Cancer stem cells in head and neck carcinomas: identification and possible therapeutic implications

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Abstract

The recurrence and/or lack of response of certain tumors to radio- and chemotherapy has been attributed to a small sub-population of cells termed cancer stem cells (CSCs). CSCs have been identified in many tumors (including solid and haematological tumors). CSCs are characterized by their capacity for self-renewal, their ability to introduce heterogeneity within a tumor mass and its metastases, genomic instability and their insensitivity to both radiation and chemotherapy. The latter highlights the clinical importance of studying this sub-population since their resistance to traditional treatments may lead to metastatic disease and/or tumor relapse. Head and neck squamous cell carcinomas (HNSCCs) are the sixth most common malignancy worldwide with the highest incidence occurring in East Asia and eastern and southern Africa. Several cellular sub-populations believed to have CSC properties have been isolated from HNSCCs but, at present, identification and characterization of CSCs remains an experimental challenge with no established or standardized protocols in place to confirm their identity. In this review we discuss current approaches to the study of CSCs with a focus on HNSCCs, particularly in the context of what this might mean from a therapeutic perspective.

Key Words: Cancer stem cells, head and neck carcinomas

1 Introduction

Head and neck carcinomas (HNCs) comprise a group of malignant epithelial tumors originating at various anatomical sites with a variety of both known and uncertain etiologies. Anatomical areas affected include the oral cavity, oropharynx, pharynx, upper respiratory track and larynx. Of all malignancies that affect the head and neck, about 90% are histological variants of squamous cell carcinoma (SCC) (Jemal et al. 2011). Based on 2012 global cancer statistics, head and neck squamous cell carcinomas (HNSCCs) are classified as the sixth most common malignancy worldwide with the highest incidence occurring in East Asia and eastern and southern Africa (Torre et al. 2015).

The majority of HNSCCs are strongly associated with environmental and lifestyle risk factors such as obesity, poor nutrition, high tobacco usage and alcohol consumption (Bruni et al. 2017; Torre et al. 2015; Vigneswaran & Williams 2014). However, an increasing incidence of HNSCCs at specific sites suggests that other etiological factors may be involved. Human papillomavirus (HPV) infection is now a well-established cause of cervical cancer and there is growing evidence that HPV is an important causative factor in HNSCCs (Bruni et al. 2017; Torre et al. 2015). The pathogenic role of HPV in subsets of head and neck tumors has been recognized by the World Health Organization through re-classification of SCC of the oropharynx (base of tongue, tonsils and adenoids) into HPV positive and HPV negative SCC (El-Naggar et al. 2017).

Despite ongoing advances in treatment regimes, the prognosis for HNSCCs has improved only marginally over the past 30 years, with the 5-year survival rate ranging from 10% - 50% depending on a number of factors including the clinical stage of the tumor at the time of diagnosis (Karimnejad et al. 2016; Yan et al. 2013). Standard therapy for HNSCCs includes a combination of surgery, chemotherapy and radiotherapy, which is determined by the histological variety, grade and clinical staging of the tumor (Zhu et al. 2015). However, irrespective of the treatment regime, patients exhibit relatively high rates of local recurrence as well as regional cervical lymph node and distant tumor metastases, which contribute to significant morbidity and mortality (Karimnejad et al. 2016; Zhu et al. 2015). Recurrent tumor and metastatic deposits are frequently resistant to the original treatment modalities utilized (Karimnejad et al. 2016; Zhu et al. 2015).

The recurrence and lack of response to further radio- and chemotherapy in these tumors has been attributed to a small sub-population of tumor-initiating cells known as cancer stem cells (CSCs). This sub-population is believed to a) have unlimited capacity for self-renewal; b) introduce heterogeneity into tumors as a result of genomic instability; and c) show resistance to traditional anti-cancer treatments with the exception of surgery (Fábián et al. 2013; Gil et al. 2008; Qian et al. 2016).

Traditional approaches to treating HNSCCs are based on a stochastic model of cancer. According to this model, HNSCCs result from the accumulation of genetic

changes via successive mutations in different cells and gradual selection of malignant clones (Gil et al. 2008). Cells in the dominant clonal population(s) possess similar tumorigenic potential (Karimnejad et al. 2016) and current therapies mainly target these cells which form the bulk of the tumor.

The CSC model suggests a unidirectional hierarchical organization of cells starting with a progenitor cell endowed with stem cell-like properties (the CSC) (Valent et al. 2012; Visvader & Lindeman 2012; Allegra 2012). The CSC model accommodates the heterogeneity seen in human tumors and postulates that this heterogeneity is due to distinctive characteristics of different CSCs present in the primary tumor. CSCs share similar features to normal stem cells, including the ability to selectively self-renew, proliferate and differentiate into the cell mass that compromises the bulk of the tumor (Valent et al. 2012; Visvader & Lindeman 2012; Allegra 2012). More importantly, it is believed that their stem cell-like features provide CSCs with a measure of protection against traditional anti-cancer therapies compared to the rest of the cells in the tumor (Fábián et al. 2013), thereby providing one possible explanation for treatment resistance and/or tumor relapse. This highlights the clinical importance of identifying and defining these sub-populations of cells.

Much effort has been invested into characterizing and identifying CSCs in various tumor types and exploring the mechanisms of their involvement in the unique reaction of certain tumors to different treatment regimes. At present, characterization and identification of CSCs is largely limited to the experimental setting, in which CSCs demonstrate (retrospectively) the capability of generating and propagating a malignant cell population. In this review we discuss current approaches to the identification and study of CSCs in HNSCCs, particularly from a therapeutic perspective.

2   Identification of CSCs

Three main strategies are used to isolate putative CSCs from solid tumors: i) tumor sphere formation assays performed under non-attached culture conditions; ii) side population assays aimed at identifying cells with greater efflux potential; and iii) fluorescence activating cell sorting (FACS) where sub-populations are sorted based on known cell surface markers. The sub-populations identified and isolated are then

subjected to further testing that assesses their tumorigenic ability and self-renewal properties *in vitro* and/or *in vivo*.

2.1 Sphere formation assays for the identification of putative CSCs populations in HNSCC

For solid tumors, the most accessible method for studying self-renewal *in vitro* is tumorsphere formation under non-attached culture conditions (Valent et al. 2012; G. Zhang et al. 2012; Fábián et al. 2013). Primary spheres are generally assessed for their ability to form secondary spheres and their ability to form colonies once plated onto adherent plates (Harper et al. 2007). This method relies on the theory that isolated cancer cells that form tumorspheres are capable of proliferation, self-renewal and greater tumorigenic capacity (G. Zhang et al. 2012). Sphere culturing is thus used to isolate, enrich and expand putative CSC populations.

The tumorsphere formation assay has been used with both HNSCC cell lines and primary tumor tissue to identify and study various sub-populations (Chiou et al. 2008; Harper et al. 2007; Zscheppang et al. 2016; Yu et al. 2010). Tumorspheres have a higher proliferative and self-renewal capacity than their parent populations *in vitro* (Chiou et al. 2008; Harper et al. 2007). They also have distinctive phenotypes compared to the parent populations, showing high positivity for markers such as cluster of differentiation (CD) 44, CD133, aldehyde dehydrogenase 1 (ALDH1) and breast cancer resistant protein (BCRP/ABCG2) (Chiou et al. 2008; Harper et al. 2007; Yu et al. 2010; Chen et al. 2009). When compared to the parent cell population, these spheres have also been shown to have greater survival abilities against ionizing radiation *in vitro* (Chiou et al. 2008; Zscheppang et al. 2016) and greater tumorigenic capacity following xenotransplantation (Chiou et al. 2008; Zscheppang et al. 2016).

Advantages of this method include lower cost and quicker results but several shortcomings have been identified. The *in vitro* system is unable to replicate the three-dimensional structure and environment of a tumor *in vivo*. Factors important for *in vivo* growth and self-renewal of some or all CSCs may not be provided by the *in vitro* conditions. Additionally, the growth of the cells *in vitro* is usually limited to several

weeks or months, during which time cells may differentiate, die or transform (Valent et al. 2012).

2.2   Side population discrimination assays for the identification of CSC in HNSCC

Another strategy used to identify putative CSC populations is based on the ability of these cells to efflux a fluorescent dye that can bind to DNA. One possible mechanism whereby CSCs remain resistant to traditional anti-cancer chemotherapeutics lies in their cell surface expression of adenosine triphosphate (ATP)-binding cassette (ABC) transporter proteins. These proteins actively translocate or efflux cytotoxic chemicals out of cells (Dean 2009). In the assay, the cells are stained with a fluorescent dye such as Hoechst 33342, followed by incubation for a period of time. When analysed via flow cytometry, the sub-population of cells with efflux capability, believed to be CSCs, will have lower fluorescence than the more differentiated tumor and stromal cells. This sub-population is termed the side population (SP).

The SP assay was first described by Goodell *et al.* when it was used to isolate a sub-population highly enriched in hematopoietic stem cells (Goodell et al. 1996). Since then, the SP assay has been used in many human tissues in an attempt to identify somatic stem cells, as well as in a variety of cancers in an attempt to identify a sub-population of cells with CSC characteristics.

The SP assay has also been used to identify putative CSC populations in HNSCCs using primary tumor cell isolates as well as established cell lines. Zhang *et al.* tested for the presence of the SP in nine oral squamous cell carcinoma (OSCC) cell lines including Tca8113, NTRC and TSCC and 11 primary OSCC specimens. They found the SP to be present in all cell lines and primary tumors (Zhang et al. 2009). Various other studies have further shown that SP cells are resistant to chemotherapeutic drugs and have greater colony formation abilities than non-SP cells *in vitro,* as well as greater clonogenicity and tumorigenicity following xenotransplantation (Zhang et al. 2009; G. Zhang et al. 2012; Li et al. 2011).

There are however, limitations to the SP assay. It is a highly technical assay which requires modification for each cell type studied (Golebiewska et al. 2011). Results show poor reproducibility due to high inter-individual variability between samples and laboratory protocols (Golebiewska et al. 2011). Even though Zang *et al.* found the SP in all OSCC cell lines and primary tumors tested, the authors reported variable percentages of SP cells within the samples ranging from 0.1% - 10% (Zhang et al. 2009). It is also important to emphasize that the ABC transporters and the SP phenotype are not exclusive to stem cells (Golebiewska et al. 2011). Since all tumors are heterogeneous in nature, there is no guarantee that the SP only represents CSCs, and may in all likelihood contain both CSCs and normal stromal cells with efflux capabilities. The SP assay, similar to the sphere formation assay, may however be useful as an enrichment step in order to obtain a more homogeneous sub-population to investigate for stem-like properties.

2.3   Identification of CSC in HNSCCs using specific markers

The third method of CSC isolation consists of sorting cells based on either phenotypic and/or transcriptional profiles. However, a consensus on unequivocal and specific markers for the identification of CSCs in all cancers types has not yet been achieved (Visvader & Lindeman 2012; Allegra 2012; Yan et al. 2013)

An extensive list of possible CSC markers has been studied in HNSCCs. Below we discuss data from the more promising markers described in the literature. **Table 1** provides a summary of the markers discussed.

2.3.1   ABC Transporters

ABC transporters form one of the largest families of membrane transporter proteins (Falasca & Linton 2012; Huls et al. 2009; Lin et al. 2006). The human ABC transporter protein family consist of 49 members, which are divided into seven subfamilies (A to G) based on similarities in gene structure, order of domains and sequence homology (Huls et al. 2009; Falasca & Linton 2012; Dean et al. 2001; Leonard et al. 2003). This diverse family plays an important role in the maintenance of cellular homeostasis

through the transport of lipids and organic anions, iron metabolism and providing tissue protection via drug resistance and metabolism (Huls et al. 2009; Erdei et al. 2014; Leonard et al. 2003; Lin et al. 2006). Expression of the transporters is tightly regulated and their classification is based on their ability to recognize various toxic agents. They are able to functionally substitute for each other, thereby emphasizing the importance of their protective role (Huls et al. 2009; Erdei et al. 2014).

BCRP/ABCG2 is expressed by many tumors and has been linked to tumor initiation and promotion as well as tumor cell proliferation (Qian et al. 2016; Ding et al. 2010). It is also an important multidrug resistance protein shown to confer cross-resistance on several classes of cancer chemotherapeutic agents (Mao & Unadkat 2015). Because of this function, ABCG2 expression has been linked to the SP assay, and is believed to be one of the main mediators of the SP phenotype along with other members of this family such as P-glycoprotein (P-gp/ ABCB1).

BCRP/ ABCG2 expression has been demonstrated in putative HNSCC CSC sub-populations (Hang et al. 2012; Li et al. 2011; Tsai et al. 2011) along with other members of the family such as ABCA3 (Li et al. 2011), P-gp/ ABCB1 (Zhang et al. 2009; G. Zhang et al. 2012; Li et al. 2011), ABCC1 (Li et al. 2011) and ABCB5 (Grimm et al. 2012).

The expression of these proteins has also been associated with poor patient prognosis. High levels of expression of both ABCG2 and ABCB5 are associated with tumor progression and recurrence (Grimm et al. 2012; Hang et al. 2012).

2.3.2 CD44

CD44 is the most well established and frequently used CSC marker, and has been intensively studied in HNSCCs. It is a cell-surface trans-membrane protein that belongs to the adhesion molecule superfamily (Qian et al. 2016). This protein regulates cell-cell interactions, cell migration and cell adhesion (Mărgăritescu et al. 2012). It is implicated in tissue development, inflammation, wound healing and is also believed to influence tumorigenesis and progression, playing a role in invasion and metastasis (Qian et al. 2016). The molecule activates a variety of receptor tyrosine kinases in many cancer types, through which it drives increased proliferation and survival of tumor cells via activation of the mitogen-activated protein kinases (MAPK)

and phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT) pathways (Mărgăritescu et al. 2012; Misra et al. 2006).

Prince *et al.* were the first to report that HNSCCs contain two distinct populations of either CD44$^+$ or CD44$^-$ cells (Prince et al. 2007). Cells of the CD44$^+$ sub-population were able to initiate tumor growth in xenographs more efficiently than their CD44$^-$ counterparts (Prince et al. 2007). In addition, tumors developed from the transplantations displayed phenotypically diverse populations of cells, indicating that the CD44$^+$ cells were not only able to initiate tumor growth, but were also able to differentiate into phenotypically diverse daughter cells (Prince et al. 2007).

Since the publication of this landmark study, CD44 has been used routinely as a marker in experiments on HNSCC CSC sub-populations (Zhang et al. 2009; Noto et al. 2013; Chen et al. 2009; Harper et al. 2007). CD44 expression has also been correlated with a greater frequency of lymph node metastasis, higher primary tumor recurrence, resistance to radiotherapy and a poor clinical prognosis in HNSCC patients (Wang et al. 2009; Han et al. 2014)**.**

More recently however, studies have called into question the sensitivity and specificity of CD44 as a CSC surface marker in HNSCCs, as it is expressed on the majority of cells that constitute head and neck tissues, including normal oral epithelium as well as potentially malignant and malignant lesions (Mărgăritescu et al. 2012; Yan et al. 2013).

2.3.3 CD133

CD133 is a transmembrane pentaspan glycoprotein specifically localized on cellular protrusions (Mărgăritescu et al. 2012). The function of this protein is not fully known although it is used as a marker of CSCs in many solid tumors including brain, colon, pancreas, prostate, liver, lung and kidney (Qian et al. 2016; Mărgăritescu et al. 2012; Okamoto et al. 2013).

The literature regarding CD133 expression in HNSCCs lacks unanimity. Tsai *et al.* demonstrated CD133 positivity in a sub-population of OSCCs which exhibited resistance to the conventional chemotherapeutic drug cisplatin *in vitro* (Tsai et al. 2011). Zhang *et al.* reported on a CD133$^+$ sub-population within OSCC cell lines which

exhibits higher clonogenicity and tumorigenicity in xenograft models when compared to their CD133⁻ counterparts (Zhang et al. 2010).

In contrast to these findings, other investigators failed to demonstrate CD133 expression in freshly isolated OSCC from patient biopsy samples (Mărgăritescu et al. 2012; Okamoto et al. 2013). In a study done by Hang *et al.*, CD133 was found in 27% of patients with esophageal squamous cell carcinoma (ESCC) in which the proportion of CD133⁺ cells in ESCCs ranged from 3% - 46%, with higher percentages being expressed in the more differentiated tumors (Hang et al. 2012).

In addition, no consensus has been reached on the prognostic value of CD133. An association between CD133 expression and poor prognosis has been reported in HNSCC patients (Chiou et al. 2008; Fan et al. 2017). In contrast, Hang *et al.* found no significant association between CD133 expression and the 5-year survival rate of ESCC patients (Hang et al. 2012).

2.3.4 CD24

CD24, also known as heat stable antigen 24 (HSA), is a mucin adhesion molecule and is expressed by pre-B lymphocytes and neutrophils (Satpute et al. 2013). It has been identified as a ligand of P-selectin, an adhesion receptor found on activated platelets and endothelial cells (Satpute et al. 2013; Modur et al. 2016). This molecule is associated with tumor growth and metastasis (Han et al. 2014) and has been shown to be expressed in HNSCCs (Zimmerer et al. 2017; Koukourakis et al. 2012; Modur et al. 2016).

Studies on HNSCC cell lines and primary tumor cells have revealed the presence of a sub-population of cells that is CD24⁺. This sub-population has been shown to have a greater ability to self-renew and differentiate and demonstrates a greater resistance to chemotherapeutic drugs such as gemcitabine and cisplatin *in vitro* (Han et al. 2014; Modur et al. 2016). In addition Zimmer *et al.* showed that CD24⁺ sub-populations, isolated from freshly isolated OSCC samples, could initiate tumor growth and accelerate angiogenesis *in vivo* (Zimmerer et al. 2017). The study showed greater functional capillary density of newly formed microvessels by the CD24⁺ sub-population compared to the CD24⁻ sub-population after the cells were transplanted into non-obese diabetic/ severe combined immunodeficient (NOD/SCID) mice (Zimmerer et al.

2017). The authors believe that CD24^{+} cells have the potential to induce and modulate angiogenesis and to stimulate other tumor cells to switch to an angiogenic phenotype (Zimmerer et al. 2017).

From a prognostic point of view, the expression of CD24 along with CD44 is associated with lower overall survival rates compared to other phenotypes (Han et al. 2014; Modur et al. 2016).

2.3.5   ALDH1

ALDH enzymes are a family of cytosolic iso-enzymes that are involved in cell differentiation and detoxification, and confer drug resistance via oxidation of intracellular aldehydes (Z. Zhang et al. 2012; Clay et al. 2010). The ALDH family member most extensively investigated is ALDH1 and it is regarded as a marker for hematopoietic stem and progenitor cells (Qian et al. 2016; Z. Zhang et al. 2012). ALDH1 has also been identified as a potential functional marker for CSCs as it is expressed in various tumor types (Clay et al. 2010).

ALDH1 expression is significantly increased in sub-populations of ESCC cell lines (G. Zhang et al. 2012; Yata et al. 2015) and in primary isolated tumors from HNSCC patients (Yu et al. 2010; Chen et al. 2009).

ALDH1[+] sub-populations from HNSCC primary cell isolates possess greater tumorsphere- and colony-forming capacity *in vitro* than their ALDH1^{-} counterparts (Yu et al. 2010; Chen et al. 2009; Yata et al. 2015). In addition, ALDH1^{+} sub-populations are highly tumorigenic when compared to ALDH1^{-} sub-populations following xenotransplantation (Yu et al. 2010; Chen et al. 2009; Clay et al. 2010; Yata et al. 2015).

Although ALDH1 is present in HNSCC sub-populations, there is controversy as to whether ALDH1 expression may be viewed as a prognostic marker. Whereas some studies associate ALDH1 protein expression with lymph node metastasis and poor survival (Wang, Zhe et al. 2012), others report that ALDH1 expression in HNSCCs is correlated with a favourable clinical outcome (Koukourakis et al. 2012).

2.3.6 BMI1

Moloney murine leukemia virus insertion site 1 (BMI1) is a member of the Polycomb (PcG) family of transcriptional repressors that mediate gene silencing by mono-ubiquitination of histone H2A (Yu et al. 2010; Chen et al. 2017). This gene plays a role in the regulation of the cell cycle and the regeneration of hematopoietic and neuronal stem cells (Yu et al. 2010).

BMI1 has been implicated in tumorigenesis in several human cancer types including HNSCC (Prince et al. 2007; Yu et al. 2010). Yu *et al.* studied BMI1 expression in sub-populations of HNSCCs and found that BMI1$^+$ sub-populations have enhanced tumorigenic abilities both *in vitro* and *in vivo* (Yu et al. 2010). When BMI1 was knocked down, there was a decrease in tumorigenicity *in vitro* while inducing overexpression of BMI1 resulted in increased tumor growth, metastatic activity and radioresistance *in vivo* (Yu et al. 2010). In another study, sub-populations of OSCCs with increased BMI1 expression exhibited resistance to the chemotherapeutic drug cisplatin *in vitro* (Tsai et al. 2011).

Studies evaluating BMI1 as a prognostic marker for HNSCCs have shown inconsistent results. Yu *et al.* reported that BMI1 expression correlates with poor overall patient survival (Yu et al. 2010). However, a recent meta-analysis could not identify an association between the expression of BMI1 in HNSCC patients and overall survival (Fan et al. 2017).

2.3.7 Oct3/4, SOX2 and Nanog

The transcription factors octamer-binding transcription factor 3/4 (Oct3/4), sex determining Region Y-box2 (SOX2) and Nanog play essential roles in the maintenance of pluripotency and self-renewal (Z. Zhang et al. 2012; González-Moles et al. 2013).

Oct3/4 belongs to the Pit-Oct-Unc (POU) transcription factor family (Bourguignon et al. 2012). It is the product of the OTF3 gene and has been shown to maintain stemness in pluripotent stem cells (Eun et al. 2017). Oct3/4 functions as a master regulator during embryonic development by interacting with other embryonic regulators such as SOX2 and Nanog to oversee a vast regulatory network that maintains pluripotency

and inhibits differentiation (Bourguignon et al. 2012). It is considered to be one of the best indicators of stemness (González-Moles et al. 2013).

SOX2 belongs to the family of high-mobility group transcription factors that play important roles in stem cell function, development and organogenesis (Bourguignon et al. 2012; Ren et al. 2016)

Nanog is another important transcription factor involved in self-renewal and the maintenance of pluripotency (Bourguignon et al. 2012). Nanog signalling is regulated by interactions between pluripotent stem cell regulators (such as SOX2 and Oct3/4) which together control the expression of a set of target genes required for pluripotency (Bourguignon et al. 2012).

These factors are known to form a self-organized core of transcription factors that maintain pluripotency and self-renewal (Bourguignon et al. 2012). All three markers have been studied in HNSCCs, and all three have been found to be highly expressed in putative CSC populations (Tsai et al. 2011; Bourguignon et al. 2012; Kaseb et al. 2016). Tsai *et al.* demonstrated that sub-populations of OSCC cells isolated via tumorsphere formation expressed elevated levels of Oct4 and Nanog and displayed increased resistance to the chemotherapeutic drug cisplatin *in vitro* (Tsai et al. 2011). Similar results were obtained by another research group who found Oct3/4, SOX2 and Nanog gene expression to be significantly higher in putative CSC sub-populations (Bourguignon et al. 2012).

Elevated expression of these markers in HNSCC has also been associated with a poor prognosis and lower overall survival rate (Chiou et al. 2008; Fan et al. 2017; Koukourakis et al. 2012).

Table 1 Markers studied with the aim of identifying CSC sub-populations in HNSCCs

Marker Name *Marker Abbreviation*	Expression in HNSCCs	Prognostic Value	Selected References
ABC Transporters			
ABC subfamily A member 2 *ABCA3*	Yes	Unknown	(Li et al. 2011; Albrecht & Viturro 2007; Vasiliou et al. 2009)
P-glycoprotein *P-gp/ ABCB1*	Yes	Unknown	(Huls et al. 2009; Lin et al. 2006; Wang et al. 2000; Zhang et al. 2009; G. Zhang et al. 2012)
ABC subfamily B member 5 *ABCB5*	Yes	Yes	(Grimm et al. 2012)
Multidrug resistance protein 1 *MRP1/ ABCC1*	Yes	Unknown	(Li et al. 2011; Borst et al. 1999)
Breast cancer resistance protein *BCRP/ ABCG2*	Yes	Yes	(Lin et al. 2006; Zhang et al. 2009; G. Zhang et al. 2012; Chiou et al. 2008; Tsai et al. 2011; Hang et al. 2012)
Extracellular Markers			
Hyaluronan receptor *CD44*	Controversial	Yes	(Qian et al. 2016; Prince et al. 2007; Mărgăritescu et al. 2012; Zhang et al. 2009)
CD24	Yes	Yes	(Koukourakis et al. 2012; Satpute et al. 2013; Zimmerer et al. 2017; Han et al. 2014)
Prominin-1 *CD133*	Controversial	Controversial	(Mărgăritescu et al. 2012; Okamoto et al. 2013; Hang et al. 2012; Tsai et al. 2011; Chiou et al. 2008)
Intracellular Markers			
Aldehyde dehydrogenase *ALDH1*	Controversial	Controversial	(G. Zhang et al. 2012; Clay et al. 2010; Koukourakis et al. 2012; Yata et al. 2015)
Moloney murine leukemia virus insertion site 1 *BMI1*	Yes	Controversial	(Yu et al. 2010; Tsai et al. 2011; Fan et al. 2017)
Nanog	Yes	Yes	(Z. Zhang et al. 2012; G. Zhang et al. 2012; Chiou et al. 2008; Tsai et al. 2011; Fan et al. 2017)
Oct3/4	Yes	Yes	(Z. Zhang et al. 2012; G. Zhang et al. 2012; Chiou et al. 2008; Tsai et al. 2011; Fan et al. 2017)
Sex-determining region Y box 2 *SOX-2*	Yes	Yes	(Z. Zhang et al. 2012; Fan et al. 2017)

2.4   Xenografts

Xenografts of candidate CSC populations into immune deficient mice remains the gold standard for verification of stem cell properties by assessing the tumor initiation capabilities of the specific isolated sub-population (Valent et al. 2012; Visvader & Lindeman 2012; Z. Zhang et al. 2012). This method has been successfully applied to the detection of CSCs in many types of human malignancies including HNSCCs (Prince et al. 2007; Wang et al. 2007; Chiou et al. 2008; Chen et al. 2009; Zhang et al. 2009).

However, the ability or failure of potential CSCs to produce a detectable malignant population in a transplanted mouse may not accurately reflect the behaviour of the cells within the patient's original tumor micromilieu and thus caution should be used when interpreting results (Valent et al. 2012; Z. Zhang et al. 2012). As the majority of xenotransplantation studies utilize immunodeficient mice, limitations of these models must be expected. These include lack of cytokines which can stimulate the growth of CSCs, the inability to precisely model the tumor-specific microenvironment and the deficiency of natural immune-surveillance (Valent et al. 2012). Humanized mice, with a more human-type hematopoietic and immune microenvironment, may have to be considered for future studies.

3   Clinical challenges of CSC in the treatment of HNSCCs

The small sub-population of CSCs within the diverse and heterogeneous cell population comprising a tumor mass may be responsible for tumor recurrence, the promotion of metastasis due to high migration capacity, as well as resistance to both cytotoxic agents and radiation therapy. Intrinsic characteristics such as an increase in ABC transmembrane proteins, a semi-quiescent state and altered apoptotic mechanisms within CSCs limit susceptibility to cell death (Clarke & Fuller 2006; Zhang et al. 2009).

Therapeutic success following radiation of solid tumors is inversely proportional to the percentage of CSCs within the tumor mass (Yaromina et al. 83AD). CSCs not eliminated by radiation are potentially responsible for non-remission and recurrence,

as they have the capacity to renew and differentiate into the heterogeneous constituents of the tumor (Wicha et al. 2006). CSCs themselves are inherently more radioresistant, employing mechanisms which may encompass increased checkpoint activation and enhanced DNA damage repair responses (Eyler & Rich 2008). Increasing the radiation dose in HNSCCs is however associated with intolerable side effects such as xerostomia (Toledano et al. 2012). This may be explained by the radiation affecting microniches in which normal salivary stem cells are located, often in close proximity to blood vessels concentrated in the salivary glands (Vissink et al. 2010). Mechanisms sparing normal tissues aimed at curtailing the dose-limiting side effects of radiation are of interest to many researchers (Vallard et al. 2016).

Central tumor hypoxia seen in larger masses may also confer a survival advantage to CSCs being treated with chemotherapeutics or radiation (Heddleston et al. 2010). In methods similar to the hypoxic maintenance of the pluripotency of embryonic stem cells, poor perfusion of larger tumor masses may promote the CSC phenotype by creating specific CSC niches. Sub-optimal blood flow not only limits the distribution of chemotherapeutic agents to cancer cells, but also decreases the oxygen tension required for free radical formation in response to radiation (Satpute et al. 2013). The over-expression of hypoxia inducible factors (HIFs) in CSCs may also contribute to radioresistance of HNSCCs (Vlashi et al. 2009). Overexpression of HIF-1-α in CSCs mediates the induction of epithelial-mesenchymal transitions (EMT) in complex interactions which confer increased mobility on CSCs, as well as ensuring the maintenance of their pluripotency (Yang et al. 2010). Interactions between stem cells and stromal components in EMT which enhance local invasion and metastasis appear to involve the Wnt/beta-catenin signalling pathway (Sato et al. 2004; Zechner et al. 2003; Takahashi-Yanaga & Kahn 2010).

Treatment of HNSCCs still mainly involves surgical removal of the primary lesion wherever possible, with particular care being taken to include liberal surgical margins together with case-specific permutations in perioperative chemo- and/or radiation therapy (Satpute et al. 2013). The latter two modalities also aim at targeting residual tumor tissue. These debulking strategies however do not ensure elimination of CSCs, and these treatments may therefore fail to ensure remission and may predispose the patient to disease recurrence, as is often seen in HNSCCs. Future strategies should

therefore aim to target these CSCs or the CSC niche specifically, which, when done in combination with traditional treatment modalities, might ensure greater long-term disease remission. Mechanisms which promote sensitivity to cytotoxic agents and radiation, prevent evasion of immune surveillance and/or inhibit EMT crosstalk pathways may also hold the key to improved treatment outcomes in HNSCCs (Méry et al. 2016). As the mechanisms targeted are often integral to normal tissue stem cell function, treatment may result in severely toxic side effects.

4   Comments and future perspectives

The main challenge remains the identification of CSC sub-populations within primary tumors. The heterogeneity of these tumors is well known (Liang & Fu 2017). Techniques such as sphere formation and the side population discrimination assay can only be used to enrich for a population that may contain both cancer cells and CSCs.

Using markers to identify putative CSC populations also has its limitations. The existence of various isoforms of surface markers used for the identification of CSCs should be considered. Different antibody epitopes as well as possible cross reactions between antibodies need to be considered when designing CSC studies to prevent false positive and/or false negative results (Mărgăritescu et al. 2012). CD44 for example has various isoforms (Spiegelberg et al. 2014), three of which (CD44-v3, v6 and v10) have been associated with increased metastasis and aggressive HNSCC progression (Wang, Wong et al. 2009). The use of different marker isoforms by different research teams may provide one explanation for the contrasting results reported. Another difficulty with regard to CSC surface markers is that many are not only expressed on CSCs but are also present on other cell types including stem cells, somatic cells and other tumor cells. The expression of these markers may also vary greatly between patients and different tumors.

It is clear that the use of a single marker is currently not sufficient to isolate a pure CSC sub-population from a given tumor. Refined methods for the identification and characterization of CSCs are therefore needed. Multiple integrated analysis

approaches such as transcriptomics and proteomics are being used to study these cells.

Proteomics is a powerful tool for identifying signalling complexes which control CSC differentiation and regulate CSC maintenance pathways (Tsai et al. 2015; Yan et al. 2013). Using mass spectrometry and liquid chromatography-mass spectrophotometry, additional markers such as activated leukocyte cell adhesion molecule (ALCAM/ CD166) and intercellular adhesion molecule 1 (ICAM1) have been identified in putative CSC populations of HNCs (Yan et al. 2013; Tsai et al. 2015). It should be noted however that the above-mentioned studies and techniques were all conducted on cancer cell lines and not on primary isolated cells from tumor biopsies.

Next generation sequencing and bioinformatics are also generating a better understanding of the cancer genome. The transcriptome includes signalling and regulatory molecules as well as essential housekeeping molecules, and in its totality reflects a cell's identity (Saliba et al. 2014). To date, most transcriptome studies are conducted at a "population level", averaging the transcriptomes of millions of cells. It is now possible to study cells at the single-cell level providing an opportunity to dissect the complexity and heterogeneity of the tumor mass in detail. Increased understanding of tumor heterogeneity now offers researchers the potential to identify and study various sub-clones within a tumor mass (Jamal-Hanjani et al. 2015). Much work remains to be done to fully elucidate the biology of CSCs in HNSCC. The development of more focused treatment strategies will require a detailed understanding of the biological processes that generate these cells which appear to initiate and drive recurrence and aggressive metastatic tumor spread. Single cell transcriptome analysis will allow for a more detailed and accurate investigation of CSCs and possible sub-populations; to the authors' knowledge, data generated using this approach in the study of CSCs in HNSCCs has not yet been published.

In conclusion, the study of CSCs in HNSCC is important due to the high rates of recurrence and metastases encountered in these patients. As a result of the complexity and heterogeneity of HNSCCs, the application of techniques such as proteomics and transcriptomics to the study of CSC sub-populations is likely to result in a more efficient therapeutic option for these patients in the long term.

5 Conflicts of interest

The authors have no conflicts of interest to declare.

6 Author Contribution

MSP conceived the project, EW drafted the first version of the manuscript, and EW, SB, SN, AEM and MSP provided intellectual input and contributed to the writing of the manuscript. All authors vetted and approved the final version of the manuscript.

7 Abbreviation List

ABC - ATP-binding cassette

ATP - adenosine triphosphate

ALCAM - activated leukocyte cell adhesion molecule

ALDH - Aldehyde dehydrogenase

AKT - protein kinase B

BCRP - breast cancer resistant protein

BMI1 - moloney murine leukemia virus insertion site 1

CD44 - cluster of differentiation

CSC - cancer stem cell

EMT - epithelial-mesenchymal transition

ESCC - esophageal squamous cell carcinoma

FACS - fluorescence activating cell sorting

HIF - hypoxia inducible factors

HNC - head and neck carcinoma

HNSCC - head and neck squamous cell carcinoma

HPV - human papillomavirus

HSA - heat stable antigen

ICAM1 - intercellular adhesion molecule 1

MAPK - mitogen-activated protein kinases

NOD - non-obese diabetic

Oct3/4 - octamer-binding transcription factor 3/4

OSCC - oral squamous cell carcinoma

P-gp - P-glycoprotein

POU - Pit-Oct-Unc

PI3K - phosphatidylinositol-3-kinase

SCC - squamous cell carcinoma

SCID - severe combined immunodeficient

SOX2 - sex determining Region Y-box2

SP - side population

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