentiate into multiple nervous system lineages. $ALDH^+$ cells tend to show high expression of induced pluripotent stem cell-related genes. Notably, targeted knockdown of ALDH1 by shRNA interference in BTICs potently disturbed their self-renewing ability. After 3 months, $ALDH^+$ cells gave rise to tumours in 93% of mice whereas ALDH cells did not. The characteristic pathology of mice brain tumours from $ALDH^+$ cells was similar to that of human brain tumours, and these cells are highly proliferative *in vivo*.

Our data suggest that primary brain tumours contain distinct subpopulations of cells that have high expression levels of ALDH and BTIC characteristics. ALDH might be a potential therapeutic target applicable to primary brain tumours.

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http://dx.doi.org/10.1016/j.ejca.2013.09.004

1. Introduction

The treatment of brain tumours remains a challenge in the neurosurgical field. Despite advances in surgery and adjuvant therapy, the overall prognosis of this disease is still dismal because of difficulty in radical excision and chemo-radiotherapy resistance. A recent concept in neuro-oncology is that brain tumours arise from a rare population of undifferentiated cells that share characteristics with normal neural stem cells (NSC) [1]. These cells have been termed brain tumour stem cells (BTSCs) or brain tumour initiating cells (BTICs), and they are responsible for tumour initiation and chemo/radiation therapy resistance in primary brain tumours.

A hematopoietic stem cell marker, CD133 (prominin 1), has been proposed to be a BTIC marker [1–4]. $CD133^+$ BTICs exhibit stem cell-like properties *in vitro* and tumour-initiating potential *in vivo* [1,2,4,5]. However, there is compelling evidence showing that $CD133^+$ cells are expressed in differentiated epithelial cells in various organs, and $CD133^-$ cancer cells also initiate tumours *in vivo* [6,7]. While $CD133^+$ may be the first BTIC marker, it is not a universal marker.

Recently, aldehyde dehydrogenase (ALDH) was identified in stem cells from both normal and cancerous tissue [8–10]. ALDH is a cytoplasmic enzyme responsible for oxidising a variety of intracellular aldehydes to carboxylic acids [11]. Their activity can be detected by western blotting, FACS analysis, immuno-staining and fluorescence microscopy [12]. Among these techniques, sorting cells via flow cytometry with Aldefluor, a fluorescent substrate for ALDH, offers several potential advantages because of its simplicity, high reproducibility and reliability in discriminating between viable and nonviable cells as well as the stability of the sorted cells [10]. ALDH has been described as a stem cell marker in various kinds of tumours including breast [13], lung [14] and colorectal cancer [15], as well as glioblastomas [9]. In the present study, we applied Aldefluor-based flow cytometry on various primary brain tumours to evaluate the potential of ALDH as a universal BTIC marker. Furthermore, biological role of ALDH in maintaining stem cell status was monitored through gene expression levels of reprogramming-related factors and selectively knocked down ALDH1 using shRNA interference.

2. Materials and methods

2.1. Isolation of brain tumour initiating cells

Brain tumour tissue samples were obtained from patients with brain tumours who underwent initial surgery at the Seoul National University Hospital. Patients with neoadjuvant therapies or recurrent tumours were excluded from this study. All eligible patients or their parents provided written informed consent approved by the institutional review board of the Seoul National University Hospital. Tissues were prospectively obtained from paediatric (mean age: 7.0 ± 5.8 years, range: 1 month to 18 years, M:F = 15:9) and adult patients (mean age: 63.5 ± 7.9 years, range: 50–73 years, M:F = 6:0) (Supplementary Table S1). Various primary brain tumours from children included medulloblastoma ($N = 9$), ependymoma ($N = 5$), atypical teratoid/rhabdoid tumour (ATRT, $N = 2$), pilocytic astrocytoma ($N = 4$), subependymal giant cell astrocytoma (SEGA, $N = 1$), pineoblastoma ($N = 1$), choroid plexus papilloma ($N = 1$) and choroid plexus carcinoma ($N = 1$). Primary adult brain tumours were glioblastoma ($N = 5$) and anaplastic astrocytoma ($N = 1$). They were mechanically chopped in Dulbecco's phosphate buffered saline (DPBS; Invitrogen, Grand Island, NY) without calcium and magnesium before enzymatic digestion. Single cells were then isolated by filtration through a 40 μm filter and were washed with red blood cell lysis buffer. Live primary tumour cells were seeded at a standard density of 15,000 cells/cm^2 in NBE media consisting of neurobasal medium (Invitrogen), 2 mM L-glutamine, N2 supplement (Invitrogen), B27 supplement (Invitrogen), 20 ng/ml of human recombinant epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF; both from Chemicon, Temecula, CA). When the cells formed primary aggregates, they were collected and plated without dissociation onto extracellular matrix (ECM)-coated flasks (ECM 1:10 dilution, Sigma-Aldrich, St. Louis, MO) and were allowed to form a primary monolayer (Cambridge protocol [16]). As the primary monolayer approached confluence, the cells were dissociated by incubation with accutase (Invitrogen) at room temperature and were washed with DPBS. To generate subsequent monolayers, the cells were seeded at a standard density of 15,000 cells/cm^2 at each passage. All experiments were conducted before the fourth cell passage (Supplementary Table S2). All cells were incubated at 37 °C in an incubator in a 5% CO_2/95% air atmosphere.

2.2. ALDH activity analysis and cell sorting by fluorescence activated cell sorting (FACS)

To measure ALDH activity, cells were obtained from freshly dissociated neurospheres and were analysed using an Aldefluor assay kit (Aldagen Inc., Durham, NC). Dead cells, cell debris, doublets and aggregates were excluded by forward and side scattering and pulse-width gating. The sorting gates were established using as the cells stained with 7-Aminoactinomycin D (7-AAD) for viability. Cells were suspended in Aldefluor assay buffer containing ALDH substrate, BAAA (1 μmol/L) for 30–60 min per 1×10^6 cells. After staining, the single cell dissociation was maintained on ice