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Cancer stem cell-like cells from a single cell of oral squamous carcinoma cell lines

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ABSTRACT

Resistance of oral squamous cell carcinomas (OSCC) to conventional chemotherapy or radiation therapy might be due to cancer stem cells (CSCs). The development of novel anticancer drugs requires a simple method for the enrichment of CSCs. CSCs can be enriched from OSCC cell lines, for example, after cultivation in serum-free cell culture medium (SFM). In our study, we analyzed four OSCC cell lines for the presence of CSCs. CSC-like cells could not be enriched with SFM. However, cell lines obtained from holoclone colonies showed CSC-like properties such as a reduced rate of cell proliferation and a reduced sensitivity to Paclitaxel in comparison to cells from the parental lineage. Moreover, these cell lines differentially expressed the CSC-marker CD133, which is also upregulated in OSCC tissues. Interestingly, $CD133^+$ cells in OSCC tissues expressed little to no Ki67, the cell proliferation marker that also indicates reduced drug sensitivity. Our study shows a method for the isolation of CSC-like cell lines from OSCC cell lines. These CSC-like cell lines could be new targets for the development of anticancer drugs under *in vitro* conditions.

1. Introduction

Worldwide, approximately 700,000 people develop head and neck carcinomas each year, especially squamous cell carcinoma of the oral cavity, pharynx, and larynx [1]. Head and neck cancer is listed in sixth place among the most common malignant tumors worldwide and is responsible for ~350,000 cancer deaths per year [2]. Unfortunately, the incidence of oral tumors is increasing around the world [3,4].

The current treatment strategy for carcinomas attempts to attack homogenous cancerous tissues. Recently, data shows that tumors are organized as hierarchical tissues containing various differentiated cells with a small subpopulation of undifferentiated cells, so-called cancer stem cells (CSCs) that are the focus of a new strategy of treatment [5–7]. In models of CSCs, these cells exclusively have the capability for tumor formation and growth, and are possibly slow-cycling cells resistant to therapies targeting fast proliferating cells [8]. This tumor-initiating cells hypothesis has recently been tested for a number of diseases including squamous cell carcinoma [9]. The isolation of CSCs makes the discovery of more efficient cancer treatment strategies possible.

Although the existence of CSCs has been demonstrated for many tumors, the isolation of these tumor-initiating cells is still difficult because a definitely unique CSC-marker is not available. One possible marker of CSCs is the surface marker CD133 [10,11]. This surface marker, also known as AC133 and Prominin-1, is expressed in glioblastoma cells [12]. A method for the enrichment of oral squamous cell carcinoma CSCs (OSCCSCs) was the cultivation of oral squamous cell carcinoma lines in serum-free cell culture medium [13]. Here, cells formed spheres and expressed pluripotent stem cell markers such as Oct-4 and the CSC-marker CD133. Interestingly, in comparison to their parental OSCC cell line, these OSCCSCs demonstrated increased cell viability after treatment with known anticancer treatments such as Paclitaxel or radiation therapy under *in vivo* and *in vitro* conditions [13,10]. CSC-like cells, which have some properties of multipotent stem cells, can also be enriched from established cancer cell lines after treatment with anticancer drugs such as cisplatin [14,15]. Interestingly, the proliferation of a myriad of CSCs depends on the activation of the WNT pathway, an important target of novel CSC drugs [14,10,9].

Harper et al. identified CSCs in head and neck squamous cell carcinoma (HNSCC) cell lines [16,17]. This study obtained colonies

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with variable morphologies from single cell suspension of HNSCC cells, which defines the degree of cell differentiation. Colonies with holoclone morphology contain undifferentiated, stem cell-like cells. The formation of paraclones or meroclones reveals cells with a higher degree of differentiation that are less likely that to contain CSCs [16,17]. Moreover, Locke et al. have demonstrated that the pattern of stem cells and differentiated cells is robust and persists even in cancer cell lines [16].

We investigated OSCC cell lines from tissues with variable degrees of differentiation for CSCs. Cell lines were evaluated for their expression of stem cell markers such as CD133, their ability to form colonies in standard medium and their potential for cultivation and enrichment of CSC-like cells under serum-free cell culture conditions. We reveal that cell lines with CSC-like qualities could be obtained from holoclone colonies of established OSCC cell lines. Cells obtained from these holoclone colonies had differentially expressed the stem cell marker CD133. In contrast to parental cell lines, WNT signaling stimulates cell proliferation of these holoclone-derived cells.

2. Materials and methods

2.1. Cell culture

OSCC cell lines PCI-4A, PCI-8, PCI-9A and PCI-13 were kindly provided by Dr. Theresa L. Whiteside (University of Pittsburgh, PA, USA). The tumors were highly (PCI-4A), moderately (PCI-8, PCI-9A) or poorly (PCI-13) differentiated. Cells were cultivated with high glucose DMEM (PAA, Pasching, Austria) supplemented with 10% FBS (Invitrogen, Darmstadt, Germany) and P/S (Gibco). The medium was changed every two to three days and the cells were passaged prior to reaching confluence. For cultivation in serum-free medium (SFM), cells were cultivated in DMEM/F-12 (Invitrogen), N2 supplement (Invitrogen), 10 ng/mL fibroblast growth factor (FGF)-2, and 10 ng/mL epidermal growth factor (EGF) (Biomol, Hamburg, Germany).

Monoclonal subpopulations were generated by diluting a cell suspension to a concentration of one cell per 100 μl. Then, 100 μl of this suspension was transferred into a single well of a 96-well plate. Wells containing two or more cells were marked and not used subsequently. After a single-cell colony had formed, cells were seeded into 6-well plates for further analyses. For the colony-forming unit assay, 100 cells were seeded into a T75 cell culture flask. After 10–14 days, the number of formed holo-, mero-, and paraclones was recorded [17].

2.2. Cell proliferation assay

To examine the influence of the Wnt/β-catenin-pathway, cells were cultivated with 100 nM of the Wnt activator BIO (6-bromoindirubin-3′-oxime, Sigma–Aldrich) or 10 ng/ml of the Wnt inhibitor Dickkopf-1 (R&D Systems, Minneapolis, USA), respectively. Cell proliferation was measured with the MTT assay after 48 h of cultivation. Here, cells were treated with 5 mg/mL MTT for 4 h at 37 °C. The cell culture medium was removed, and cells were lysed by the addition of 0.1 M HCl/isopropyl alcohol. The metabolized MTT was evaluated by optical density (OD) in a spectrophotometer at 540 nm.

2.3. Drug sensitivity assay

5×10^3 cells/well were plated in 96-well plates, allowed to attach overnight, and the chemotherapeutic agent was added. Cell cultures were incubated at 37 °C for 48 h and the number of viable cells following drug treatment was assessed using a WST-1 proliferation assay Kit (Roche, Mannheim, Germany).

2.5. Reverse transcription (RT)-PCR

To isolate RNA, the cell lines were processed according to the RNA isolation kit NucleoSpin RNA II (Macherey–Nagel, Düren, Germany) manual. Reverse transcription of 1 μg total RNA into cDNA was performed with the QuantiTect Reverse Transcription Kit (Qiagen). Isolation of total RNA from the Formalin-fixed paraffin-embedded tissue specimens was done using the RNeasy FFPE Kit (Qiagen, Hilden, Germany).

RT-PCR was done using the GoTaq® Green Master Mix (Promega, Madison, WI, USA) and the Biometra T3000 Thermocycler (Biometra, Göttingen, Germany). Primers are listed in Table S1 (Supplementary Materials). Oligonucleotides were purchased from Metabion (Martinsried, Germany). Tris/borate/EDTA (TBE) agarose gel electrophoresis was used for PCR product analysis.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed using the LightCycler® FastStart DNA Master^{PLUS} SYBR Green I Kit or the LightCycler® TaqMan® Master (Roche, Mannheim, Germany). The LightCycler 2.0 was used for thermocycling. Primers used were CD133-forward: TCCACAGA AATTTACCTACATTGG and CD133-reverse: CAGCAGAGAGCAGATG ACCA. The corresponding probe was #83 (Universal ProbeLibrary Roche). Oligonucleotides were purchased from Metabion (Martinsried, Germany) and Eurofins MWG (Ebersberg, Germany). Samples were measured in triplicate. The gene expression of GAPDH, a housekeeping gene, was used for normalization. The ΔΔCt calculation method was used for the relative quantification of gene expression.

2.6. Immunohistochemistry

For immunohistochemistry, formalin-fixed paraffin-embedded tissues were stained with antibodies against CD133 (New England Biolabs, Frankfurt, Germany) and Ki67 (Dako, Carpinteria, CA, USA) using the Dako Liquid DAB+ Substrate Chromogen System according to the manufacturer's protocol. We analyzed the intensity and distribution of the immunohistochemical staining of CD133. Here, 8 biopsies of oral mucosa-derived squamous cell carcinoma and 24 biopsies of normal oral epithelium were analyzed.

2.7. Flow cytometry analysis

Single cell suspensions were incubated with a monoclonal antibody CD133/293C3 (Miltenyi Biotec, Bergisch Gladbach, Germany) for 45 min at 4 °C, washed once in PBS with 2 nM EDTA and 0.5% BSA. Immunoglobulin G (IgG) isotype-matched antibody (Miltenyi Biotec) was used as the negative control. Flow cytometry analyses were done using the FACS Canto II (Becton Dickinson, Heidelberg, Germany).

3. Results

3.1. Characterization of OSCC cell lines

Four different OSCC cell lines (for details see materials and methods) were investigated for the expression of stem/progenitor cell markers, the existence of CD133 subpopulations, sphere formation and colony forming efficiency. A RT-PCR analysis demonstrated the expression of markers that are associated with stem cells such as OCT4, ABCG2, CD133, Nestin, cytokeratin 15, and CD44 (Fig. 1A). Interestingly, some stem/progenitor cell marker genes such as CD133 and Nestin are expressed at higher levels in

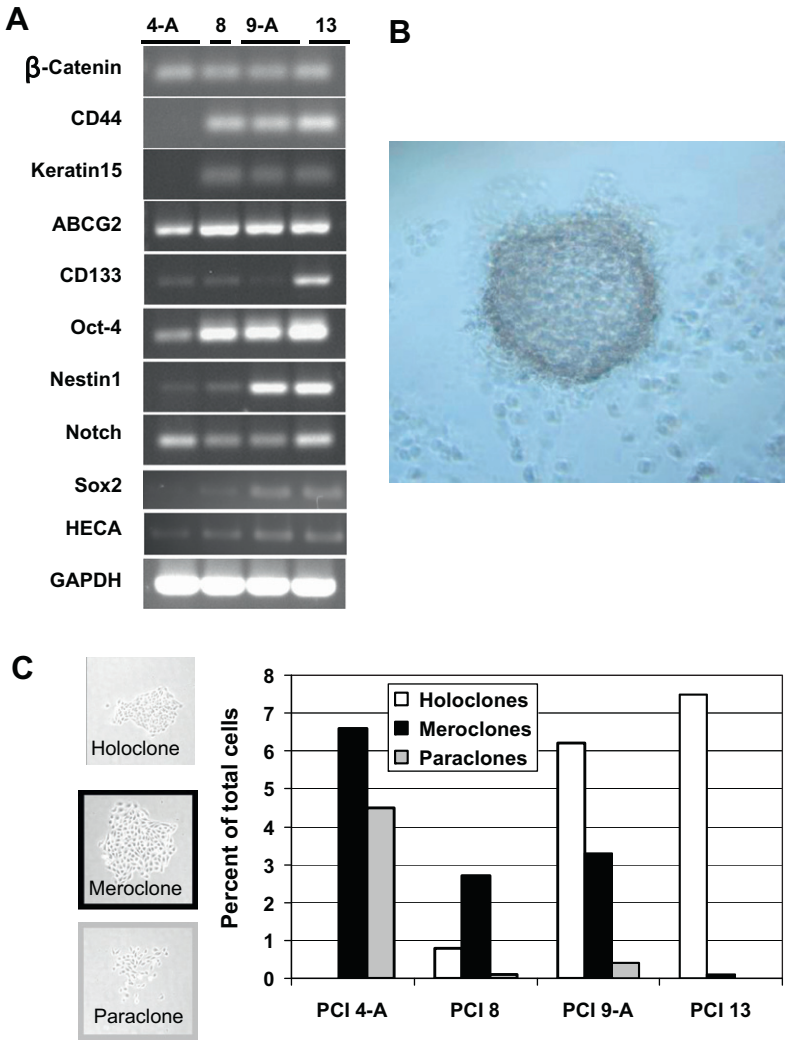

Fig. 1. For the characterization of four different OSCC cell lines, the following analyses were performed: (A) RT-PCR analysis of CSC markers; (B) formation of sphere-like cell clusters after cultivation in serum-free cell culture medium (the figure shows cell line PCI 13); and (C) percentage of colony forming efficiency (CFU) with para-, mero- and holoclones of all investigated cell lines.

the PCI-13 cell line, which was derived from less differentiated tissue of this study. However, a FACS analysis revealed that none of these cell lines contained a subpopulation of CD133 positive cells (data not shown). However, OSCC PCI-13 cells formed spheres under SFM cell culture conditions (Fig. 1B). These cell clusters did not proliferate, and they died after 5 days of cultivation (data not shown). In contrast, every cell line contained single cells that formed colonies with holoclone, meroclone and paraclone morphologies. The distribution of holoclone colonies, indicating stem/progenitor cells, correlates with the grade of tissue differentiation. Here, cell line PCI-13 showed the highest number of holoclone colonies (Fig. 1C).

3.2. Isolation of monoclonal cell lines from holoclone colonies, and the expression of CD133, Ki67 in normal and tumor tissues

For enrichment of CSC-like cells, we prepared monoclonal cell lines from holoclone colonies of the parental cell line PCI-13. These monoclonal cells had a significantly lower cell proliferation rate than cells of the parental cell line (Fig. 2A), but they differentially expressed stem cell markers such as CD133 and Notch1 (Fig. 2B). In FACS analysis, monoclonal cell lines were revealed to contain an larger fraction of $CD133^+$ cells (Fig. 2C). These results confirm that monoclonal cell lines contain CSC-like cells. However, putative OSCCSC markers such β-Catenin [16,9], CD44 [18,19] and CK15 [16] were not upregulated (Fig. 2B). Stem cell markers are upregulated in cancer tissues [13] and therefore we investigated dormitory the expression of CD133 and Ki67 in cancer and control tissues (Fig. 3A). Tumor tissue slices contained 31 (±20)% CD133 positive cells and 18.6 (±12.5)% Ki67 positive cells. The staining intensity ranged from moderate to strong for CD133 and moderate for Ki67. 20 (±10)% and 5.8 (±2.5)% of normal oral epithelial cells showed a positive staining for CD133 and Ki67, respectively. CD133 staining intensity ranged from weak to moderate, but overall was weaker than staining in OSCCs. Staining intensity of Ki67 in normal tissues was similar to that of tumor tissue cells. Interestingly, areas positive for the cell proliferation marker Ki67 in both control and cancer tissues showed little to no staining for CD133. CD133-stained cells are therefore slow-cycling cells, which is a possible feature of CSC-like cells. Furthermore, a real-time RT-PCR analysis revealed that the expression of CD133 is significantly upregulated in cancer tissues (Fig. 3). Our results suggest that CD133 is upregulated in slow proliferating cells of OSCC tissues.

3.3. Influence of WNT signaling and Paclitaxel on cell proliferation

OSCCSC proliferation is sensitive to the activation of WNT signaling [9]. Therefore, we investigated the proliferation of a

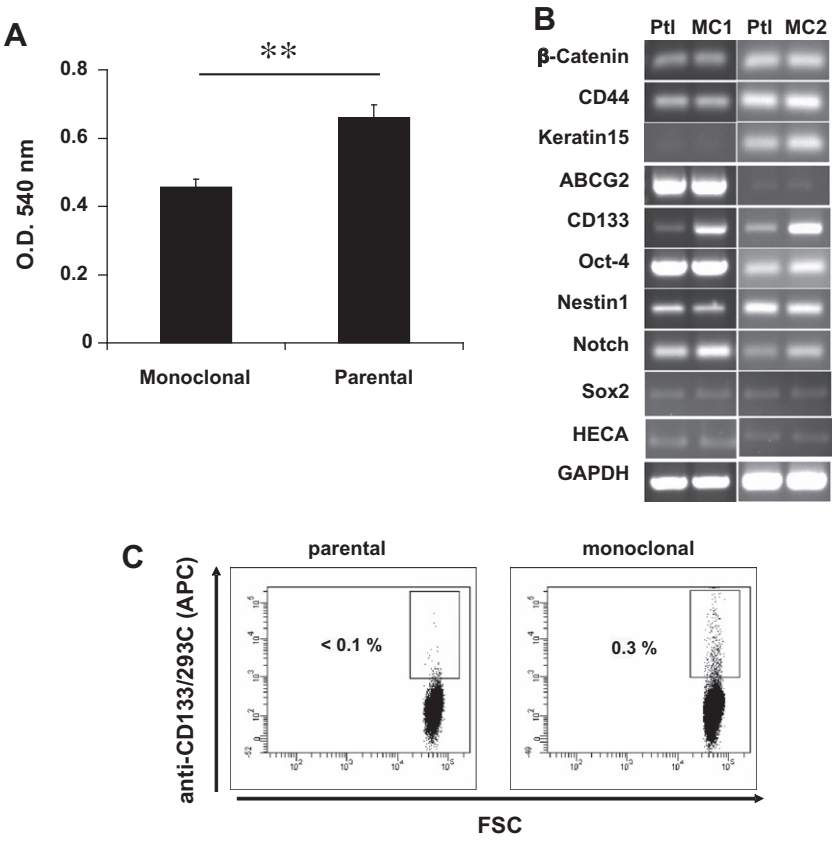

Fig. 2. Comparison between monoclonal cell lines and the parental OSCC cell line 13. Cell proliferation (A), gene expression of CSC markers (B) and the percentage of CD133$^+$ cell population (C) were estimated in monoclonal cell lines (MC1, MC2) and their parental cell line. For statistics, Student *t*-test was applied: *t*-test $^{**}p < 0.01$.

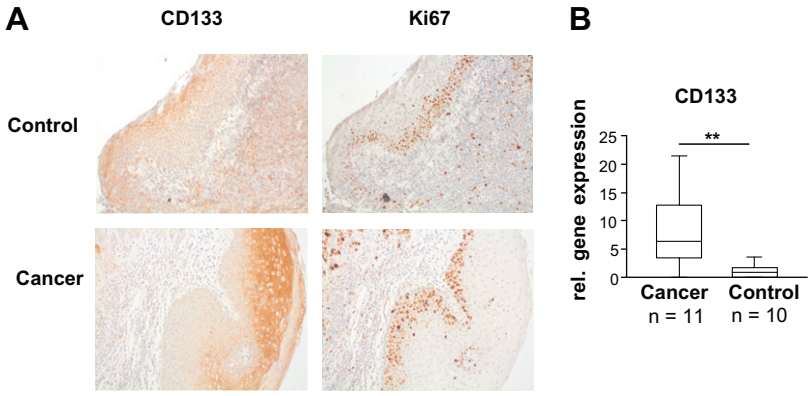

Fig. 3. A: Immunohistochemistry of CD133 and Ki67 in OSCC and normal tissues. B: Gene expression (real-time RT-PCR) of CD133 in OSCC and normal tissues. For statistics, Mann–Whitney test was applied: $^{**}P < 0.01$.

monoclonal cell line and its parental cell line after stimulation and inhibition of WNT signaling (Fig. 4). The proliferation of monoclonal cells was significantly increased after stimulation of WNT and slightly decreased after inhibition of this pathway. No significant differences were detected upon modulation of WNT signaling in parental cells. This result supports the hypothesis that the monoclonal cell line contains enriched numbers of CSC-like cells.

We cultivated monoclonal and parental cell lines in the presence of Paclitaxel to evaluate the influence of chemotherapeutics on cell proliferation/vitality. The monoclonal cell line was less sensitive to Paclitaxel than the parental cell line (Fig. 4B).

4. Discussion

The isolation and characterization of CSCs is an ambitious target of tumor biology. Previous studies showed that CSCs could persist in established cell lines that were derived from cancer tissues [14,16,13,10,17]. Chiou et al. [13] isolated and cultivated CSCs as spheroid cell clusters similar to neural stem cells, which differentially expressed typical pluripotent stem cell markers such as Oct-4. Oct-4 is purported to be a reliable marker for CSCs [13,20,21]. Investigated cell lines of our study formed sphere-like cell clusters, but these cells could not be propagated under

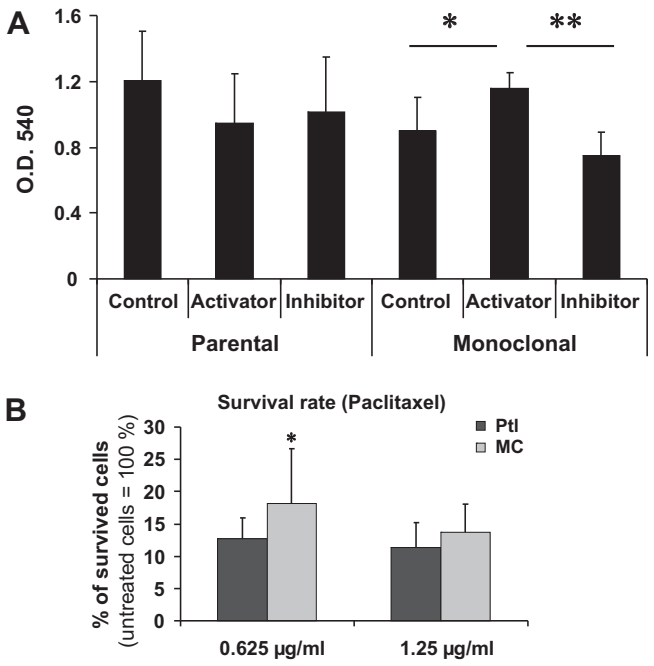

Fig. 4. The influence of activation or inhibition of the WNT pathway (A) and of the cytotoxic drug Paclitaxel (B) on proliferation of the monoclonal cell line (MC) and the parental cell line (Ptl). For statistics, Student *t*-test was applied: *t*-test $^{**}p < 0.01$; *t*-test $^{*}p < 0.05$.

serum-free cell culture conditions. This is probably specific to the cell lines of our study, which were derived from a pool of squamous cell carcinoma cell lines. Whiteside and colleagues have isolated 21 cell lines from fresh tumor specimens to study OSCC [22]. These cell lines were isolated in the 1980s, and in this study they express only low levels of CSC markers such as CD44 or CD133. Interestingly, CD133 was detected at the mRNA transcript level (RT-PCR) but not at the protein level (FACS analysis) in the PCI-13 cell line. However, the expression level of pluripotent stem cell marker Oct-4 correlates well with the tumor cell line's differentiation grade. The same is true for the number and the morphology of colonies derived from these four different cell lines.

Mackenzie and colleagues demonstrated that under *in vitro* conditions, colonies similar to those of normal keratinocytes form a continuous gradient extending from morphologies that they define as holoclone, paraclone and meroclone. Here, cells isolated from holoclone colonies, unlike those from meroclones and paraclones, were able to generate all colony types, which pinpoint holoclone-derived cells as CSC-like cells [16,17]. The PCI-13 cell line, which was derived from a poorly-differentiated tumor with the lowest grade of differentiation in our study, produced the greatest number of holoclone colonies. Although this could be an indicator for CSC-like cells, this cell line, unlike the PCI-4A, PCI-8 and PCI-9A cell lines, was not tumorigenic in a first study with athymic mice [22]. We isolated monoclonal cell lines from holoclone colonies of the PCI-13 cell lines. These monoclonal cell lines show typical properties of CSCs under *in vitro* conditions, including a reduced cell proliferation rate in comparison to parental cells that depends on the activation of the WNT pathway. This pathway has been previously implicated in the maintenance of CSCs, tumorigenesis and progression [14,9,23]. Although the effect was small, Paclitaxel toxicity was reduced in monoclonal cell lines in comparison to the parental cell line, also a characteristic of CSC-like cells [10].

Monocolonal cell lines differentially expressed the CSC-marker CD133. Although $CD133^-$ cells could be tumor-initiating cells in lung cancer cell lines [14,24], CD133 is a reliable CSC marker for human OSCCSCs [13,10]. Our study suggests that CD133 is a marker for OSCCs unlike other prospects such as CD44, CK15 and β-Catenin [17–19]. Transcripts for CD44, CK15 and β-Catenin were not upregulated in monoclonal cells of our study. Mack and Gires showed in a comparative immunohistochemical study using cancer tissues and healthy controls that CD44 is not upregulated in head and neck cancer tissues [25]. Similar results were previously obtained after the isolation of CSCs with serum-free cell culture medium [13]. Recently, Richard and Pillai describe the hyperplasia of CSCs in different stages of OSCC [26]. Although our random sample is small, we found a significantly greater expression of CD133 in cancer tissues by real-time RT-PCR. However, CD133 immunohistochemical staining was almost exclusively observed in suprabasal layers of histologically normal oral mucosa, not in the basal layer as was expected. Squamous cell carcinoma showed a similar distribution pattern, especially in biopsies with keratin-forming squamous cell carcinomas; positive staining was observed in cell layers bordering on the central keratin pearl. Similar to the expression of CD133 transcripts, intensity and number of $CD133^+$ cells were increased in OSCC. Here, the cell staining of the cell proliferation marker Ki67 indicates that $CD133^+$ cells are slow-cycling cells, which could be an explanation for an enhanced resistance to anticancer drugs. Interestingly, Kemper et al. [27] showed that CD133 expression is similar in colon CSCs and differentiated colon cancer cells, although CD133 cell staining with a CD133/AC133 or CD133/293C antibody is diminished after differentiation of CSCs. They suggest that differential glycosylation hides epitopes of CD133 antibodies [27]. For our study we have used a peptide-specific antibody for immunohistochemistry and the CD133/293C antibody for FACS analyses. We find that both protein and mRNA expression were upregulated in monoclonal cell lines and OSCC tissues in comparison to parental cells and control tissues. These results suggest that CD133 is a marker for OSCC cells with CSC-like properties that is probably independent of the glycosylation status of CD133.

In conclusion, our study shows that although CSC-like cells could not be enriched in SFM from investigated cell lines, CSC-like cells were obtained from holoclone colonies of OSCC. These monoclonal cell lines differentially express CD133, which is also upregulated in OSCC tissue cells. Here, $CD133^+$ cells are slowly-proliferating cells ($Ki67^-$) and are not located in the basal layer, which is the niche of hypothesized epithelial stem cells. $CD133^+$ cells from cancer tissues or monoclonal-derived CSC-like cells isolated from established OSCC cell lines are interesting targets for the future evaluation of anticancer drugs.

Conflict of interest statement

None declared.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.02.084.

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