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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.

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