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A perspective on challenges and opportunities in characterizing oral cancer stem cells

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1. ABSTRACT

Cancer stem cells (CSCs) or tumor-initiating cells (TICs) represent a minority population of cells in a tumor that can self-renew and re-create the heterogeneity of the entire tumor. Cell lines, patient-derived tumor cells, and patient-derived xenografts have all been used to isolate presumptive CSC populations from different tumor types. Because of their purported roles in tumor recurrence and prognosis, numerous efforts have centered around reliably identifying CSCs using cell surface markers, and in using genomics tools to identify molecular features unique to these cells. In this brief review, we will discuss different markers, CD44, ALDH1, CD271 and others that have used for identifying and isolating CSCs from primary head & neck and oral squamous cell carcinomas. In particular, we focus on the challenges associated with these experiments as this will be useful to researchers attempting similar isolations. We also discuss some important molecular features gleaned from studying these CSCs such as the expression of stem cell-related markers, loss of cell adhesion and terminal differentiation markers, and the presence of both epithelial and epithelial-to-mesenchymal transition (EMT) features.

2. INTRODUCTION

The concept of cancer stem cells often abbreviated as CSCs arose from studies on leukemic cells. John Dick and colleagues found that not all leukemic cells are equal in terms of their contribution to tumor formation. Leukemic cells expressing the cell surface protein CD34, widely considered as a marker of hematopoietic stem cells and progenitors, formed tumors frequently when injected into nude mice, while CD34-negative cells formed fewer or no tumors (1). This gave rise to the concept of a sub-

population of cells with stem cell-like properties that are potent tumor-initiating cells as indicated by their ability to self-renew and reform the original tumor in immunocompromised mice (2).

Very soon, the concept of CSCs found traction in solid tumors (3). Multiple reports emerged suggesting that most if not all tumors may have this sub-population of cells with stem cell-like properties (4–6). Most often, such sub-populations were isolated using Fluorescence Activated Cell Sorting (FACS), after labelling cells with a cocktail of antibodies against cell surface 'markers' that would enable selection of specific sub-populations of interest (7). However, the identification and isolation of such cells continue to be fraught with technical difficulties (8), some of which are:

1. In the haematopoietic system, the identities of stem and progenitor cells, as established by marker-based isolation and downstream functional characterization, is well known. However, in most solid tissue types, these identities are not well established.

2. Isolation of cells from solid tissues typically involves mechanical and/or enzymatic disruption of cell-cell and cell-matrix connections which can affect the display of cell surface proteins including any markers that may be used for cell isolation.

3. The purity of the isolated cell sub-populations depends on the specificity of the cell surface marker and the antibodies used for the process of isolation. Because tumors are typically an admixture of multiple cell types, in most cases, it is challenging to ascertain and ensure without ambiguity that no other cell types in the tissue being studied express the putative CSC marker.

In this review, we will specifically address these challenges as applied to oral cancers (9, 10). Once a putative CSC marker- expressing population of cells is isolated, the gold standard of testing the "stemness" and tumor-initiating potential of this cell population continues to be transplantation experiments in immunocompromised mice (11). From SCID (Severe combined immune deficient mice) to NOD-SCID (Non-obese diabetic SCID mice), and now NOD-SCID-IL2Rgamma null (or NSG) mice, the mouse models that are used in these experiments have progressed and added to the robustness of this assay. Other surrogate experiments such as testing sphere-forming ability in ultra-low attachment plates, growth and differentiation into multiple lineages in organoid cultures etc. have also been used to score stemness (12, 13). While these continue to be useful, the gold standard baton of testing has remained with mouse transplantation experiments.

3. ORAL CANCER STEM CELLS- IDENTIFICATION AND ISOLATION

Oral cancers or oral squamous cell carcinomas (OSCC) are a prevalent subtype of the larger, anatomically more diverse group of head and neck squamous cell carcinomas (HNSC). Oral cancers include cancers of the mouth, buccal cavity, lips, oral cavity, and tongue (14). Oral cancers are a part of the large class of head and neck cancers. Oral cancers account for a significant number of mortalities in the Indian subcontinent (15). Largely attributed to tobacco chewing, the affected region of the oral cavity in this subtype is the Gingivo-buccal sulcus or GBS. Accounting for ~60% of all reported oral cancer cases, this prevalent subtype is often referred as the Indian variety of oral cancer (16, 17). The primary mode of treatment is surgery with or without radiation, and in some instances, this is combined with chemotherapy. Loco-regional recurrences, however, are frequent and maybe as high as 60% according to some reports (18, 19).

The idea that a few cells with stem cell properties, which may have escaped surgical excision and adjuvant therapy, could self-renew and re-initiate the tumor resulting in recurrence, has found some support in this field (9, 10). These spurred experiments aimed at identifying cell surface markers which could mark such tumor-initiating cells

and help in the isolation and characterization of head and neck CSCs.

3.1. Isolation of CSCs from oral cancers using the cell surface marker CD44

The first study reporting the isolation of putative head and neck CSCs used CD44 as the marker for isolating this sub-population (5). CD44 is a receptor expressed on the cell surface that binds hyaluronan (HA). CD44 binding to HA is believed to have a role in CSC homing and adhesion, although there is no evidence that CD44 has a direct role in self-renewal or pluripotency (20). The primary reason for testing CD44 in HNSC was that it had been previously used successfully to mark CSCs in another epithelial tissue, *e.g.,* breast tumors (3, 4). There was also evidence from immunohistochemistry of head and neck tumor sections that CD44 stained only the basal or poorly differentiated tumor cells, and its staining pattern was mutually exclusive to that of involucrin, a well-known terminal differentiation marker in these epithelia (5, 21). Although this has been corroborated by more recent studies, ambiguities have persisted (22). Some studies have found CD44 expression in most layers of the mucosal epithelium, and this has cast doubts on its usage as a CSC-specific marker in head and neck epithelia (23).

The use of CD44 to isolate head and neck CSCs presents yet another challenge. CD44 is widely expressed on the surface of hematopoietic cells, and head and neck cancers are typically well-infiltrated by immune cells. In fact, studies that have reported FACS profiles of cells from primary patient HNSCs have estimated the percentage of mature lineage marker-positive or Lin^+ cells (includes hematopoietic and stromal cell populations) to be as high as 50-60% in many tumors (21, 24). There are two consequences to this observation-

1. Since many Lin^+ cells will also be $CD44^+$, these will have to be effectively excluded if CD44 has to be used as a selection marker for isolating the minority CSC sub-population.

2. This essentially also meant that only half the cells (or lesser) recovered from tumors post dissociation represented epithelial tumor cells.Thus, if the goal is to isolate the minority $CD44^+$ CSC population from HNSCs or OSCCs surgically excised from patients, it would be wise to start with tumours that show a good proportion, we suggest 40% or above, of epithelial tumour cells (Figure 1).

In general, across different epithelial cancers, two strategies have been used to remove the non-epithelial, Lin^+ cells. The first one employed a cocktail of antibodies (namely, CD2, CD3, CD10, CD18, CD31, CD64, CD140b) to mark and remove tumour-associated leukocytes, endothelial cells, and some other stromal cells. The second strategy employed CD45 and CD31 to mark and remove the tumor-associated leukocytes and endothelial cells, respectively. Both these strategies have been successfully used in a slew of studies for removing several “contaminating” non-epithelial cell types. Table 1 provides a representative listing of these studies. Because of the large numbers of Lin^+ cells in HNSCs, any marker-based isolation (not just CD44) of the CSC subpopulation in HNSCs would first have to mark and resolve the Lin^- population. Studies that have reported and used markers besides CD44 to isolate the CSC population in HNSCs are discussed in the next section.

3.2. Isolation of CSCs from oral cancers using other markers

Markers such as ALDH1, CD271, and SSEA-4 have also been used to isolate presumptive CSCs from head and neck cancers. Assays based on aldehyde dehydrogenase isoform 1 (ALDH1) activity have been described to isolate both murine and human stem cells from various organs (25). This enzyme is highly expressed in many stem and progenitor cells as well as in some tumor cells. For instance, normal and cancer human mammary epithelial cells with increased ALDH1 activity have stem/progenitor properties (26). In HNSCs, $ALDH1^{high}Lin^-$ cells comprised a sub-population of mostly $CD44^+Lin^-$ cells that were highly tumorigenic suggesting that

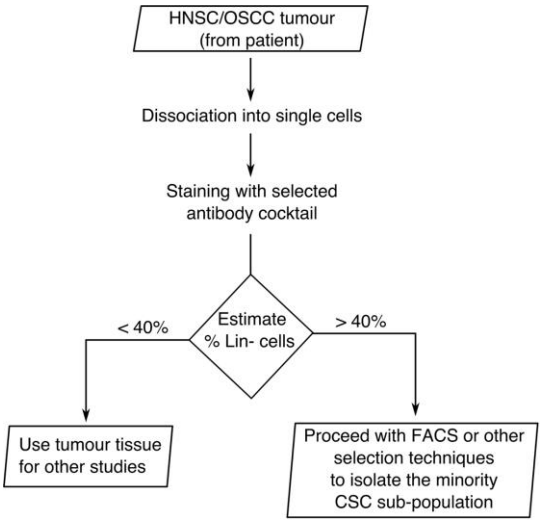

Figure 1. A flowchart indicating the sequence of processes used for isolating the minority cancer stem cell population from patient-derived primary HNSC/OSCC tumours. Fresh, surgically excised HNSC or OSCC tumour tissues have to be dissociated into single cells followed by staining with a selected cocktail of antibodies. The antibodies are chosen to exclude the lineage marker-positive, Lin^+ cells, and to specifically stain the required sub-population of cells, in this case, the CSCs. Since the CSC population represents a very small minority of tumour cells, we recommend that the it is best to start with as high numbers of Lin^- cells as possible. Based on our experiments, we recommend isolating the CSC sub-population from HNSC/OSCC tumours in which Lin^- cells represent at least 40% or more of the derived cells.

ALDH1 activity could be a highly selective marker for CSCs in HNSC (27, 28).

Could the choice of markers be guided by stem cell markers that may have been characterized in normal head and neck tissues? In normal human oral epithelium, a subpopulation of cells within the basal layer of the epithelium with stem cell-like properties has been shown to express a cell surface molecule, CD271. CD271, also known as the low affinity nerve growth factor (NGF) receptor or p75NTR, is a neurotrophin receptor with known roles in cell migration, differentiation, and survival in the nervous system. Although its role in oral epithelia is still unclear, CD271 appears to specifically identify the tumor-initiating subpopulation within the $CD44^+$ compartment of HNSCs. A few hundred $CD44^+$ $CD271^+$ Lin^- cells formed tumors more effectively when injected into nude mice than either $CD44^+$ $CD271^-$ Lin^- or $CD44^-$ $CD271^-$ Lin^- cells (29). Similar studies were carried using SSEA-4 or stage-specific embryonic antigen-4 which is a marker for pluripotent stem cells and embryonic stem cells. Cell lines double-positive for CD44 and SSEA-4 exhibited the highest tumorigenicity compared with the remaining sub-populations or parental cells (30).

Thus, all the putative CSC markers discussed so far in this section; CD271, ALDH1, and SSEA-4 appear to be expressed in a sub-population of $CD44^+Lin^-$ HNSC cells (Figure 2). However, as far as we are aware, it remains to be determined if there is an overlap between CD271 and SSEA-4 expression, and ALDH1 activity or if they represent different sub-populations of cells.

4. CORRELATION BETWEEN ORAL CSC NUMBERS AND CLINICAL PROGNOSIS

The CSC hypothesis gained significant traction because it provided a plausible explanation for tumor recurrence that was observed across all cancer types. This in turn led to the thought that higher CSC numbers in a tumor could perhaps imply a greater chance of recurrence. This observation, if true, would have significant clinical implications in terms of duration and kind of treatment regimen, and prognosis. Thus, studies in different cancer types including HNSCs have attempted to correlate CSC numbers with prognosis. Several studies have performed immunohistochemical analysis of CD44 staining in HNSC tissue sections and found that stronger CD44 staining correlated with poor overall survival (31). However, we are not aware of many studies where actual numbers of CSCs, based on FACS or related techniques, was correlated to disease outcome. In one such study of 31 patients with HNSC, higher numbers of $CD44^+$ Lin^- cells and the success of xenograft implantation in nude mice, were significantly correlated with advanced T classification and recurrence (24). This suggested that in HNSCs, the frequency of the presumptive CSC sub-population i.e., $CD44^+$ Lin^- cells, was associated with a worse prognosis.

5. MOLECULAR FEATURES OF ORAL CSCs

While it is clear that *not all* $CD44^+Lin^-$ cells are tumor-initiating, several reports, as discussed in the previous sections, seem to agree that the

Table 1. A representative listing of studies that have described the use of different antibodies for marking and excluding the lineage marker-positive, Lin^+ cells

S. No.	Species	Tumour tissue	Antibody cocktail used to mark the non-epithelial Lin^+ cells	% Lin^+ (if reported)	Method of separation used	Reference
1	Human	HNSC	CD2, CD3, CD10, CD18, CD31, CD64, and CD140b	no	FACS	(5)
2	Human	Breast	CD2, CD3 , CD10, CD16, CD18, CD31, CD64, and CD140b	no	FACS	(4)
3	mouse	Breast	CD45, CD31, Ter 119, and CD140a	no	FACS	(42)
4	Mouse	Breast	CD5, CD45R/B220, CD11b, GR 1, 7/4, and Ter^-119	no	Magnetic selection (MACS)	(43)
5	Mouse	Breast	CD45, Ter119, CD31, and CD140a	no	Stem Cell technologies kit	(44)
6	Human	Melanoma	CD45, CD31, and glycophorin A	50±33%	FACS	(45)
7	Mouse	Retina	B 220, CD3, CD4, CD8, CD11b, GR-1, and Ter119	no	Magnetic selection (MACS)	(46)
8	Human	Colon	CD3, CD10, CD16, CD18, CD45, and CD64	no	FACS	(6)
9	Human	Breast	CD45, CD15, CD14, CD19 (leucocyte cocktail), CD31, CD146, CD105 (endothelial cell cocktail), Cadherin5, and CD34	no	Dynal beads	(47)
10	Human	OSCC-GB	CD45, CD31	50 - 70%	FACS	(21)
11.	Human	HNSC	CD45, CD31, TE-7 (fibroblast)	no	FACS	(29)

$CD44^+Lin^-$ subpopulation is significantly enriched for tumor-initiating CSCs ((28), see also Figure 2). Thus, not surprisingly, several studies have used $CD44^+Lin^-$ cells to discern the molecular features of the oral CSC compartment. Some significant features that have been gleaned from these studies are listed and discussed in the sub-sections below.

5.1. Expression of stem cell- and pluripotency- related genes, and inducers of cellular proliferation

Prince *et al.* described expression of high levels of the Polycomb Repressor Complex1 component, *BMI1* in $CD44^+Lin^-$ cells of primary HNSC tumors (5). PRC1 is a well-known chromatin remodeler that acts as an epigenetic repressor of multiple genes involved in embryonic development and self-renewal of somatic stem cells. In HNSC, there is evidence that BMI1 mediates its tumor-promoting effects through upregulation of the mitotic kinase Aurora A (32). Similarly, higher expression of *OCT4A*, a transcription factor that is also expressed in undifferentiated, pluripotent cells and sustains stem cell properties, was associated with the enrichment of cancer stem-like cells and increased resistance upon exposure to chemotherapeutic agents (33). Of note, the chemotherapeutic drug cisplatin increased the fraction of $BMI1^+$ and $OCT4A^+$ populations in HNSC cell lines *in vitro*.

Some upstream kinases that signal to accelerate cell proliferation, including in stem cells, have been shown to have a role in regulating the tumorigenicity of the CSC compartment in HNSC. For instance, the c-Met receptor upon binding to Hepatocyte growth factor (HGF), which is secreted by several mesenchymal cells including fibroblasts, results in the activation of multiple signaling pathways related to proliferation, invasion, and resistance to cell death. Knock-down of c-Met in $ALDH1^+$ Lin^- HNSC cells inhibited tumor formation in a xenograft model and increased mice survival (34). Similarly, glycogen synthase kinase 3ß (GSK3ß), a kinase known to regulate cell cycle progression, was required to maintain HNSC CSCs in a self-renewing state, and consequently, inactivation of GSK3ß promoted their differentiation (35).

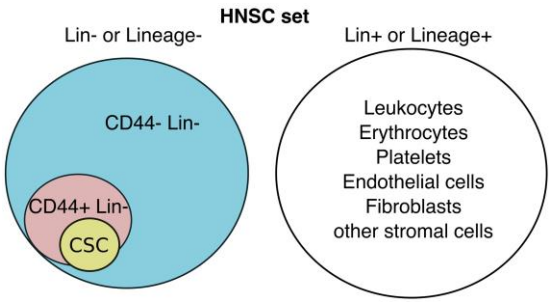

Figure 2. The different Lin$^-$ and Lin$^+$ cell types constituting an HNSC tumour. Most reports agree that the CD44$^+$ Lin$^-$ subpopulation is significantly enriched for tumor-initiating CSCs as depicted in the Venn diagram. CD271, ALDH1, and SSEA-4 are expressed by subsets of CD44$^+$ Lin$^-$ cells and could represent the putative CSC compartment. A part of this venn diagram is adapted from reference 28.

5.2. Loss of cell adhesion and terminal differentiation markers

In general, the ability of CSCs to form non-adherent "spheres" is regarded as a key characteristic of their stemness. Using cells sorted from three established HNSC cell lines, it was observed that the number of 'orosphere' (sphere cultures of oral cancer cells in non-adherent plates, akin to 'mammospheres') colonies generated from ALDH1$^+$ CD44$^+$ cells was greater than from ALDH1$^-$ CD44$^-$ cells (36). In addition to this, some specific cell-cell adhesion molecules have also been implicated. A transcriptomics analysis of CD44$^+$ Lin$^-$ and CD44$^-$ Lin$^-$ cells derived from primary OSCCs identified significantly lowered expression of the cell-cell adhesion molecule, protocadherin 18 (PCDH18) in the CD44$^+$ Lin$^-$ subpopulation (21). Interestingly, lower *PCDH18* expression correlated with poorer overall survival in The Cancer Genome Atlas (TCGA)-HNSC data highlighting it as a potential negative prognostic factor in this cancer.

A key protein that maintains the proliferative potential of cells in the basal layer of squamous epithelia is TP63, wherein it controls the expression of basal markers (e.g., keratins 5/14 (K5/14)) amongst other genes (37, 38). Immunohistochemical analysis of TP63 and CD44 indicated that these proteins are co-expressed in undifferentiated, basal layer-like cells in HNSC tissue sections (21, 39). Conversely, terminal differentiation markers such as involucrin and TGM1 (transglutaminase1) are not expressed by these CD44$^+$ cells. These observations support the hypothesis that the CD44$^+$ epithelial tumor cells in HNSCs represent poorly differentiated, TP63-expressing, basal-like cells.

5.3. Duality- Presence of epithelial and epithelial-to-mesenchymal transition (EMT) features

Biddle *et al.* showed that cell lines derived from human oral and cutaneous squamous cell carcinomas as well as cells freshly generated from OSCC tumors contained two CD44$^+$ CSC sub-populations. One, termed the non-EMT CSCs, was proliferative and retained epithelial characteristics, whereas the other, termed EMT CSCs, was migratory and had mesenchymal features (40). Interestingly, it appeared that the two sub-populations could switch between their phenotypic states through EMT and the reverse process of mesenchymal-to-epithelial transition (MET). More recently, Pascual *et al.* described a sub-population of CD44$^+$ cells in human oral carcinomas that did not overexpress mesenchymal genes (similar to the non-EMT CSCs described above) and were slow-cycling. These cells also expressed high levels of the fatty acid receptor CD36 and related lipid metabolism genes (41). In particular, these CD44$^+$ CD36$^+$ cells were potent metastasis-initiators suggesting perhaps that EMT might not be strictly required for the metastatic process in OSCCs.

6. CONCLUDING REMARKS

Notwithstanding the challenges and controversies that continue to exist in the isolation of CSCs from solid tumours, a remarkable amount of information has emerged from the characterization of CSCs. We anticipate this progress to continue given the emergence of new and sensitive high-throughput techniques that will aid in the characterization of CSCs. For instance, single cell transcriptomics can enable studies from very small numbers of cells and hence will be particularly relevant to CSCs. On the functional front, improvements to mouse models that will support the robust growth and tracking of

transplanted human patient tumour cells, should make it easier to test the tumour-initiating properties of putative CSCs besides offering a parallel to study and understand tumour progression. Such advances have the potential to provide a better and more nuanced understanding of CSC biology which can in turn spur the discovery of newer markers and with it opportunities for making a broad clinical impact.

7. ACKNOWLEDGMENTS

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Abbreviations: CSC: cancer stem cell; TIC: tumour-initiating cell; FACS: fluorescence activated cell sorting ; SCID: severe combined immune deficient; NOD-SCID: Non-obese diabetic SCID; NSG: NOD-SCID-IL2Rgamma null; OSCC: oral squamous cell carcinoma; HNSC: head and neck squamous cell carcinoma; GBS: Gingivo-buccal sulcus; HA: Hyaluronan; Lin: Lineage marker; HGF: Hepatocyte growth factor; EMT: epithelial-to-mesenchymal transition; MET: mesenchymal-to-epithelial transition

Key Words: Oral squamous cell carcinoma, Gingivo-buccal sulcus, Head and neck squamous cell carcinoma, Cancer stem cells, CD44, ALDH1, Review

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