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Stem cell profiling in head and neck cancer reveals an Oct-4 expressing subpopulation with properties of chemoresistance

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SUMMARY

Objectives: In the past decade cancer, including head and neck squamous cell cancer (HNSCC), is increasingly being regarded as a stem cell associated disease which arises from cells with the property of stemness. According to the cancer stem cell (CSC) theory, only a specific subpopulation of cancer cells has the ability to initiate and perpetuate cancer growth, especially under treatment. In this article we describe a subpopulation of cells within HNSCC that expresses the stemness factor Oct-4, which leads to apoptotic resistance after exposure to chemotherapeutic agents.
Materials and methods: Permanent cell lines and HNSCC tissue were analyzed for expression of stem cell markers using flow cytometric, immunohistochemical approaches and an antibody based protein array. Chemotherapeutic agent-induced growth inhibition, also known as "enrichment", was determined by the colorimetric cell proliferation assay (MTT-based) and putative stem cell markers were investigated by flow cytometry.
Results: Various potential CSC markers were identified in heterogenic expression profiles in permanent cell lines and solid tumors. Our data suggest the Oct-4A isoform as a marker of stemness in HNSCC and the enrichment of cancer stem-like cells by various chemotherapeutic agents was associated with a significantly higher expression of Oct-4.
Conclusion: This data suggests that many potential CSC markers are expressed on different expression levels in HNSCC. Among these markers Oct-4(A) plays a pivotal role in the detection of cancer cells with enhanced chemoresistance and provide evidence for the existence of cancer stem-like cells in HNSCC.
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Introduction

Head and neck squamous-cell carcinoma (HNSCC) is one of the most common solid neoplasms, occurring often among middle-aged tobacco and alcohol abusers [1,2]. The molecular mechanisms responsible for tumor aggressiveness and its reaction to chemo- and radiation therapies remain mostly unknown [3–5].

A possible explanation for these facts could be the cancer stem cell hypothesis, postulating each tumor contains a rare subpopulation of suggestive cancer stem cells (CSC), sharing the three most representative features; self-renewal, colony formation and pluripotency [6–8]. The first description of a hierarchical tissue organization of cellular replacement by stem cells was taken by the hematopoietic system [9–11]. However, cancer stem-like cells have been identified in many tumors including carcinomas of the biliary [12], brain [13], breast [14], colon [15], esophagus [16], liver [17], lung [18], pancreas [19], stomach [20], and prostate [21].

The transcription factor Oct-4 (Octamer binding transcription factor 4, also established as Oct-3 or POU5F1) was first described as a gene exclusively expressed in pluripotent lineages. Oct-4 is a transcription factor of the POU family that is expressed in undifferentiated, pluripotent cells, including human embryonic stem and germ cells [22–27]. Oct-4 is sufficient to reprogram human somatic cells to pluripotent stem cells with other stem cell factors (SOX2, NANOG, LIN28) or directly generate pluripotency in adult neural stem cells in a one-factor induced way [28,29]. Moreover, Oct-4 has been shown to support tumor growth in a dose dependent manner and has an important role in chemoresistance [30,31].

The human Oct-4 gene encodes two variants known as Oct-4A and Oct-4B. Importantly, only the Oct-4A isoform sustains stem cell properties and is localized to the nucleus, whereas the Oct-4B isoform cannot be assigned to stem cell properties and is expressed in the cytoplasmic compartment of the cell [32–35].

In this work we investigated the expression of putative stem cell markers (CD34, CD44, CD59, CD117, CD133, AFP, E-cadherin, GATA-4, Goosecoid, HCG, HNF-3ß/FoxA2, Nanog, Oct-4, Otx2, PDX-1/IPF1,

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Snail, Sox2, Sox17, TP63/TP73L, and VEGFR-2/KDR/Flk-4) including Oct-4A in permanent HNSCC cell lines and solid HNSCC-tumors, as well as the reaction of Oct-4+ cells to different chemotherapeutic agents.

Materials and methods

Cell culture

The HNSCC cell lines used here were generated from primary tumors: a hypopharyngeal carcinoma (PCI-I; Pittsburgh Cancer Institute, PA, USA) and an oropharyngeal carcinoma (BHY; 'Deutsche Sammlung für Mikroorganismen und Zellkulturen', Braunschweig, Germany). Tumor lines designated 'UT-SCC' were all established at the Department of Otorhinolaryngology, Head and Neck Surgery (University of Turku; Turku, Finland). The UTSCC lines were paired and generated from the primary (A) and metastatic lymph nodes (B) of the same patients: a cutis nasi tumor (UT-SCC 12A/B), a tonsillar carcinoma (UT-SCC 60A/B) and a tongue carcinoma (UT-SCC 74A/B). The cells were cultured in serum-free high-glucose DMEM (DMEM; PAA, Pasching, Austria), supplemented with 2.5% HEPES buffer, 1% sodium pyruvate, 1% L-glutamine, 1% nonessential amino acids.

Human tissue samples

After written informed consent was provided the tumor tissue specimens were obtained during the standard surgical procedure. The tissue specimens were transported in sterile saline and processed immediately after excision. The use of human tissues for research purposes was approved by the ethics committee of the University of Lübeck.

Preparation of single-cell suspensions

The HNSCC specimens were washed several times and finely minced into small fragments and then placed in a digestion solution which consisted of the digestive enzymes collagenase type II (31.5 mg/ml, GIBCO, Eggenstein, Germany), hyaluronidase (3.99 mg/ml, Sigma, Munich, Germany) and dispase (33.4 mg/ml, Sigma, Munich, Germany) in addition to sterile serum-free high-glucose DMEM (PAA, Pasching, Austria), supplemented with 2.5% HEPES buffer, 1% sodium pyruvate, 1% L-glutamine, 1% nonessential amino acids and 10% of PAA's antibiotic/antimycotic solution (PAA, Pasching, Austria) undergoing shaking at 37 °C for 3 h. The resulting cell suspensions were filtered through a 70 μm and 40 μm nylon cell strainer (Falcon; Becton Dickinson Labware, Heidelberg, Germany), centrifuged, washed and resuspended several times with phosphate-buffered saline (PBS) (PAA, Pasching, Austria). Single cell suspensions were used for the MTT assays and flow cytometric analysis.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide-based cell assay (MTT)

Cell proliferation was determined by a quantitative colorimetric MTT assay. This assay determines viable cell numbers based on the mitochondrial conversion of MTT. The growth kinetics under different treatment with cytostatic agents were correlated to control wells (normalized to 100%) and are represented as mean ± SD in a fitted curve. Using the single exponential function of OriginPro 8 software (OriginLab Corp, Northampton, MA) we obtained nonlinear fitted dosage curves (in accordance with the formula: $y = A1 * \exp(-x/t1) + y0$, also used for calculating ED50 and ED90).

Flow cytometry

Cells were grown to confluence, trypsinized, pelleted, and resuspended in PBS solution. Single-cell suspensions of the tumor tissue specimens and the permanent cell lines were stained with specific antibodies on ice for 15 min followed by washing with a solution of PBS. For intracellular staining of Oct-4, cells were treated with 0.1% Saponin. Next Oct-4 cell suspensions were mixed with PE-conjugated anti-CD34, -CD44, -CD117 and -CD133 and FITC-conjugated anti-CD59 as directed by each manufacturer. Hence, a directly labeled antibody of Oct-4A for flow cytometry was not distributed, we exclude Oct-4B positive PBMCs by the FITC-conjugated lineage cocktail 1 marker mix (anti-human-CD3, -CD14, -CD16, -CD19, -CD20, - CD56) for excluding Oct-4-positive lymphocytes, monocytes, eosinophils and neutrophils. The three isotypes PE IgG1/IgG2a, APC IgG1/Igg2a and FITC IgG1/IgG2b were used as negative controls. Data was analyzed by flow cytometry on a FACSCanto (Becton Dickinson) equipped with FACS DIVA software.

Proteome profiler array

We investigated the relative protein expression levels regarding 15 different stem cell markers and transcription factors in tumor tissue samples and permanent cell lines in order to obtain a comprehensive pattern of the stemness proteome by using R&D's human pluripotents stem cell array. The kit contains a nitrocellulose membrane with spotted capture and control antibodies. 200 μg of total cell proteins were transferred onto the membrane and incubated at 4 °C overnight with the human pluripotent stem cell array. Results were revealed by scanning and analyzing the mean spot pixel density from the *array membrane* by using ImageJ software (National Institutes of Health, Bethesda, USA). Each generated average pixel intensity was related to the average pixel intensity of 6 positive controls.

Immunohistochemistry

We used the immunohistochemical method for detection of Oct-4 in tumor tissue samples. It was our aim to confirm qualitatively the quantity of detected stem cells via the other methods.

Small pieces of intraoperative tumor tissue samples were frozen in optimal embedding media (Miles Inc. Elkhart, Indiana, USA). Then 6-μm sections were cut using a cryostat microtome (Leica cryotom CM3050S, Nussloch, Germany), mounted on glass slides, and stored at −80 °C until use. The sections were dried at room temperature (22 °C), incubated in methanol for 20 min and rehydrated and rinsed in PBS. For the intracellular staining regarding Oct-4A and Nanog, cells were permeabilized with 0.1% Triton-X-100 (Sigma–Aldrich Chemie GmbH, Steinheim) in PBS. The primary antibodies, anti-Oct-4A mouse antibody (Epitomics, Inc., Burlingame, California, USA) and Nanog (Abcam, Cambridge, Massachusetts, USA) were diluted 1:100 (20 μg/ml) with an antibody dilution buffer (containing blocking buffer) (DCS LabLine, DCS, Hamburg, Germany) and incubated in a humidified chamber at room temperature for 60 min. After washing in PBS the corresponding secondary antibodies were incubated for 45 min. The secondary antibody goat anti-mouse Cy2 (Dianova, Hamburg, Germany) was diluted 1:100 and the secondary antibody goat anti-rabbit Cy3 (Dianova, Hamburg, Germany) was diluted 1:200. The tissue samples were rinsed three times in PBS. Negative controls were identical to positive controls, except that incubation with primary antibodies was replaced with buffer. The nuclei were stained using DAPI (1 μg/ml, Roche Diagnostics, Mannheim, Germany). The samples were rinsed three times for 5 min in PBS and were embedded afterwards in Fluoromount G (Southern

Biotechnologies Associates, Birmingham, Alabama, USA). Samples were evaluated on a 200 M microscope (Zeiss, Göttingen, Germany) by either differential interference contrast (DIC) microscopy or fluorescence microscopy, using different fluorescent filter sets according to the fluorescent staining (AHF Analysentechnik AG, Tübingen, Germany). Cells were photographed using an AxioCam MRm Rev.3 FireWire and the AxioVision Rel. 4.7 software (Zeiss, Jena, Germany).

Statistical analysis

For statistical analysis, data obtained from independent experiments were compared using a Mann-Whitney U Test for paired comparisons, using SPSS software (SPSS, Inc., Chicago, USA). Values of $p < 0.05$ were considered statistically significant.

Results are expressed as means ± standard deviation (SD).

Results

Flow cytometric expression profiling regarding potential surface stem cell markers

The cell surface phenotype of five head and neck cancer cell lines, as well as five single-cell suspensions of solid head and neck tumors, were analyzed by flow cytometry concerning their expression profiles of various potential stem cell surface proteins (Fig. 1A).

Since it was demonstrated, that stem cell markers were not only expressed in stem cells (for example Oct-4B in peripheral blood mononuclear cells [34–36], we used the lineage marker mix (anti-human-CD3, -CD14, -CD16, -CD19, -CD20, -CD56) to exclude lineage positive cells like lymphocytes, monocytes etc. We could not detect any lineage positive cells in the cell lines studied. In solid HNSCC tumors a small number of cells were identified as lineage positive cells which were not considered in the calculation of expression level (data not shown).

As shown in Fig. 1A, all permanent HNSCC cell lines showed a high expression of CD44 (mean 78.0%, SD 8.9) and CD59 (mean 95.7%, SD 3.8). Only a minority of cancer cells expressed the markers CD34 (mean 1.8%, SD 0.7), CD117 (mean 4.8%, SD 5.6), CD133 (mean 1.3%, SD 0.6), and Frizzled 3 (FZD3, mean 0.7%, SD 0.4). In contrast, the Wnt transmembrane receptor Frizzled 7 (FZD7) was identified in a heterogeneous population of different HNSCC cell lines (mean 37.6%, SD 24.8). Interestingly, the primary tumor cell lines UT-SCC 12A and UT-SCC 60A showed a significant higher expression of FZD7 compared with their corresponding metastatic cell lines UT-SCC 12B and UT-SCC 60B ($P = 0.001$).

Comparing to the expression of candidate CSC markers in permanent HNSCC cell lines, there was only a small discrepancy towards solid HNSCC tumor-derived cell cultures. As expected, the variability of the stem cell marker expression was greater in the polymorphic tissue of solid tumors, although all counted cells were negative for the lineage marker mix. Compared to the permanent cell lines, all investigated markers could also be identified, but altogether the populations and the numbers of positive cells were on a lower level (Fig. 1B). The highest expressed markers we detected in the single-cell suspensions were CD44 (mean 54.0%, SD 19.3) and CD59 (mean 58.2%, SD 14.1). Similarly to the HNSCC cell lines,

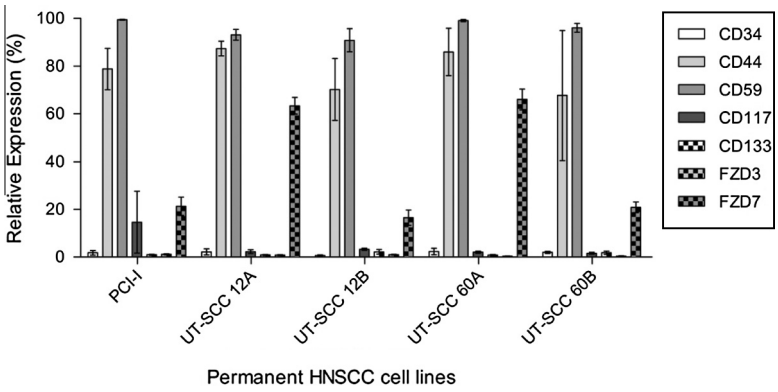


Figure 1A. Expression profiling of potential surface CSC marker in permanent HNSCC cell lines. Expression profiles of CD34, CD44, CD59, CD117, CD133, Frizzled-3 (FZD3), and FZD7 in different permanent HNSCC cell lines (PCI-I, UT-SCC 12A/B, UTSCC 60A/B). Illustrated is the mean with standard deviation in error bars ($n = 3–4$) and represents the number of positive cells compared to total number of all measured cells (5×10^5–10^6 cells).

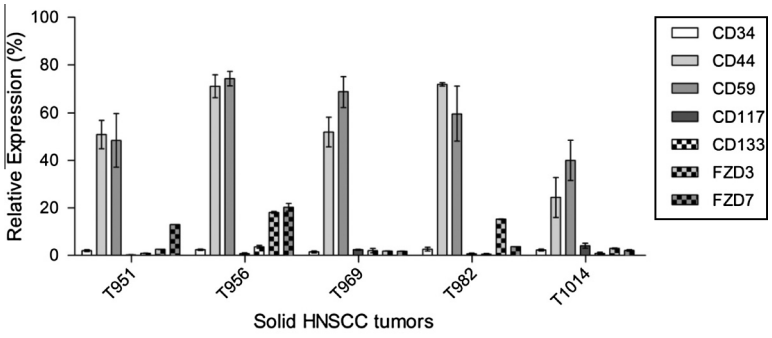


Figure 1B. Expression profiling of potential surface CSC marker in solid HNSCC tumors. The vertical bars (mean, SD, $n = 3–4$) show the expression of the seven potential CSC markers, including CD34, CD44, CD59, CD117, CD133, FZD3, and FZD7 in five solid HNSCC tumors. Pictured in bars is the number of cells with proof of the potential stem cell marker compared to all investigated cells.

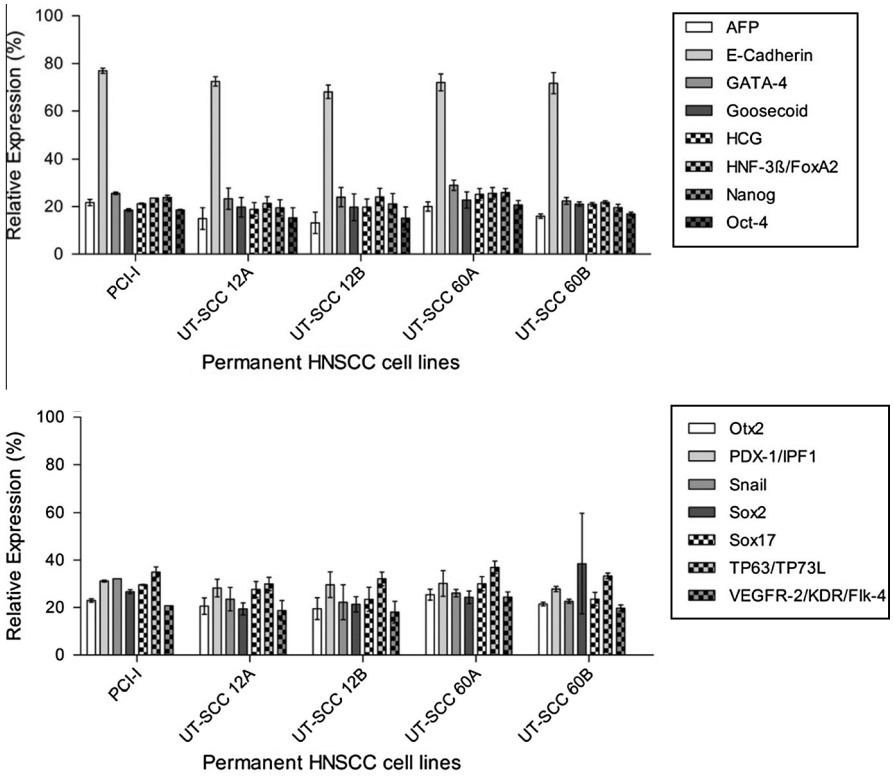


Figure 1C. Expression profiling of embryonic stem cell marker in permanent HNSCC cell lines. Bars (mean, SD, $n = 4$) display the expression level of fifteen embryonic stem cell marker in figure (AFP, Ecadherin, GATA-4, Goosecoid, HCG, HNF-3ß/FoxA2, Nanog, Oct-4, Otx2, PDX-1/IPF1, Snail, Sox2, Sox17, TP63/TP73L, and VEGFR-2/KDR/Flk-4) in various permanent HNSCC cell lines, including PCI-I, UT-SCC 12A/B, UTSCC 60A/B.

we observed an inhomogeneous expression of FZD7 (mean 8.1%, SD 8.2). Only one potential CSC marker, which we demonstrated on a higher expression level in solid tumors than in permanent cell lines, was FZD3 (mean 8.1%, SD 7.9). The remaining three markers, namely CD34 (mean 2.1%, SD 0.4), CD117 (mean 1.6%, SD 1.6), and CD133 (mean 1.6%, SD 1.3) were found in low dimensions.

Proteomic profiling regarding embryonic transcription factors and markers

The expression of characteristic stem cell-related transcription factors and markers was evaluated by using the antibody based proteome profiling array from R&D System. We simultaneously screened for the expression of 15 transcription factors and markers in protein lysates prepared from 5 HNSCC cell lines and 4 solid primary tumors tissue samples (T), respectively (Figs. 1C, 1D). This quantitative transcription factor analysis revealed a detection of all 15 markers in all HNSCC tissue samples, derived both from cell lines and solid tissue. The level of detected markers was similar in all samples of permanent cell lines. Only E-cadherin, the best characterized member of the cadherin-family, mainly expressed in epithelial adherent junctions, but also in embryonic stem cells, was quantitatively assessed with an approximate average of 72.2% in cell lines. The levels of the other markers were between 9.1% (AFP in UT-SCC 12B) and 39.1% (TP63/TP73L in UT-SCC 60A). In the solid tumor samples we found the highest expression levels for E-cadherin with percentage levels up to 80.2% (T1005). The percentage expression rate of the other embryonic transcription factors and markers were in a range between 6.5% (VEGFR-2/KDR/Flk-4 in T800) and 29.0% (Snail in LN801). In general, the samples of the fresh tissues showed more heterogeneous expression level in comparison to permanent cell lines.

In addition, in Fig. 1E the results of 2 primary solid tumors with their corresponding lymph node metastases (LN) are represented. There are no statistically significant differences in terms of the expression of each stem cell marker between the primary solid tumors and their metastases.

It has been shown, that the embryonic transcription factor Oct-4 alone is sufficient to induce pluripotency in neural stem cells and is essential for maintenance of pluripotency, which is a characteristic property of stem cells [28–31]. Hence, we focused predominantly on this protein in following investigations.

Identification of an Oct-4A subpopulation in immunocytochemistry and an Oct-4$^+$/lineage$^-$ subpopulation by flow cytometry

As mentioned above, Oct-4 has been identified in HNSCC cell lines as well as in solid tumor cells. However, the proteome profiling array cannot distinguish between the two isoforms Oct-4A and Oct-4B. Since Oct-4B was detected in PBMCs this distinction is important because Oct-4B cannot sustain stemness properties and is located in the cytoplasmic compartment of cells. For this reason further investigation using immunocytochemistry (Fig. 2) and flow cytometry (Fig. 3) were performed. The antibody we used in immunocytochemistry specifically recognizes Oct-4A and not Oct-4B. Oct-4A immunocytochemistry on permanent HNSCC cell lines as well as primary tumor tissue sections illustrate expression of Oct-4A alone (Fig. 2B and F) and together with Nanog in a coexpression manner in a permanent HNSCC cell line (UTSCC-12B, Fig. 2C and D). Especially, in DAPI overlayed samples the nuclear localization of the proteins, typically for Oct-4A (Fig. 2D and G), can be seen. In this work we could not detect a coexpression of Oct-4A and Nanog in solid HNSCC tumors, similar to the permanent cell lines.

To determine the expression level of Oct-4A we choose flow cytometry and combined the common Oct-4 antibody (APC) with

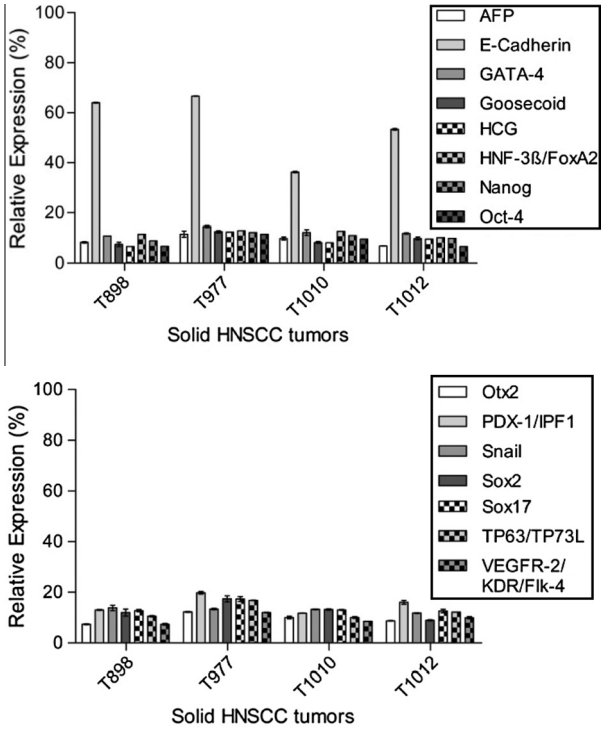


Figure 1D. Expression profiling of embryonic stem cell marker in solid primary HNSCC tumors. This graph (mean, SD, $n = 4$) show the expression profiles of fifteen embryonic stem cell marker, including AFP, E-cadherin, GATA-4, Goosecoid, HCG, HNF-3ß/FoxA2, Nanog, Oct-4, Otx2, PDX-1/IPF1, Snail, Sox2, Sox17, TP63/TP73L, and VEGFR-2/KDR/Flk-4 in solid primary HNSCC tumors (T).

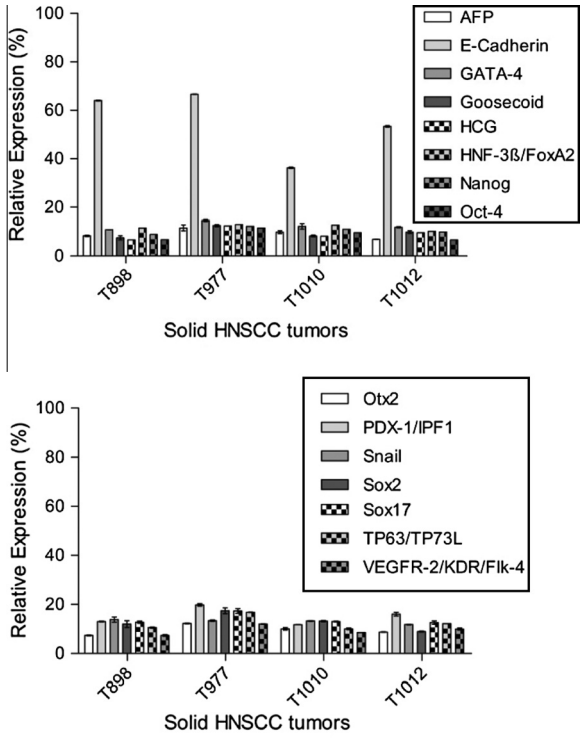


Figure 1E. Expression profiling of embryonic stem cell marker in solid primary HNSCC tumors and their lymph node metastases. Bars (mean, SD, $n = 4$) represent the expression of fifteen embryonic stem cell marker (AFP, E-cadherin, GATA-4, Goosecoid, HCG, HNF-3ß/FoxA2, Nanog, Oct-4, Otx2, PDX-1/IPF1, Snail, Sox2, Sox17, TP63/TP73L, and VEGFR-2/KDR/Flk-4) in solid primary HNSCC tumors (T) and the corresponding lymph node metastases (LN).

the lineage marker mix (FITC) in order to exclude Oct-4B positive PBMCs. This procedure was necessary since a directly labelled Oct-4A antibody was not distributed. Fig. 3 displays a solid tumor (T951) in two dot plots, the isotype control and the measurement after staining with Oct-4 (APC) and lineage (FITC). For better recognition, the small Oct-4A$^+$/lineage$^-$-subpopulation in dot plot B was circled. Five solid tumor samples were examined and revealed a heterogeneous expression on a low expression level between 0.2% (T1014, SD 0.1) and 10.8% (T956, SD 4.6).

Enrichment of Oct-4 expressing cells after exposure to chemotherapeutic agents

An important property of CSC is the resistance in response to chemotherapeutic agents. Investigating this property of CSC seemed to be the next step, since we had found Oct-4A positive potential CSC in HNSCC. Therefore we exposed the cell lines to the three predominantly cytostatic agents used in current HNSCC: 5-fluorouracil, cisplatin, as well as paclitaxel. The cell viability was determined using a MTT proliferation assay 48 h after addition of respective doses of the cytostatic agents (untreated respectively DMEM, 10, 50, 100, and 200 μM) and fitted into a dose-dependent toxicity curve (Fig. 4). The aim was to achieve different levels of cytotoxicity with the drugs (see the red[2] arrow in Fig. 4). Only permanent cell lines could be treated with chemotherapeutic agents, the number of vital cells in a single cell suspension of solid tissues was too low after enrichment with chemotherapeutic drugs. The Oct-4 population of untreated PCI-I cells, cultured in DMEM, shows an average cell rate of 21.2% (SD 9.3, $n = 11$, Fig. 5). Importantly, exposure to 200 μM 5-fluorouracil for 48 h increased the mean of Oct-4 positive cells from 21.2% to 33.8% (SD 5.5, $P = 0.0057$, Fig. 5), whereas the mean number of vital cells were decreased to 62.0% (SD 5.0, Fig. 4). In the cisplatin-treated fraction with a reduction to 30.4% (SD 10.4, Fig. 4) of all PCI-I-cells we observed a slightly further increase of Oct-4 positive cells (mean: 36.5%, SD 9.0, $P = 0.0044$, Fig. 5). The most impressive increase detected in the paclitaxel-treated fraction was a treatment with 10 μM Paclitaxel which led to a reduction of 18.7% OR which was reduced down to 18.7% (SD 8.9, Fig. 4) of all cells as well as a pronounced increase of Oct-4$^+$-cells (mean: 57.9%, SD 13.0, $P = 0.0011$, Fig. 5). These results are shown by the cell line PCI-I, comparable results with other cell lines also reveal an increase of Oct-4 after exposure of chemotherapeutic agents.

These results confirm a relative increase of Oct-4 positive cells dependent on the toxicity effects of the cytostatic remedy and an increased resistance to chemotherapeutic agents.

Discussion

The concept of CSC proposes that a small population of aberrant cells are responsible for the maintenance of the malignant tissue of every tumor. In almost every type of solid cancer a potential subpopulation with stem cell properties was demonstrated [12–21].

In permanent HNSCC cell lines, as well as solid tumor tissue, only a small subpopulation (<4.8%) expressed the markers CD34, CD117, and CD133, as previously described [35,37,38]. Regarding the expression of CD44 there exists divergent data in primary tumors and permanent cell lines, regardless of the grade of malignancy and tumor stage: there are reports of expression rates below 10% [22,39], but also expression rates of more than 75% have been described [24,25].

Wnt signaling plays an important role in embryogenesis and several human diseases including cancers. Numerous studies

[2] For interpretation of color in Fig. 4, the reader is referred to the web version of this article.

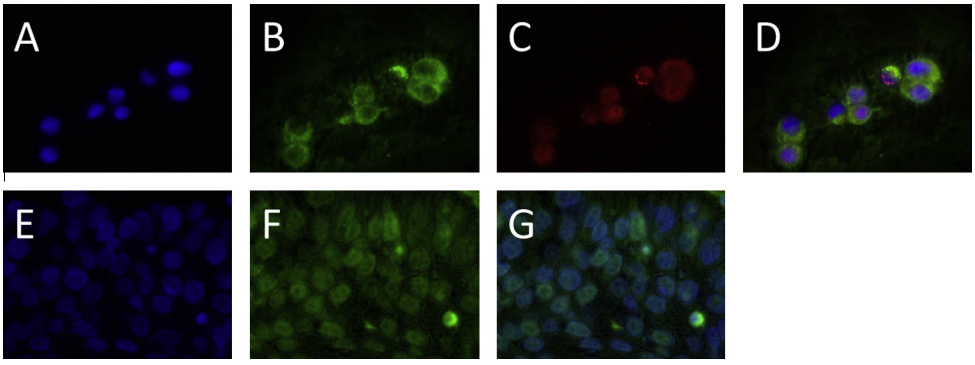


Figure 2. Expression of Oct-4A in coexpression with Nanog in the permanent HNSCC cell line UT-SCC 12B (A–D) and solely in a solid HNSCC tumor (E–H). Permanent HNSCC cell lines and solid HNSCC tumor tissues were first incubated with primary antibodies (Oc-4A and Nanog) and then probed with Cy2 and Cy3 secondary antibodies. The nuclei were counterstained with DAPI (4,6-diamidino-2-phenylindole dihydrochloride; blue; panel A). Panel B shows Oct-4A staining in a nuclear localization (Cy2; green fluorescence), panel C demonstrates Nanog in a nuclear localization (Cy3; red fluorescence). Panel D is the overlay (merge of panel a–c) and demonstrates the coexpression. Solid HNSCC tumor tissue was also stained with DAPI (panel E) and Oct-4A (Cy2, green fluorescence) (panel F) in a nuclear localization. Panel G is the overlayer of DAPI and Oct-4A (merge of panel E + F).

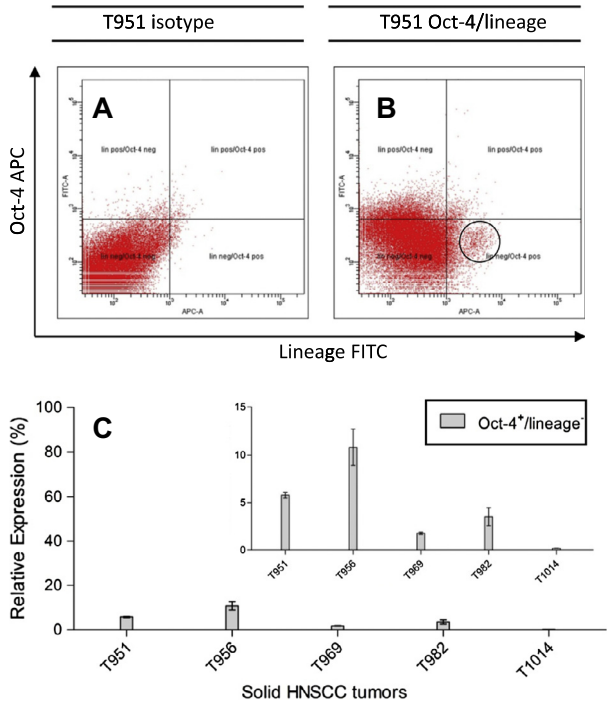


Figure 3. Identification of an Oct-4$^+$/lineage$^-$ subpopulation in solid HNSCC tumors (T951) using flow cytometry. As displayed in dot-plot A, an IgG-Isotype FITC and APC control was used as a negative control. Dot-plot B revealed a small Oct-4 positive and lineage negative population with an average expression profile of 5.8% (n = 8, SD 0.7). In graph C bars (mean, SD, n = 8) shows the percentage expression of the Oct-4$^+$/lineage$^-$ subpopulation in five solid HNSCC tumors. The mean expression rate varies between 0.2% (T1014) and 10.8% (T956) and clarifies a heterogeneous expression of this embryonic stem cell marker at a low expression level.

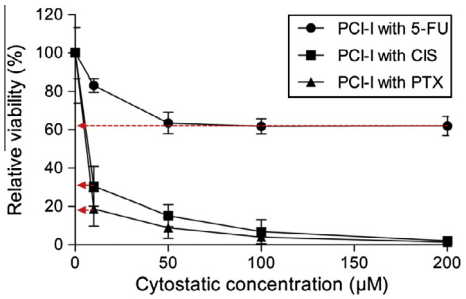


Figure 4. Dose-dependent cytotoxicity of the cell line PCI-I under treatment with 5-fluorouracil (5-FU), cisplatin (CIS), and paclitaxel (PTX). The graph with points and connecting (mean, SD, n = 3) lines show three different cytotoxicity levels of the various cytostatic drugs. A treatment with 200 µM 5-fluorouracil leads to a reduction of 38% (SD 5.0) of all vital cells after 48 h, 10 µM cisplatin to 30.4% (SD 10.4), and 10 µM paclitaxel to 18.7 (SD 8.9).

demonstrate that aberrant activation of the Wnt pathways lead to cancer development and progression [40–43]. FZD7 has previously been found with an overexpression in solid HNSCC tissue and dislocated an aberrant activation of the Wnt signaling pathway in HNSCC [44]. Moreover, it has been shown that FZD7 is a novel surface antigen expressed on human embryonic stem cells and interacts with the stem cell marker Oct-4 [45]. Recently, novel investigations on colon cancer cells revealed that transfection with FZD7-siRNA leads to significantly decreased cell viability and invasion activity [46]. These findings suggest that FZD7 plays a pivotal role in carcinogenesis and represents a potential therapeutic target. In this work, we observed an inhomogeneous expression of FZD7 in both investigated tissue types, namely permanent cell lines and solid tumors, with average expression rates of 37.2% and 8.1%, respectively [44].

Whereas many analysed proteins have been identified and extensively studied before, we demonstrated at first a significant expression level of some stem cell proteins in this study, for example GATA-4, Goosecoid, HNF-3ß/FoxA2, Otx2, PDX-1/IPF1, as well as Sox17. Having found expressions of Oct-4, Nanog and Sox2 it strengthens the hypothesis of being the interactions key factor in maintaining the pluripotency which is often transcribed concomitantly [29,35,47]. Recently, Kim et al. have shown that Oct-4 is sufficient to generate pluripotent stem cells and emphasized the pivotal role of this transcription factor in stem cells [28]. Moreover, it has been reported that the Oct-4 gene encodes two different spliced variants, called Oct-4A and Oct-4B. Whereas Oct-4A controls the transcription of various stem cell genes in the nucleus, Oct-4B is localized in the cytoplasm and is unable to sustain self-renewal and pluripotency [32–35].

In terms of Oct-4, Oct-4B positive PBMCs in the cell lines were excluded using the lineage marker mix. As demonstrated in Fig. 5, incubating all three drugs at different concentrations led to significant increase of a subpopulation of Oct-4 positive cells. Moreover, there seems to be a direct relationship between the toxicity of the cytostatic agents and the quantity of Oct-4 positive cells. Indeed, it has been shown that Oct-4 is essential for survival of embryonic stem cells in response to stress [48]. Hu et al. proved that Oct-4

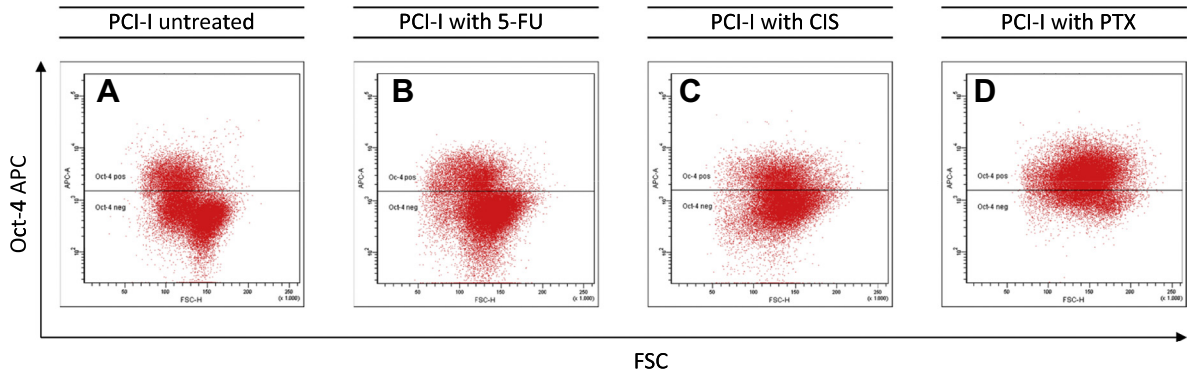


Figure 5A. Oct-4 expression rate of the cell line PCI-I under treatment with 200 μm 5-fluorouracil (5-FU), 10 μM cisplatin (CIS), and 10 μM paclitaxel (PTX). Representative flow cytometric dot-plots showing expression of Oct-4 after 48 h incubation with DMEM (A), 200 μM 5-fluorouracil (B), 10 μM cisplatin (C), and 10 μM paclitaxel (D).

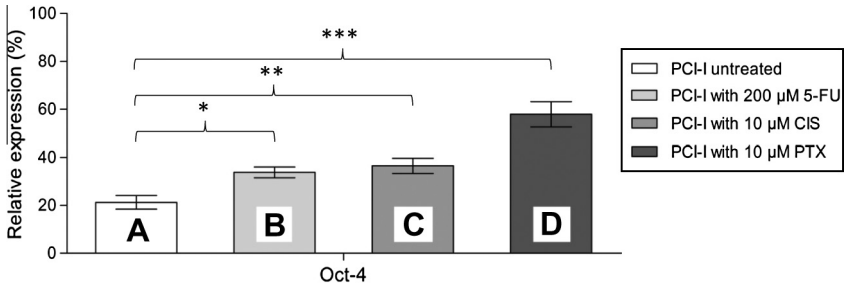


Figure 5B. Oct-4 expression profile of the cell line PCI-I under treatment with 200 μm 5-fluorouracil (5-FU), 10 μM cisplatin (CIS), and 10 μM paclitaxel (PTX). Bars illustrate the expression rate of PCI-I after an incubation period of 48 h in DMEM (A), 200 μM 5-fluorouracil (B), 10 μM cisplatin (C), and 10 μM paclitaxel (D). $^{*}p = 0.0057$; $^{**}p = 0.0044$; $^{***}p = 0.0011$.

maintain the survival of potential cancer stem cells partly through Oct-4/Tcl1/Akt1by inhibiting apoptosis [49]. In conclusion, these investigations suggest Oct-4 as a promising therapeutic target against chemoresistant cells of HNSCC.

Conflict of interest statement

The authors have no actual or potential conflict of interest.

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