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Research Article

Identification, expansion and characterization of cancer cells with stem cell properties from head and neck squamous cell carcinomas

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ABSTRACT

Head and neck squamous cell carcinoma (HNSCC) is a major public health concern. Recent data indicate the presence of cancer stem cells (CSC) in many solid tumors, including HNSCC. Here, we assessed the stem cell (SC) characteristics, including cell surface markers, radioresistance, chromosomal instability, and *in vivo* tumorigenic capacity of CSC isolated from HNSCC patient specimens. We show that spheroid enrichment of CSC from early and short-term HNSCC cell cultures was associated with increased expression of CD44, CD133, SOX2 and BMI1 compared with normal oral epithelial cells. On immunophenotyping, five of 12 SC/CSC markers were homogenously expressed in all tumor cultures, while one of 12 was negative, four of 12 showed variable expression, and two of the 12 were expressed heterogeneously. We showed that irradiated CSCs survived and retained their self-renewal capacity across different ionizing radiation (IR) regimens. Fluorescence *in situ* hybridization (FISH) analyses of parental and clonally-derived tumor cells revealed different chromosome copy numbers from cell to cell, suggesting the presence of chromosomal instability in HNSCC CSC. Further, our *in vitro* and *in vivo* mouse engraftment studies suggest that CD44+/CD66− is a promising, consistent biomarker combination for HNSCC CSC. Overall, our findings add further evidence to the proposed role of HNSCC CSCs in therapeutic resistance.

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is an epithelial tumor caused by multiple genetic alterations. Risk factors for HNSCC include tobacco smoking, alcohol consumption, and/or human papillomavirus infection [1,2]. The number of new cases of HNSCC in the USA in 2016 is estimated to be more than 61,000 and HNSCC is estimated to result in 3000 deaths [3]. Prognosis remains poor, with approximately a 50% 5-year overall survival (OS) rate [4,5]. This low survival rate is due to a number of factors, including local recurrence, distant metastasis, and therapeutic resistance [2,6].

The cancer stem cell theory suggests that a subpopulation of cells in the tumor possesses stem cell properties with the potential to self-renew and generate the entire heterogeneous tumor bulk in a unique 'hierarchic' pattern [7,8]. The 'hierarchic' model of CSC suggests that CSCs are a distinct subpopulation of cells that drive the tumor. These cells reside on the top of the tumor cell hierarchy and divide symmetrically and asymmetrically in a similar pattern to normal stem cells (SC) [8,9]. However, another model, the stochastic model, has been proposed to explain how cancer drives tumor development. This model proposes that a tumor forms as a result of random oncogenic mutations [8,10]. We and others believe that the hierarchic and stochastic models of cancer are not mutually exclusive, adding another level of complexity to our understanding of the biology of cancer [11]. CSCs were shown first in hematopoietic cancers [12] and later in solid tumors, such as glioma [13], lung cancer [14], breast cancer [15], colon cancer [11,16] and HNSCC [6,17,18]. Various terms have been used to describe cancer stem cells, including cancer initiating cells,

Abbreviations: CFE, colony forming efficiency; CIN, chromosomal instability; CSC, cancer stem cell; ELDA, extreme limiting dilution assessment; FISH, fluorescence *in situ* hybridization; HNSCC, head and neck squamous cell carcinoma; IR, ionizing radiation; NSCLC, non-small cell lung carcinoma; OS, overall survival; SC, stem cell; SP, side population

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functional tumor stem cells, tumor initiating cells, and cancer stem-like cells.

The literature reveals that several different markers have been used to identify CSC in HNSCC. No single cell surface marker or approach has been shown to be optimal for identifying CSC in HNSCC. However, a number of markers have been applied by multiple research groups, including CD133 [19–23], CD44 [6,17,24–27] and ALDH1 enzymatic activity [18,20,28,29]. CD44 has been the most common marker used to identify HNSCC CSC [17,27]. CD133 has been shown by some researchers to be a consistent marker [19,20]. ALDH1 is a promising marker verified by a number of groups [18,20,28–30]. Overall, the variability of cell surface markers used in CSC research has led many researchers to focus on the identification of other antigens and antibodies that might be more reliable and consistent biomarkers of CSC.

Radiotherapy is currently an important treatment modality for HNSCC, used either alone or in combination with chemotherapy for primary and recurrent cancers [31]. Radiotherapy will remain a crucial component of therapy for HNSCC because these tumors tend to locally infiltrate important surrounding structures. Surgery is often difficult due to the anatomical location. We and others have shown that the markers, CD133, CD44, and SOX2 are biomarkers of radioresistance in tumors [32–34]. Breast CSC have been shown to survive various radiation therapy protocols, and subsequent to some protocols, show higher self-renewal [35]. The side population (SP) cells from breast cancer cell lines were found to be more resistant to radiation than the non-SP cells [36]. SP isolation depends on the characteristic property of CSC of Hoechst dye efflux by multidrug resistance transporters [36,37]. Bao et al. showed that CD133-positive glioma CSCs repaired radiation-induced DNA damage more efficiently than CD133-negative cells, and might be the source of tumor recurrence after radiation therapy [13]. CD133-positive non-small cell lung carcinoma (NSCLC) CSCs are also radioresistant, due to DNA repair defects manifested as increased basal γH2AX staining and decreased phosphorylation of various protein kinases, including ATM and KAP1/TRIM28 [38]. In prostate cancer, CD44-positive CSCs are radioresistant compared to parental cancer cells [39]. CD44 knockdown enhances radiosensitivity [34]. Furthermore, Ghisolfi et al. showed that irradiation enriched CSCs in hepatocellular carcinoma cell lines [40]. A few groups have begun to examine radioresistance in HNSCC CSC, showing radioresistance using a number of different markers. Chen et al. found evidence that an ALDH1-positive subpopulation exhibited increased radioresistance compared to ALDH1-negative cells [30]. On the other hand, Wilson et al. showed no significant differences in radiosensitivity between CSC isolated from HNSCC cell lines using several different CSC isolation techniques [17]. We have recently demonstrated that CSCs (SOX2+) showed fewer and different types of chromosomal segregation defects after ionizing radiation treatment than non-CSCs (SOX2-), suggesting that CSCs may undergo altered behavior, including therapeutic resistance as a result of chromosomal instability [32]. Overall, radioresistance in HNSCC CSCs is a promising field of research that we chose to explore.

To characterize CSC from primary tumors, we enriched CSC sequentially on stromal feeder layers and later on Matrigel™-coated culture dishes or ultra-low attachment plates. The stromal feeder layer provides cellular interaction for CSC and promotes epithelial cell growth. This system has been used previously by our group to isolate and expand CSC from human metastatic colon cancer for at least five passages [11]. To understand and characterize CSC populations within HNSCC, we examined cells derived from five HNSCC. We examined the expression of 21 different cell surface markers, assessed self-renewal, chromosomal instability (CIN), radioresistance of CSC *in vitro,* and confirmed the tumorigenicity of HNSCC CSC *in vivo*.

2. Materials and methods

2.1. Tumor cell acquisition, preparation and establishment of short-term cell strains

This study was approved by the University of Pittsburgh Institutional Review Board and the University of Pittsburgh Animal Care and Use Committee. Following informed consent, HNSCC were collected after surgical removal from five different patients. Tumor specimens were minced into multiple small fragments with scalpels and digested using a two-step collagenase dissociation method. Tissues were further dissociated with a 20 min trypsin incubation, followed by tumor cell collection. Four specimens were analyzed by flow cytometry. Later, tumor cells were cultured on stromal monolayers of previously irradiated (80 Gy) rodent epithelial feeder layer cells in SC medium composed of serum-free Dulbecco's minimum essential (DMEM/F12) medium supplemented with N2, B27, L-glutamine, Gentamicin (20 mg/ml) (all from Thermo Fisher Scientific, Pittsburgh, PA), human recombinant epidermal growth factor (EGF; 20 ng/ml, Sigma-Aldrich), and human basic fibroblast growth factor (bFGF; 20 ng/ml, Sigma-Aldrich). TERT-transfected human oral keratinocytes (OKF6/TERT-1), were kindly provided by Dr. James Rheinwald, Brigham and Women's Hospital, Harvard Institutes of Medicine [41] and were cultured in Keratinocyte-SFM supplemented with 25 μg/ml bovine pituitary extract, 0.2 ng/ml EGF, 0.3 mM $CaCl_2$, and penicillin-streptomycin (Thermo Fisher Scientific).

2.2. Spheroid self-renewal assessment

Primary/secondary spheroid enrichment analysis was done to compare the frequencies of primary spheroids formed from short-term cell tumor cultures compared to secondary spheroids formed from dissociated primary spheroids in order to assess the self-renewal capacity of the enriched CSC. Equal numbers of cells derived from short-term HNSCC CSC cultures (primary) and cells derived from primary enriched spheroids (secondary) were suspended in SC medium on ultra-low attachment six-well plates (Corning, New York). To form secondary spheroids, primary non-adherent spheroids were selected using a 37 μm Reversible Strainer (STEMCELL Technologies Inc., Vancouver, British Columbia, Canada), centrifuged at 800 × *g* for 4 min, and then mechanically dissociated and re-plated. Large spheroids (more than 100 cells) from primary and secondary cultures were counted and photographed using a phase contrast photomicroscope (Leica Microsystems, Wetzlar, Germany).

2.3. Extreme limiting dilution analyses of CSC

To determine the frequency of CSCs capable of colony formation *in vitro*, we used extreme limiting dilution analysis as described previously [11]. Tumor cells were stained with HLA-ABC and distributed by flow cytometry to 96-well plates starting at one cell per well to 1000 cells per well in 100 μl of medium and cultured on irradiated stromal cells. After three to four weeks of culture, micro-cultures were scored for the presence of colonies by direct microscopic examination. Determination of the frequency of colony-forming CSCs was obtained by the minimum Chi square method derived from the Poisson distribution relationship between the colony-positive CSC wells and the logarithm of the percentage of the colony-negative CSC wells. The L-Calc statistical software program from STEMCELL Technologies Inc. (http://www.stemcell.com/technical/28425_l-Calc.pdf) was used to calculate the frequency of colony forming tumor cells.