CD133 and ALDH1 as Prospective Markers for Cancer Stem Cells in Ileal Carcinoids

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Abstract

In recent years increasing evidence supporting the stem cell hypothesis have emerged and cancer stem cells (CSCs) have been reported to have been isolated in a wide variety of cancers. This CSC population has been attributed to contribute to the therapeutic resistance of many cancers and it is thus of great interest to find new therapeutic options targeting these cells. In ileal carcinoids however not much is known regarding the existence and properties of CSCs. The aim of this study was to identify, isolate and characterize cancer cell populations of ileal carcinoids sorted with fluorescent-activated cell sorting (FACS) using putative cancer stem cell markers. The FACS markers included the six cell surface receptors CD26, CD44, CD56, CD117, CD133 and CD166 as well as an intracellular assay indicating the activity of the enzyme aldehyde dehydrogenase 1 (ALDH1). The CD133 and ALDH1 markers did respectively in both primary patient cultures and a cell line help to identify distinct cell subpopulations which when cultured on low-attachment plastic ware gave rise to sphere-like formations. Analysis of the populations by quantitative real-time PCR (qRT-PCR) showed significant differences in the expression of mRNA relevant to CSC.

Introduction

The term “carcinoid“ (“karzinoide”) was in 1907 introduced by Siegfried Oberndorfer whom in the early 1950s also was the first to describe the carcinoid syndrome. (Modlin, et al. 2004) Modern chemo and radio therapies targeting carcinoids generally have poor effect both alone and in combination (Modlin, et al. 2006), leading to surgical resection being the only curative therapy. (Nilsson, et al. 2010) At the time of diagnosis, 26-30% of all patients have distant metastases (Modlin, et al. 2007) making radical surgery impossible to perform.

Both chemo and radio therapeutic resistance of several cancers have been attributed to a specific subset of cells called cancer stem cells (CSCs) (Eyler and Rich 2008) and hope has been raised that targeting these cells might overcome this therapeutic resistance. (Clarke 2005; Ratajczak 2005; Sales, et al. 2007; Soltysova, et al. 2005) The CSCs, as postulated in the CSC theory, are able to self-renew, give rise to differentiated progeny, and to initiate tumors in vivo. (Clarke, et al. 2006) The study of leukemia stem cells (Lapidot, et al. 1994) and later brain as well as breast cancers. (Al-Hajj, et al. 2003; Singh, et al. 2003) led to the initial understanding of these cells and showed the possibility to isolate them based on cell surface marker expression.

Aldehyde dehydrogenase 1 (ALDH1) is an enzyme having the role of oxidating intracellular aldehydes (Sophos and Vasiliou 2003; Yoshida, et al. 1998) and that could have the responsibility to oxidize retinol to retonic acid in stem cells. (Chute, et al. 2006) Sorting cells based on high ALDH activity has been shown to enrich for CSC in several cancers (Douville, et al. 2009; Ginestier, et al. 2007), including carcinoids. (Gaur, et al. 2011) Another marker frequently observed to enrich for CSC is the often used CD133 (also referred to as prominin-1) marker. The human CD133 protein is of 865 amino acids, with a gene conserved in a wide variety of animals. However, little is known about the biological function of the marker. Although its function is mainly unknown it is the most commonly used marker used to prospectively identify cancer stem cells ever since its ability to enrich for CSC was first discovered. (Singh et al. 2003; Singh, et al. 2004; Wu and Wu 2009) However, CD133 has in several publications been shown to be expressed in differentiated epithelia in a variety of organs. (Immervoll, et al. 2008; Karbanova, et al. 2008; Lardon, et al. 2008; Oshima, et al. 2007; Sagrinati, et al. 2006; Schmelzer, et al. 2007)

Using ALDH1 and CD133 as markers in fluorescent-activated cell sorting (FACS), cell subpopulations were isolated. To investigate these populations, the mRNA expression of several genes was analyzed using qRT-PCR. The genes from which the mRNA is transcribed are believed to either be important parts of reprogramming somatic cells to induced pluripotent stem cells; SOX2, OCT4, KLF4, CMYC, NMYC, NANOG, LIN28 (Patel and Yang 2010), be key transcription factors involved in enteroendocrine cell differentiation; PAX4 (Larsson, et al. 1998), PAX6 (Larsson et al. 1998), NKX2-2 (Desai, et al. 2008), SOX9 (Formeister, et al. 2009), be mature neuroendocrine markers; CHGA and SSTR2, putative stem cell markers; LGR5 (Barker, et al. 2007), CD133 (Sei, et al. 2011), DCAMKL1 (May, et al. 2009), neurogenin (Sei et al. 2011) or used for validation of the FACS; CD26.

In the present study, it is demonstrated that both the CD133 and the ALDH population give rise to distinct sub populations with significantly altered mRNA expression.

Material and methods

Cell culturing

The two ileal cell lines; GOT1, established in the same laboratory in 2001(Kolby, et al. 2001) and KRJ1(Pfragner, et al. 1996), were cultured in uncoated 25 and 75 cm^2cell culture flasks (Nunclone; Nuncbrand, Roskilde, Denmark). GOT1 was maintained in RPMI 1640 supplemented with 10% foetal bovine serum (FBS), 5 µg/mL transferrin, 5 µg/mL insulin, 5 mM L-glutamine, 200 IU/mL penicillin and 200 mg/mL streptomycin and KRJ1 was maintained in DMEM-Hams F12 supplemented with 10% FBS, 5mM L-glutamine as well as 200 IU/mL penicillin and 200 mg/mL streptomycin. All cells were incubated at 37 ºC in a humidified atmosphere of 5% CO_2 with media change twice each week.

Primary cell cultures of ileal carcinoids were earlier established from biopsies collected at the time of surgery from three patients with ileal carcinoids

involving lymph node metastases. Tumour tissue was minced with scissors into 1 mm pieces and digested in 50 mL RPMI 1640 containing 2 mg/mL collagenase I (Sigma) and 24 µg/mL DNAse (Sigma) for 1–3 h at 37ºC. The reaction was stopped by the addition of equal volume of medium, and red blood cells were removed by treatment with lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA) for 1 min. The samples were aliquoted in ampullas with CryoStor CS-5, 5% DMSO (Biolife Solutions) and put in -20°C freezer before stored in liquid nitrogen. For experiments with primary cell cultures, ampullas containing 14.7, 13.0 and 8.0 million viable cells for each patient respectively were used. The harvesting and preparation of primary cell cultures as described was performed by the Ola Nilsson research group at Sahlgrenska Cancer Center at Sahlgrenska Academy, University of Gothenburg.

Two days before cell sorting the ampullas containing the primary cells were thawed in 37ºC water bath. The cell suspension was rapidly suspended in warm media and washed before it was seeded onto the same kind of plastic ware and culture media as the GOT1 cells, except instead supplemented with 4% FBS since earlier observations in light-microscope have suggested that lower concentration of FBS inhibits growth of fibroblasts.

After FACS the primary cell cultures were maintained in stem cell media containing DMEM-Hams F-12 supplemented with B-27 Serum-Free Supplement (1:50; Invitrogen), N-2 Serum-Free Supplement (1:100; Invitrogen), 5 mM L-glutamine, 200IU/mL penicillin and 200 mg/mL streptomycin on 24 or 96 well clear flat bottom low-attachment plastic ware (Costar Corning Life Sciences). Also an addition of 2µg/mL heparin, 20 ng/mL EGF (Invitrogen) and 10 ng/mL FGF (Millipor) was added every 2-3 days and the stem cell media changed once every week.

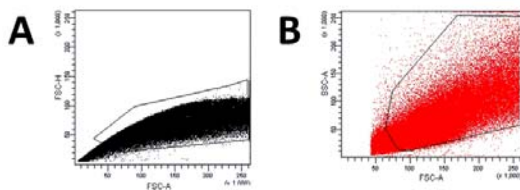

Figure 1 In effort to exclude cell debris, aggregates and non-viable cells all populations were, before sorted with fluorescent-activated cell sorting, gated with regard to their morphology. (A) First, all cells were gated as illustrated in a forwards scatter plot. (B) Subsequently, the cells were gated in a forward versus side scatter plot as shown.

Fluorescence-activated cell sorting

The ALDH1 enzymatic activity was measured using an Aldefluor kit (Stemcell Technologies, Vancouver, CA). Cells were trypsinized to remove cells from the cell culture flask two days after seeding, washed and resuspended in 1 mL Aldefluor assay buffer. The Aldefluor buffer contains BODIPY- aminoacetaldehyde (BAAA), 1 mol/L, an ALDH1 substrate which, after 40 minutes of incubation at 37°C in the intracellular presence of ALDH1 forms the fluorescently labeled product BODIPY-aminoacetate (BAA), which is retained in the cells. An ALDH1 inhibitor, 0.3 mmol/L diethyl-aminobenzadehyde (DEAB) was before the incubation added to a small aliquot of cell suspension to serve as negative control. Following incubation the cells were washed and resuspended in Aldefluor buffer. The test sample was stained with the labeled antibodies at the following dilutions; 1:10 CD26 (eBioscience; cat.:12-0269-71; clone 2A6; PE), 1:10 CD44 (BD Biosciences; cat.: 560532; clone G44-26;APC-H7), 1:20 CD56 (BD Biosciences; cat.: 557747; clone B159; PE-Cy™7), 1:10 CD133 (Miltenyi Biotec; cat.: 130-090-826; APC) and 1:20 CD166 (BD Biosciences; cat.: 562131; clone 3A6; PerCP-Cy™5.5). The sample was then incubated with the antibodies at 4 ºC for 20 minutes, washed and then resuspended in Aldefluor buffer. Analysis was performed in a BD FACSDiva and five different populations were sorted based on light scatter, CD133-staining and Aldefluor-staining.

Before sorting, several so-called gates were used to define which cell populations to sort. Firstly, all populations were gated based on the forward light scatter of the cells in attempt to exclude cell debris and aggregation in the sample (Fig. 1A.). Secondly, these cells were all subject to gating with regard to their morphology. This was performed with regard to both the cells forward and side light scatter to further eliminate debris, aggregated and non-viable cells (Fig. 1B). In a plot showing the fluorescence of anti-CD133, a CD133-positive sub population was observed in 2/3 patients, which was gated for. This population, as well as a negative control that was both CD133 and CD26 negative, were sorted. (Fig. 2C) The negative control is referred to as "CD133-". In the Aldefluor-plot another sub population, suggesting high ALDH1 activity, was observed and isolated. This population is referred to as the "ALDH+" population and was sorted together with a negative control; "ALDH-". For all populations later subject to qRT-PCR analysis 1250 cells of each population were sorted and for experiments comparing growth conditions and sphere forming, 5000 and 10000 cells were sorted. The ALDH- population included the cells with lower ALDH1 activity than the ALDH+ population, but excluded the cells with the lowest concentration

since these in pre-experiments were shown to be non-viable when cultured.

Quantitative real-time PCR

Subsequently to FACS, using the TaqMan® Fast Cells-to-C_T™ kit, mRNA was extracted from different populations of sorted cells, reversely transcribed to cDNA and analyzed using 7500 Fast Real-Time PCR System (Applied Biosystems). Primer and probes specific for the targets SOX2 (Hs01053049_s1 and Hs00602736_s1), SOX9 (Hs00165814_m1), POU5F1 (OCT4; 00999632_g1), KLF4 (Hs00358836) ,MYCN (Hs00232074_m1), MYC (Hs00905030_m1), NANOG (Hs02387400_g1), LIN28 (Hs04189307_g1), LGR5 (Hs00173664), NEUROD1 (Hs00159598_m1), NEUROG3 (Hs00360700), NKX2-2 (Hs00159616_m1), DCLK1 (00178027_m1), PAX4 (Hs00173014_m1), PAX6 (Hs01088112_m1), CHGA (Hs00154441_m1), SSTR2 (Hs00990356_h1), CD26 (Hs00175210_m1), CD133 (Hs01009250_m1) and the endogenous controls ACTB (Hs99999903_m1) and GAPDH (Hs99999905_m1) were used (Applied Biosystems).

Table 1 Screening for distinct populations in primary ileal carcinoid cell and the ileal carcinoid cell line GOT1.

Samples	CD26	CD44	CD56	CD117	CD133	CD166	ALDH1
Primary cell culture	+	+	+	+	+	+	+
GOT1	+	+	+	N/A	+	+	+
KRJ1	N/A	N/A	N/A	N/A	N/A	-	-

Samples from three primary ileal carcinoid cell cultures and the ileal carcinoid cell lines GOT1 and KRJ1 were analyzed by fluorescence-activated cell sorting. The table present whether any cells present in the samples expressed respective cell surface receptor/showed aldehyde dehydrogenase 1 (ALDH1) activity based on its fluorescent signal. Samples with marker expression are marked '+', whereas those with an absent signal are marked '-'. Markers for samples not investigated are indicated with 'N/A' and not all experiments were replicated.

Analysis and automatic threshold setting was performed with 7500 Fast System Software version 1.3.1. Target genes with amplification curves in lack of a linear phase were omitted. The statistical significance between gene expressions in different populations was calculated using the two-tailed unpaired *t*-test in GraphPad Prism 5 (Graphpad Software, San Diego, CA, USA). P-values of <0.05 were considered significant and marked by one asterisk, if below 0.01 marked with two and if below 0.001 with three asterisks.

Ethics

These studies were approved by the Regional Ethical Review Board in Göteborg, Sweden (document no. 648-05).

Results

Using CD133 and ALDEFLUOR as markers in FACS give rise to distinct subpopulations, whereas other markers do not

Two ileal carcinoid cell lines, GOT1 and KRJ1, as well as primary ileal cell cultures from three patients were analyzed using FACS. The fluorescently labeled prospective CSC markers CD26, CD44, CD56, CD117, CD133 and CD166 were used. Additionally, one intracellular assay was used; the Aldefluor assay. The Aldefluor assay marks the activity of the enzyme ALDH1 which can be quantified based upon fluorescence and has frequently been used to enrich for CSCs.

Screening concluded that, for all primary cell cultures, cells expressing the investigated markers were present (Table 1). Similarly, the cell line GOT1 had cells expressing all markers, except for CD117 which was not assessed in the cell lines. For KRJ1, results were obtained only for the CD166 and ALDH1 markers, both negative.

Although several markers were expressed for the patient samples and GOT1, only the ALDH1 and CD133 markers allowed identification of separate and distinctly positive subpopulations in primary cells (Fig. 2) and the GOT1 cell line. These populations distinguish themselves by having a fluorescence intensity greater than the rest of the cells and thereby forming a separate, more marker positive, cluster of cells in the graphs. The two subpopulations were sorted together with a bulk of cells as well as negative controls (CD133- and

ALDH- respectively; please refer to the material and methods section for details).

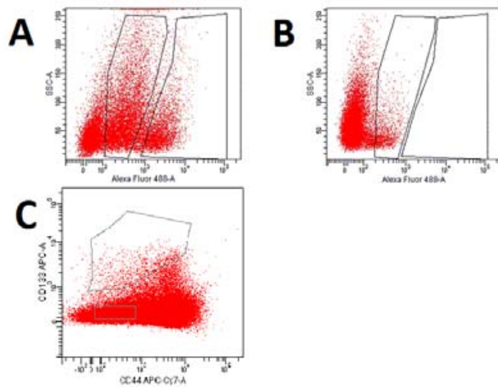

Figure 2 The CD133 and ALDH1 markers, the latter indicated by cells treated with the ALDH1 inhibitor DEAB, gave rise to separate and distinct positive subpopulations in patient samples. (A) A group of cells (gate to the right) is distinguished from the other ALDH1-expressing cells. (B) Sample treated with DEAB, an ALDH1-inhibitor. The sample showed very low ALDH activity. (C) The sorted population CD133+ as indicated by the gate.

Sorted populations give rise to sphere-like formations when cultured in serum-free media

The cell populations CD133+, CD133-, ALDH+, ALDH- and bulk cells, sorted from FACS as previously described, were cultured on non-adherent and uncoated plastic ware in a serum-free media supplemented with the stem cell factors heparin, EFG and FGF. When cultured under these conditions, the cells gave within a few days rise to sphere-like formations. Spehres could be seen several weeks after seeding (Fig. 3A-D) and several spheres have successfully been cultured for >70 days. It was noted, although no quantification experiment was performed, that compared to the cell populations that were negative for CD133 and ALDH respectively as well as the bulk population, the CD133+/ALDH+ cells formed fewer and smaller spheres when grown at the same plating densities.

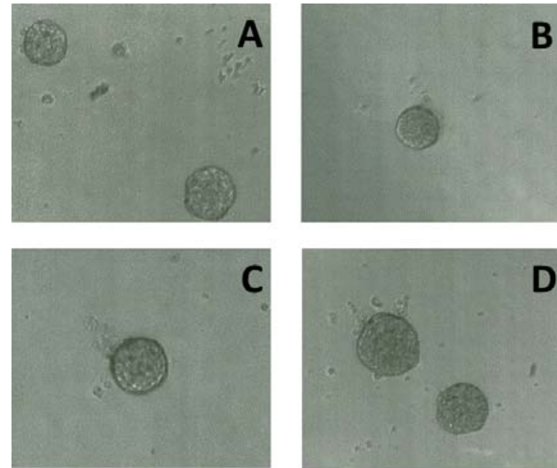

Figure 3 FACS-sorted cell populations gave rise to sphere-like formations within few days of culture under stem cell promoting conditions. (A) Two spheres after 27 days in culture on uncoated plastic ware deriving from a cell population sorted with FACS as CD133+, at a plating density of 10 cells/µL. (B) A sphere from population sorted as CD133 – after 20 days in culture on non-adherent plastic ware at 10 cells/µL plating density. (C) A sphere after 14 days of culture on non-adherent plastic ware, sorted as 'bulk' cells at 10 cells/µL plating density. (D) Two spheres from a population sorted as CD133^{-} as observed after 31 days of culture on non-adherent plastic ware, plated at 50 cells/µL density.

The CD133+ and ALDH+ subpopulations differ in mRNA expression compared to negative controls and bulk

The cell populations ALDH+, ALDH-, CD133+ and CD133- from both the GOT1 cell line and the primary cell cultures that were sorted by FACS were analyzed using quantitative real-time PCR (qRT-PCR). These included CD133+, CD133-, ALDH+, ALDH- and bulk cells sorted from patient samples and the GOT1 cell line respectively. All samples were gated based on morphology in effort to exclude cell debris, aggregates and non-viable cells.

For the CD133+ population it was shown that NKX2-2 and CD26 were significantly ($p < 0.05$) up-regulated compared to the CD133- population (Fig. 4A). The ALDH+ sample showed no significant differences compared to the ALDH- and bulk populations (Fig. 4B).

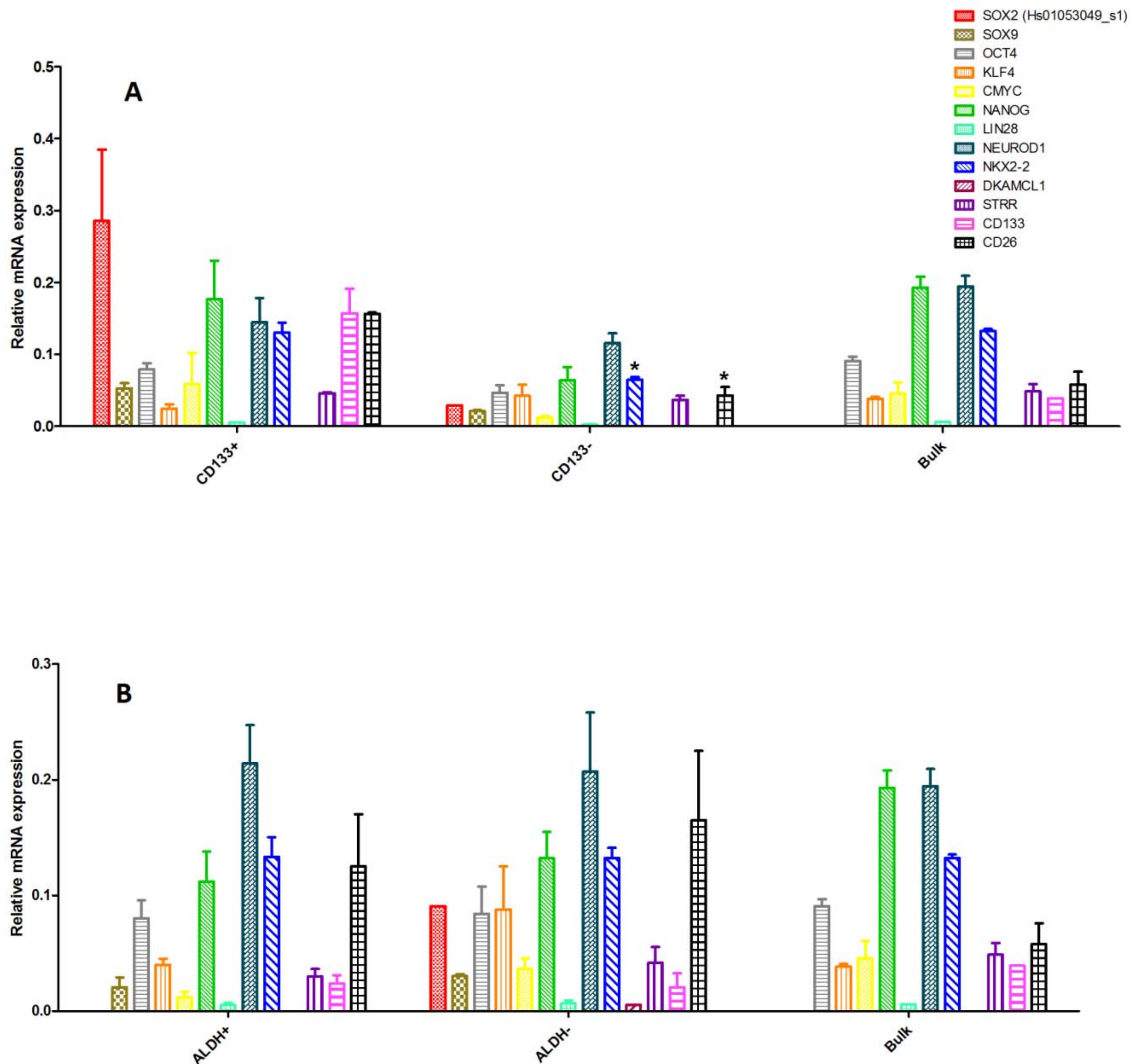

Figure 4 qRT-PCR analyses of several genes showing relative mRNA expression for primary cell cultures sorted by FACS into different populations depending on the cell expression of CD133 or ALDH1 activity. The values are calculated as $2^{-\Delta CT}$ and are given relative to the housekeeping genes ACTB and GAPDH as the mean value ± standard error of mean. The data was obtained from three primary cultures derived from three different patient tumours, each sorted with FACS one time and subsequently analyzed by qRT-PCR in triplicate. Results from all markers for all replicates were not obtained due to experimental difficulties registering low levels of mRNA, though this is considered in the standard error of mean as well as significance calculation. (A) Significant differences are calculated compared to the CD133+ population. Asterisks indicate significant difference where * corresponds to $0.01<P<0.05$. The CD133+ population showed significantly higher mRNA expression for NKX2-2 and CD26 compared to the CD133- population. (B) No significant differences were observed when compared to the ALDH+ population.

SOX2 and SOX9 were expressed in the CD133+ and CD133- populations, but were not detected in the bulk population. Furthermore, CD133 was not detected in the CD133- population.

The GOT samples were also subject to analysis by qRT-PCR, showing significant differences for several samples and genes. The CD133+ population showed a significant down-regulation of SOX2 and NANOG compared to both the bulk and CD133- populations (Fig. 5A). Also, significantly lower expression was observed for OCT4 and LIN28 when compared to bulk population and SSTR2 compared to the CD133- population.

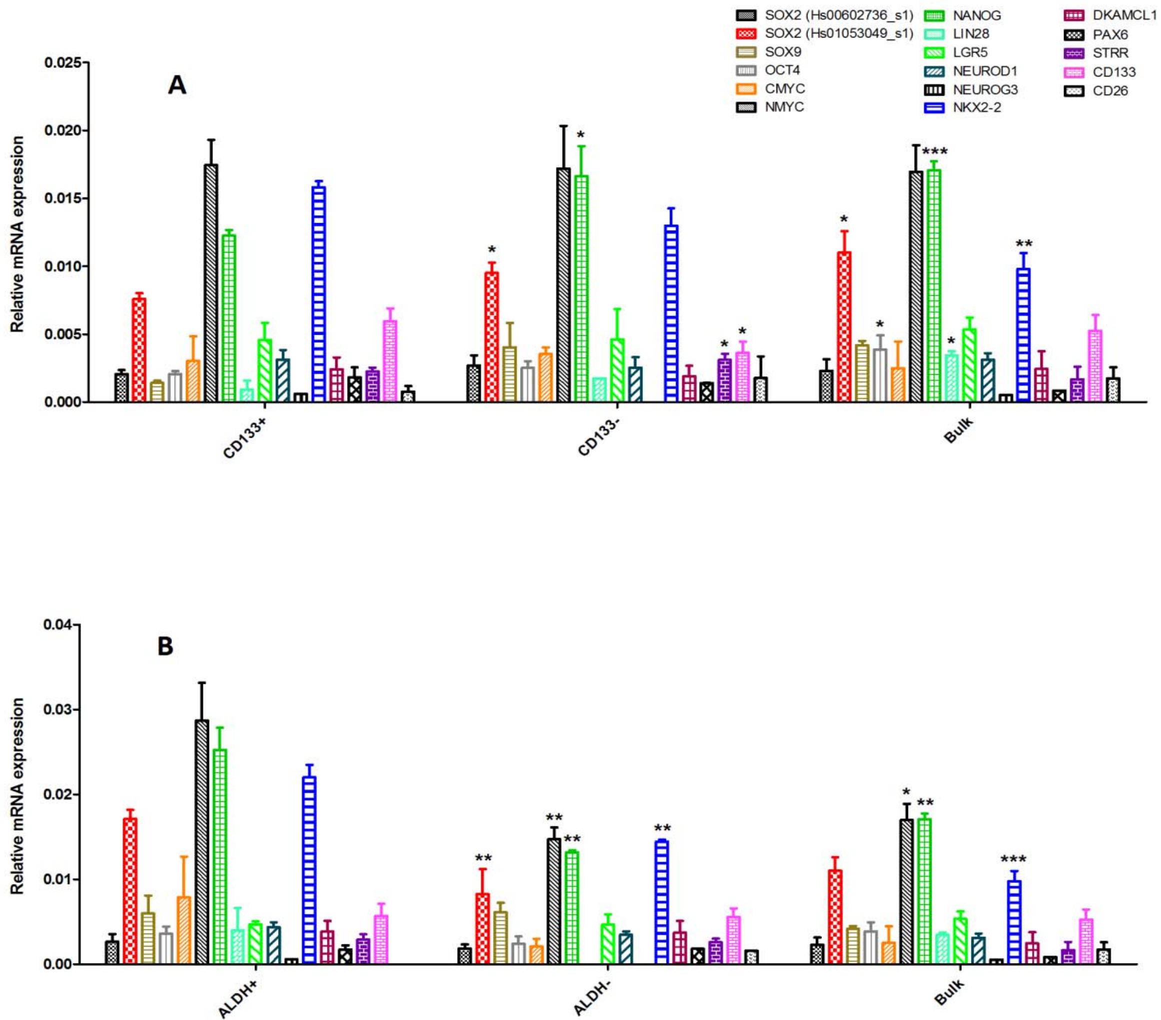

Figure 5 qRT-PCR analyses of several genes showing relative mRNA expression for GOT1 sorted by FACS into different populations depending on the cell expression of CD133 (A) or ALDH1 (B). The values are calculated as $2^{-\Delta CT}$ and are given relative to the housekeeping genes ACTB and GAPDH as the mean value ± standard error of mean. Significant differences are calculated compared to the CD133+ (A) or the ALDH1 population (B). Asterisks indicate significant difference where * P<0.05, ** P>0.01 and *** P>0.001. All data was obtained from the GOT1 cell culture sorted by FACS in triplicate, each separate sorting then analyzed by qRT-PCR also in triplicate. Results from all markers for all replicates were not obtained due to experimental difficulties registering low levels of mRNA, though this is considered in the standard error of mean as well as significance calculation.

Also, the CD133+ population had significantly higher expression of NKX2-2 compared to the bulk population and CD133 compared to the CD133- population.

For the ALDH1 sorted populations, ALDH+ had, compared to both the ALDH- and bulk populations, significantly higher expression of NMYC, NANOG and NKX2-2 (Fig 5B). SOX2 showed significantly higher expression when compared to ALDH- population alone.

For NEUROG3, no expression was detected in either CD133- or ALDH- populations. LIN28 was undetected in ALDH- only, and CD26 undetected in the ALDH+ population.

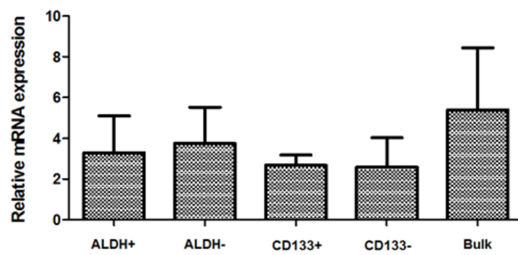

Figure 6 The results from qRT-PCR analysis of the expression of CHGA mRNA for single cell cultures, deriving from patient tumours sorted into different populations using FACS depending either on CD133 or ALDH1 expression. The values are calculated as 2-ΔCT and are given relative to the housekeeping genes ACTB and GAPDH.

Furthermore, the mRNA expression of the differentiation marker CHGA was investigated for all populations using the same technique, but no significant differences were observed (Fig 6).

Discussion

This study shows that several commonly used CSC markers are expressed in ileal carcinoids from primary cell lines established from resected tumours as well as in an ileal carcinoid cell line. Furthermore, it is shown that subsets of cells express high amounts of CD133 and ALDH1 respectively and that these subpopulations significantly differ in their expression of mRNA compared to other tumour cells.

Several commonly used cell surface markers were used to analyze single cell suspensions from resected tumours. Some of these were also used to analyze the GOT1 and KRJ cell lines, please refer to table 1 to see which ones. The markers included CD26, a cell surface glycoprotein with intrinsic dipeptidyl peptidase IV activity believed to have a significant role in tumor pathogenesis and progression (Pro and Dang 2004; Thompson, et al. 2007) which has been identified as cancer stem cell marker in colon cancer (Pang, et al. 2010); CD44, a protein long suspected to have a vital role in intestinal tumourgenesis that recently has been identified as a marker of CSCs in colorectal cancer (Dalerba, et al. 2007; Pang et al. 2010); CD56, or NCAM, a neural cell adhesion molecule reported as stem cell marker with an important role in e.g. hepatocellular carcinoma (Colombo, et al. 2011; Tsuchiya, et al. 2011); CD117, or C-kit, a surface marker for embryonic stem cells, hematopoietic stem cells, and mesenchymal stem cells that maintains cells in their undifferentiated state and confers the ability to self renew (Hassan and El-Sheemy 2004; Lu, et al. 2007; Palmqvist, et al. 2005) and CD166, or ALCAM, that has a myriad of functions and is a cancer stem cell marker in e.g. colorectal cancer (Levin, et al. 2010). When analyzing the primary cell cultures and the cell lines for the above mentioned markers, no group of cells could be distinguished as more expressing more of any marker than the rest of the positive cells.

However, CD133, a cell membrane glycoprotein (Mizrak, et al. 2008) that originally was developed for the identification of endothelial progenitor cells (Hilbe, et al. 2004) and that has been frequently used for identifying tumour cells with CSC characteristics (Bohl, et al. 2011; Singh et al. 2003; Singh et al. 2004; Wu and Wu 2009) generated a distinguishable subpopulation in FACS analysis. This was also the case for the activity of ALDH1, an enzyme suggested to be a powerful marker for e.g. both benign and malignant stem cells in breast tissue (Ginestier et al. 2007) as well as in ileal carcinoids. (Gaur et al. 2011) Therefore, these two populations, with appropriate negative controls and bulk cells were chosen for further analysis.

To avoid comparing positive subpopulations to dead cells expressing low levels of marker, viable gating was performed by excluding cells that were suspected to be misshaped based on the cells forward and side light scatter. Culturing the lowest ALDH1-expressing subsequent to FACS showed that these cells were unable to proliferate and died in a matter of days. This population has in earlier studies (Gaur et al. 2011) been used to compare sphere-forming capacity to the ALDH+ population, something that can be questioned with regard to this. To avoid the same mistake, cells with intermediate ALDH1 activity were used as comparison to the ALDH+ population. The cells showing intermediate ALDH1 activity were shown to proliferate when cultured subsequent to FACS.

The sphere-forming assays are often used to show the potential of cells to act as stem cells when removed from their in vivo niche. In this study, cells gone through FACS formed spheres when cultured in serum-free media supplemented with stem cell factors EFG and FGF. Amongst the populations studied, the CD133+/ALDH+ populations were observed to form notable fewer and smaller spheres compared to populations grown at the same plating densities. This is interesting seeing as one would expect a stem cell population to behave the opposite. However, these observations were made with cells not cultured at clonal density. In order for a proper sphere-forming assay to be successful, cells must be grown at very low density, or better, to ensure true clonality they should be plated as single-cells. This, in order to

rule out the probable event that spheres aggregate and fuse. (Mori, et al. 2006; Singec, et al. 2006) However, when cells were plated as single-cells they failed to proliferate, possible due to the lack of autocrine/paracrine signals normally released by cells into the medium. It has even been suggested that results from sphere-formation-assays with cells not grown at clonal density are impossible to interpret and aldehyde dehydrogenase activity has earlier not significantly enriched for neurosphere-forming cells. (Corti, et al. 2006; Obermair, et al. 2010) Furthermore, it has been suggested quiescent stem cells may not be detected by sphere-forming assays. (Pastrana, et al. 2011)

Following FACS-mediated cell sorting the CD133+, CD133-, ALDH+, ALDH- and bulk populations were subject to analysis by qRT-PCR. For the patient samples, a significant increase of NKX2-2 and CD26 expression was observed in the CD133^{+} population. Also, in contrast to other populations where no expression was identified, the CD133+ population expressed SOX2, SOX9 (no expression identified in bulk population) and CD133 (in contrast to the CD133- population). The absence of CD133 in the CD133- population and the significant increase of CD26 in the CD133+ population, the latter also clearly observed in the FACS analysis, act to verify the FACS.

For the ALDH+ population no expression was observed for the SOX2 and DKAMCL genes in contrast to observed expression in the ALDH- population. Similar to the CD133+ population SOX9 expression was observed in ALDH+ but not in the bulk population. No expression in any of the analyzed patient populations was observed for NMYC, LGR5, NEUROG3, PAX4 and PAX6.

For the ileal carcinoid GOT1 cell line, the CD133+ population showed significant down-regulation of several genes; SOX2, OCT4, NANOG, LIN28 and STRR2. The preceding four have all been used to reprogram somatic cells to induced pluripotent stem cells, where SOX2 and OCT4 have been shown to be essential. (Patel and Yang 2010) SSTR2 is expressed in most gastroenteropancreatic neuroendocrine tumors. (Sclafani, et al. 2011) On the contrary, NKX2-2 and CD133 were shown to be significantly up-regulated. NKX2-2 is considered to be key transcription factors involved in enteroendocrine cell differentiation (Desai et al. 2008) and the up-regulation of CD133 serves to confirm the effectiveness of the FACS sorting. In the CD133+ population NEUROG3 was expressed, but in the CD133- population expression was not identified.

Also the ALDH+ population of the GOT1 cell line showed significant differences in mRNA expression compared to the ALDH- and bulk populations. For this population several genes were up-regulated; SOX2, NMYC, NANOG and NKX2-2. In addition, LIN28 and NEUROG3 expression was, in contrast to the ALDH- population, identified. SOX2, working in pair with OCT4, is believed to be an essential gene for inducing pluripotency of somatic cell. This gene pair has been showed to be even more effective in combination with LIN28/KLF4 or CMYC/NANOG. (Patel and Yang 2010) NMYC and CMYC belong to the same family of transcription factors and could have similar or complementary functions. Therefore it is interesting to note that SOX2, NMYC and NANOG all are significantly up-regulated in the ALDH+ population. In the ALDH+ population no expression of CD26 was observed where it was observed in both the ALDH- and bulk populations. In all investigated GOT1populations no expression of either PAX4 or KLF4 was shown.

In summary, this study has provided evidence for the existence of two subpopulations showing high activity of ALDH and high expression of CD133 respectively, with significantly altered mRNA expression compared to the other cells of the tumour. The ALDH+ population seems, in contrast to the CD133+ population, up-regulate several mRNAs putatively important for stem cells – especially those earlier reported to induce pluripotency in somatic cells. This pattern is most obvious in the GOT1 cell line, which might be explained by notable larger variance between patients. With larger data set the same pattern might be revealed also in patients.

Declaration of interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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