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Review Article

Diversity of cancer stem cells in head and neck carcinomas: The role of HPV in cancer stem cell heterogeneity, plasticity and treatment response

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ABSTRACT

Head and neck squamous cell carcinomas (HNSCC) resulting from oncogenic transformations following human papillomavirus (HPV) infection consistently demonstrate better treatment outcomes than HNSCC from other aetiologies. Squamous cell carcinoma of the oropharynx (OPSCC) shows the highest prevalence of HPV involvement at around 70–80%. While strongly prognostic, HPV status alone is not sufficient to predict therapy response or any potential dose de-escalation. Cancer stem cell (CSC) populations within these tumour types represent the most therapy-resistant cells and are the source of recurrence and metastases, setting a benchmark for tumour control. This review examines clinical and preclinical evidence of differences in response to treatment by the HPV statuses of HNSCC and the role played by CSCs in treatment resistance and their repopulation from non-CSCs. Evidence was collated from literature searches of PubMed, Scopus and Ovid for differential treatment response by HPV status and contribution by critical biomarkers including CSC fractions and chemo-radiosensitivity.

While HPV and CSC are yet to fulfil promise as biomarkers of treatment response, understanding how HPV positive and negative aetiologies affect CSC response to treatment and tumour plasticity will facilitate their use for greater treatment individualisation.

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Head and neck cancers represent approximately 3.8% of cancers globally where greater than 95% of these are squamous cell carcinoma (HNSCC) [1,2]. HNSCC has been typically associated with tobacco and alcohol use but the human papillomavirus (HPV) has emerged as a discrete aetiology, particularly in the oropharyngeal areas of the tonsils and base of the tongue. Oropharyngeal squamous cell carcinoma (OPSCC) is recognised as a subset among HNSCC with a strong association to HPV oncogenesis where prevalence rates are as high as 80% [3]. Cancers resulting from high-risk HPV genotypes typically present in a younger demographic, often with good performance status and little or no smoking history [4,5]. Clinically, HPV positive OPSCC show significantly better treatment outcomes compared to the HPV negative status, such that HPV involvement is a strong positive prognostic indicator. Generally however, no distinction is made for the involvement of HPV in standard treatment protocols that are based largely on staging, raising the question of possible over-treatment in HPV positive patients [6]. Given the younger average age of the HPV-positive patients, there is the likelihood of a longer post-treatment life with the debilitation of late effects from cancer treatment and additional risk of secondary cancers. Translation of HPV status as a biomarker for potential dose de-escalation is hampered however by a lack of evidence of the mechanisms of sensitivity to treatment and the associated contributions of clonogenic cancer stem cells (CSCs) to treatment outcome. CSCs are fundamental to tumour progression, metastases and recurrence. They are the most treatment resistant of tumour cells and are adversely responsive to therapy, accelerating replication and tumour repopulation [7,8]. Therefore, CSC elimination is essential to lasting tumour control but achieving this requires overcoming their treatment resistance and the tumour plasticity that can replace them from the non-CSC population.

HNSCC is treated by multidisciplinary teams according to several factors including type, stage, location, patient performance status, molecular characteristics of the tumour. Early stage tumours represent about one third of all head and neck cancer cases and are most commonly managed with surgery [1,9], although radiation therapy is an attractive alternative when surgery is likely to result in severe side effects, and/or disfigurement. Locally advanced HNSCC requires more aggressive treatment

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which could involve any combination of radiotherapy, chemotherapy, surgery or targeted therapies. Current management typically consists of systemic chemotherapy with concurrent radiotherapy [10]. Although the combination demonstrates better clinical responses and overall survival than radiotherapy alone, significant adverse effects have been observed [11,12]. Cellular plasticity and heterogeneity of CSCs add to the challenge of tracking and eliminating this resistant subpopulation [13]. How the aetiology of HPV oncogenesis affects these capacities in CSCs is unclear and evidence from comparisons of CSCs between the HPV statuses is limited.

To shed light on this issue, this review will discuss current clinical and pre-clinical evidence of the role of CSCs in treatment outcomes in HNSCC and compares their contribution to malignancy in terms of their HPV status. Firstly, the role of HPV in the oncogenesis of HNSCC is examined, as well as the clinical evidence that demonstrates a better treatment response in these patients. Secondly, this review will look at the issue of treatment resistance in CSCs, from chemotherapy and radiotherapy perspectives, and examine characteristics that make them the most refractory of tumour cells. Finally, evidence from studies investigating CSCs in terms of their HPV status is reported.

The search strategy for this review was conducted using literature-mining of PubMed, Ovid and Scopus based on key words and terms with the aim of reporting clinical and preclinical findings of the role of CSCs in head and neck cancers (including oropharyngeal cancers) in terms of the aetiologies of HPV positive and negative cancers. The final search was limited to articles in English and published after 2004. 310 abstracts were surveyed for relevance and duplication, and 154 papers were reviewed for this review.

HPV in head and neck cancers

In 2007 the World Health Organisation's International Agency for Research on Cancer concluded the human papillomavirus to be an independent cause of HNSCCs [14]. A survey of cancer incidence trends across 5 continents found falling rates of tobacco-associated oral and lung squamous cell carcinomas at the same time observing a rise in HPV related OPSCC. This increase was most pronounced among younger men and in economically developed nations, signalling a growing health concern in this area [15–17].

Upon infection, disruption of the epithelial mucosa allows the virus to access the basal layer of the mucosa where stem cells are located, and current evidence implicates the infection of stem cells in viral maintenance and progression. Infected cells are then subject to the expression of viral products and potential oncogenic transformation. The actions of two early viral proteins, E6 and E7, confer oncogenic transformation in mucosal cells from integrated viral DNA by the inactivation of the cell's major tumour suppressor proteins p53 and retinoblastoma protein (Rb) [18]. E6 mediates the degradation of p53, disrupting its control of cell cycling and apoptosis, as well as influencing telomerase production and cell replication. E7 inactivates Rb which also prevents apoptosis, promotes malignant transformation and elevates the expression of p16, a cell cycle regulator and surrogate marker for HPV oncogenicity [19-21]. Furthermore, cellular plasticity and the inducement of stemness may be influenced by these oncoproteins through unregulated Nanog expression resulting from p53 disruption, and subsequent inability of Rb to regulate pluripotency transcription factors Oct-4 and SOX2 [18,22]. Whether HPV infection of mucosal stem cells is the initiator of oncogenesis or whether the involvement of progenitor cells and de-differentiation also plays a part remains unclear. The gene for the tumour suppressor protein p53 is one of the most frequently mutated in HNSCC but a distinguishing feature of HPV positive oncogenesis is that expression of this gene is characteristically unaffected. For example, TP53 mutation is present in 82% of HNSCC associated with tobacco use, compared to only 3% for HPV positive HNSCC [23].

Progression of infection into HPV positive tumours demonstrates a preference for particular anatomical sites in the head and neck. While detection methods vary, around 70–80% of OPSCCs are identified as HPV positive while only 1.3–7% of other HNSCCs are positive. Even among oropharyngeal subsites, HPV shows a preference for carcinogenesis at the base of the tongue and tonsils [3,24]. A global trend of increasing HPV incidence in head and neck cancers is consistent with the rising proportion of OPSCC among HNSCC [16] as well as increased HPV involvement in OPSCC cases [25,26].

More than 120 different HPV genotypes have been identified to date. An important subset among the HPV species is the α-papillomavirus group which are the high-risk HPV types including HPV16 and HPV18, considered the most dangerous [18]. Most significant of all HPV types in HNSCC is HPV16 with a prevalence of >80% in HPV positive HNSCC and >90% in HPV positive OPSCC [16].

Testing for HPV in HNSCC has been largely unstandardized and different methods have been applied to the verification of HPV causation. The present gold standard is the detection of HPV transcriptional activity for E6 and E7 mRNA [27]. This analysis has previously required RNA extraction from sample tissue and polymerase chain reaction (PCR) amplification, an exacting technique mostly limited to research laboratories [19,28]. The development of fluorescence *in situ* hybridisation (FISH) enables the visualisation of HPV E6 & E7 transcripts in a test feasible for clinical application that can verify transcriptional activity. As mentioned previously, the elevated expression of p16 is used as a surrogate marker for HPV integration into the host genome. This is detected via immunohistochemical (IHC) analysis, well suited to clinical applications and while sensitivity of p16 testing for HPV involvement is high (95–100%), specificity is only around 85–95% [21]. Approximately 7–20% of p16 positive HNSCC is found to be HPV negative as elevated expression of p16 is not exclusively linked to HPV transcriptional activity and the percentage of p16 positive/HPV negative cases is varied by anatomical region [27,29]. The prevalence of p16 positive/HPV negative cases suggests that causes other than HPV, such as senescence, are linked to p16 expression [30]. While p16 IHC is a practical option for clinical analysis and p16 positive HNSCC show comparable survival rates irrespective of HPV status [31], greater specificity for patient stratification by HPV involvement can be achieved by additional analysis for E6/E7 RNA assessment where FISH demonstrates a specificity of 88–100% [21,27,32].

Clinical outcomes by HPV status

The most important distinction of the HPV positive aetiology is the significantly better clinical response to radio-chemotherapy. Since the awareness of HPV involvement as a biomarker in HNSCC, comparative analysis demonstrates consistently better patient outcomes by clinical end-points, summarised in Table 1. Translating this treatment sensitivity into patient stratification for the de-escalation of treatment in HPV positive patients is the subject of a number of clinical studies. If heightened treatment sensitivities in an identifiable patient cohort can be used to recalculate the dose for tumour control, considerable benefits in lessened treatment morbidities can advantage patient recovery [6]. However, translation of the therapeutic sensitivity observed in HPV positive patients to greater treatment individualisation still requires further evidence of mechanisms of chemo and radiosensitivity in HPV positive patients.

Table 1
Hazard ratios for overall survival and locoregional control for HNSCC: HPV+ vs HPV−.

Author/Year	*n*	Pathology/ stage	Treatment	Follow up months	Clinical end point CI 95%	Comments
Fakhry 2008 [33]	96	HNSCC III/IV	Induction Ctx Paclitaxel + Std fx Rtx	60	HR for OS = 0.35 (0.15–0.80) HR for OS of OPSCC only = 0.29 (0.11–0.74, 95% CI)	HR by HPV status Tumour sites: laryngeal oropharyngeal
Lassen 2009 [34]	156	HNSCC I–IV	Std fx Rtx	60	HR for OS = 0.44 (0.28–0.68) HR for LRF = 0.35 (0.19–0.64)	HR by p16 status Tumour sites: pharynx & supraglottic larynx
Ang 2010 [29]	323	OPSCC III–IV	Cisplatin with either std fx Rtx or boost Rtx	60	HR for OS = 0.42 (0.27–0.66) HR for PFS = 0.40 (0.29–0.57)	HR by HPV status Tumour sites: oropharyngeal
Rischin 2010 [35]	185	HNSCC III–IV	Std Rtx plus cisplatin with or without tirapazamine	24	HR for OS = 0.36 (0.17–0.74) HR for LRC = 0.39 (0.20–0.74)	HR by p16 status Tumour sites: oropharyngeal
Lassen 2011 [36]	794	HNSCC I–IV	Nimorazole with either std fx Rtx or accel. fx Rtx	60	HR for OS = 0.54 (0.42–0.68) HR for LRC = 0.58 (0.43–0.78)	HR by p16 status Tumour sites: pharynx, larynx & oral cavity
Posner 2011 [37]	111	OPSCC	Std Rtx + cisplatin, docetaxel	60	HR for OS = 0.20 (0.10–0.38)	HR by HPV status Tumour sites: oropharyngeal
Fakhry 2014 [38]	181	OPSCC III & IV	Std Rtx & Accel Rtx plus cisplatin cetuximab	24	HR for OS = 0.49 (0.34–0.70)	HR by p16 status Tumour sites: oropharyngeal
Linge 2016 [39]	158	HNSCC Locally advanced	Std and Hyperfx Rtx with cisplatin or mitomycin-c	60	HR for LRC by p16 status = 0.30 ($p = 0.021$) HR for LRC by HPV16 status = 0.39 ($p = 0.072$)	Tumour sites: oropharynx, hypopharynx & oral cavity
Weinberger 2006 [40]	79	OPSCC II–IV	Rtx/Surgery	60	HR for OS by = 0.19 (0.1–0.7) HR for PFS by = 0.20 (0.1–0.6)	Tumour sites: oropharyngeal
Lohaus 2014 [41]	215,121	HNSCC II–IV OPSCC	Postoperative Rtx with cisplatin	47	HR for OS by HPV status = 0.33 (0.17–0.62) HR for OS by p16 status = 0.38 (0.21–0.68) HR for LRC by HPV status = 0.13 (0.03–0.54) HR for LRC by p16 status = 0.24 (0.08–0.68)	Tumour sites: oral cavity, oropharynx & hypopharynx
Rosenthal 2015 [42]	170	OPSCC III to IV	Rtx alone or Rtx with cisplatin of cetuximab	60	HR for OS, Rtx alone = 0.40 (0.21–0.74) HR for LRC, Rtx alone = 0.30 (0.16–0.58) HR for OS, Rtx + cetuximab = 0.16 (0.07–0.36) HR for LRC, Rtx + cetuximab = 0.12 (0.05–0.30)	Tumour sites: oropharyngeal

HR = hazard ratio, OS = overall survival, LRC = locoregional control, LRF = locoregional failure, PFS = Progression free survival, fx Rtx = fractionated radiotherapy, Ctx = chemotherapy, Std = standard.

HPV status and chemotherapy

The administration of cisplatin concomitantly with radiation remains the most commonly accepted schedule in HNSCC due to generally better clinical outcomes. Studies on induction (neoadjuvant) chemotherapy demonstrated markedly superior outcomes in the HPV positive group [43,44]. A retrospective subset analysis from the international phase III TAX 324 trial, in which patients were treated with docetaxel, cisplatin, and fluorouracil, examined the clinical outcome for oropharyngeal cancer patients treated with induction chemotherapy followed by carboplatin-radiotherapy [37,45]. This analysis revealed vastly superior 5-year overall survival for HPV positive patients as compared to HPV negative patients (82% vs 35%, $p < 0.0001$) and lower locoregional failure rate (13% vs 42%, $p = 0.0006$). These results are confirmed by a more recent UK phase II trial of induction chemotherapy followed by concomitant IMRT and platinum-based chemotherapy, with an impressive 2-year locoregional progression free survival of 98.6% for HPV positive as compared to 76.8% for HPV negative patients [44].

Due to the high responsiveness of HPV positive HNSCC to both radiation and chemotherapy, dose de-escalation in order to reduce treatment-related adverse events is hotly debated. The future role of chemotherapy in HPV positive OPSCCC is argued based on the fact that the main aim of treatment de-escalation should consist of reduced adverse events and better quality of life. The promising results of the epidermal growth factor receptor-targeting chimeric antibody, cetuximab (Erbitux), shown in the past when given concurrently with radiotherapy [46] were taken further by the EXTREME trial (ErbituX in first-line Treatment of REcurrent or MEtastatic head and neck cancer) that investigated the impact of HPV status on outcome in patients receiving chemotherapy with or without cetuximab [47]. The trial showed that the addition of cetuximab to platinum-based chemotherapy improved survival in patients with recurrent and/or metastatic HNSCC irrespective of the HPV status.

To date, the clinical effectiveness of cetuximab in HPV positive patients is inconclusive. This is mainly due to the inconsistent results between HPV status and EGFR expression/activation [48,49]. However, to better understand the value of anti-EGFR therapy in HPV positive patients, several phase III trials are currently ongoing which randomise HPV positive patients to receive either cisplatin or cetuximab with concurrent radiotherapy: RTOG 1016, NCT01302834 [50], De-ESCALaTE HPV, NCT01874171 [51] and TROG 12.01, NCT01855451 [52]. Beside evaluating and comparing toxicities in the two arms, the aim of the trials is to assess the feasibility to substitute cisplatin with cetuximab should the endpoints be similar.

While chemotherapy adds to both early and late normal tissue toxicity, the role of platinum-based drugs in the management of HPV positive HNSCC should not be marginalised as:

1. Clinical trials show that the addition of chemotherapy to radiation improves outcome in all HNSCC patients, including the HPV positive patients. A meta-analysis of 93 randomised trials indicated a clear benefit of concomitant chemo-radiotherapy in HNSCC patients treated with curative intent [53].
2. Cisplatin was shown to enhance radiation response by increasing apoptosis through cell cycle dysregulation and down-regulation of HPV E6 and E7 proteins *in vitro* [54]. The study suggested dose de-escalation in HPV positive patients by avoidance of cisplatin is not justified. Instead, the reduction in the total radiation dose should be considered.
3. Induction chemotherapy significantly increases progression free and overall survival in HPV positive patients and shows a reduced risk of distant metastasis in locally advanced HNSCC patients [55]. While the role of induction chemotherapy is still controversial, it is agreed that for HPV positive patients this approach should be separately investigated.
4. Perhaps most importantly, chemotherapy is able to tackle the disseminated disease. Distant metastases in HPV positive OPSCC involve a greater number of subsites that are seldom seen in HPV negative HNSCC [56]. It was suggested that treatment de-intensification should also consider the risk of distant metastases, which was shown to be higher in HPV-positive T4 and/or N3 stages than in early-stage cancer patients [57]. Furthermore, among the low-risk HPV-positive patients, subgroups with N2b (with smoking history) and N2c exhibited higher rates of distant metastases when treated with radiation only compared to those patients that received concurrent chemo-radiotherapy [57]. The biological advantage of HPV positivity might therefore be hindered by tobacco exposure, leading to a higher degree of tumour heterogeneity and thus an increased risk of disseminated disease.

It is to be noted that the eight edition of the AJCC Cancer Staging Manual (TNM classification from the American Joint Committee on Cancer) has included three major changes to head and neck cancer staging that incorporate the following: [1] the depth of invasion to oral cavity cancers, [2] novel pathologic and clinical staging system for high-risk HPV+ associated OPSCC, and [3] extranodal extension in nodal characterisation in high-risk HPV− and non-nasopharyngeal carcinoma [58]. This latest edition of the AJCC Cancer staging manual highlights the importance of determining high-risk HPV status for the purpose of staging, using the surrogate marker p16, and recommends testing on all OPSCC.

A meta-analysis of randomised clinical trials provides strong evidence of improved outcome in HPV positive HNSCC patients treated with platinum-based chemotherapy combined with radiation [6]. A review of the current literature suggests that a plausible treatment approach for HPV positive patients is induction chemotherapy, followed by dose de-escalated radiotherapy in combination with either traditional (cisplatin-based) chemotherapy or cetuximab, if deemed a successful replacement for cisplatin by the currently proceeding randomised trials [59].

HPV status and radiation: in vitro studies

Radiosensitivity of tumour cells can be examined by a variety of methods including DNA repair, apoptosis and clonogenic survival. *In vitro* studies comparing radiosensitivity in cell lines and patient tissue samples, by clonogenic assay, have found the HPV positive tumour cells are more radiosensitive than HPV negative tumour cells (Table 2). While Rieckmann et al. [60] observed no change in apoptosis among the HPV positive cell lines, elevated levels of unrepaired DSBs along with a higher frequency of G2 arrest were recorded compared to HPV-negative cells. Other studies also found higher accumulation of HPV positive tumour cells in the more radiosensitive G2 phase [61,62], but contrary to Rieckmann, they observed strong radiation-induced apoptotic responses in HPV positive cells. Overexpression of p16 in HPV positive HNSCC is reported to play a role in the radiosensitivity of these tumours independently of its effect on cell cycling. Increased radiosensitivity in HPV positive HNSCC resulting from inhibition of homologous repair of DSBs has been reported [63]. Foci formed by the repair protein RAD51 at the site of DSBs are critical to homologous repair, representing the greatest possibility of cellular survival following DSB. The overexpression of p16 however, was shown to result in down-regulation of cyclin D1 which is involved in the recruitment of RAD51 to the site of DSBs. This obstruction of cellular ability to

Table 2
Radiosensitivity of HNSCC cell lines, measured by clonogenic assay, stratified by HPV status.

Author/Year	HPV+ cell lines	HPV− cell lines	Mean SF HPV+	Mean SF HPV−	Comments
Arentz 2014 [61]	UM-SCC-47 UM-SCC-104 93-VU-147T UPCI-SCC-15	UD-SCC-1 UM-SCC-6 UM-SCC-11B UT-SCC-33	SF2 = 0.19	SF2 = 0.34	$p = 0.01$. HPV+ cells showed impaired DNA repair and Sig more apoptosis
Dok 2014 [63]	UPCI-SCC-154 UPCI-SCC-90	SQD9	SF2 = 0.28	SF2 = 0.72	
Rieckmann 2013 [60]	UT-SCC-45 UD-SCC-2 UPCI-SCC-154 UM-SCC-47 93-VU-147 T	HSC4 Cal33 UT-SCC-5 FaDu SAT	SF3 = 0.28	SF3 = 0.45	$p = 0.028$.
Kimple 2013 [62]	UD-SCC-2 UM-SCC-47 UPCI-SCC-90 93-VU-147 T	UM-SCC-1 UM-SCC-6 UM-SCC-22b SCC-1483	SF2 = 0.22	SF2 = 0.59	$p < 0.0001$
Spanos 2009 [64]	UPCI-SCC-90 UM-SCC-47	UM-SCC-1 UM-SCC-19 UM-SCC-84	SF6 = 0.48	SF6 = 0.08	
Gupta 2009 [65]	UPCI-SCC-90 UM-SCC-47	SQ20B	SF2 UM-SCC-47 = 0.55 UPCI-SCC-90 = 0.13	SF2 SQ20B = 0.74	

SF*x* = survival fraction where *x* is radiation dose in Gy.

enlist RAD51 in the DNA repair process, resulting from elevated p16, was found to affect intrinsic radiosensitivity. Consistent with these findings, greater radiosensitivity in HPV positive cell lines is also reported by elevated numbers of unrepaired DSBs using immunofluorescent staining for γ-H2AX foci [60]. While evidence of radiosensitivity in HPV positive HNSCC correlates with clinical findings, a caveat is that the *in vitro* analysis is not specific in terms of CSC which represents each cancer's most malignant cells and the target of tumour control.

Cancer stem cells in head and neck cancers

CSCs typically represent a small subpopulation among tumour cells distinguished by an ability to self-renew indefinitely as well as generate the differentiated progeny that possess only transient proliferative capacity. Consequently, tumour recurrence is theoretically possible from a single CSC surviving treatment. While characterised as typically quiescent, CSCs are drivers of tumour progression and are supported by a microenvironmental niche that facilitates maintenance of stemness and function in hypoxic states [66–68]. Cellular plasticity in cancer, an ability of cells to change identity and role, is not limited to CSCs, but can involve non-CSCs where phenotypic transition between these states means numbers of CSCs in tumour populations are not fixed, but responsive to environment and treatment. De-differentiation may be promoted among non-CSCs, reverting cells to a state of stemness in response to treatment, increasing CSCs numbers and intratumoural heterogeneity while accelerating repopulation [69,70]. Elevated expression of transcription factors promoting epithelial–mesenchymal transition (EMT) is associated with stemness and represents a feature of CSC plasticity. EMT involves the loss of epithelial polarity and connectivity, allowing transition of carcinoma cells to a migratory mesenchymal phenotype, promoting metastatic spread. Programmed cell death by anoikis is normally the result of epithelial detachment but CSCs demonstrate resistance to this, enabling transition to other sites [66,71,72]. Capacity for accelerating tumour repopulation by CSCs also results from alterations in divisional dynamics as a response to tumour volume loss. CSC replication usually involves asymmetrical division where one daughter cell enters the differentiation pathway and the other retains stemness. In response to treatment however, this may change to symmetrical division where both daughter cells are CSCs. As with de-differentiation, this increases the clonogen pool, facilitating an accelerated repopulation [73–76].

Identification of CSCs among tumour populations generally involves molecular markers for features of stemness. Testing marker tumourigenesis *in vivo* uses transplantation assays where a tumour subpopulation is segregated by selective phenotypic markers. Cell populations expressing, or absent for these are tested for their propagation potential by frequency of tumour development for seeding density. Subsequent serial tumour transplantations of CSCs from resulting tumours can confirm the persisting tumourigenesis of the phenotype and reproduction of the cellular heterogeneity of the parent tumour [77,78]. Numerous CSC markers have been investigated but not all have been verified clinically or validated for both HPV statuses (Table 3) [13].

CSCs were originally identified in HNSCC by the cell adhesion molecule CD44 [79]. Study of the distribution of CD44 in HNSCC has questioned its specificity for stemness due to an abundant expression in healthy tissue including the mucosal epithelium [80]. Aldehyde dehydrogenase (ALDH) is a metabolic enzyme that catalyses the oxidisation of aldehydes to carbolic acid and potentially plays an antioxidant role, protecting cells from oxidative stress and thereby contributing to chemo- and radio-resistance. ALDH is frequently used for CSC isolation in numerous solid tumour types and also in conjunction with CD44 to identify a CSC phenotype in HNSCC [68,81] where it is considered a primary marker for stemness irrespective of aetiology [82]. Other markers with strong associations to stemness are c-Met, Bmi-1, CD98 and 26S proteasome activity where diminishment of this function can be identified by ZsGreen-cODC, a fluorescent fusion protein which is indicative of tumourigenic cells by its accumulation. Importantly, each of these markers is found to identify putative CSCs in both HPV statuses of HNSCC, allowing comparison of CSCs by HPV status [83-87].

Cancer stem cells and chemotherapy in head and neck cancers

Cisplatin is the most common chemotherapeutic agent employed for treatment of HNSCC but a persisting problem yet to be resolved is cisplatin-resistance. Whether intrinsic or acquired, drug resistance is a major factor in the failure of chemotherapy in general and cisplatin-based treatment in particular [107–109]. Cellular mechanisms behind resistance to cisplatin are multifactorial and can lead to decreased DNA-adduct formation, enhanced DNA repair, decreased induction of apoptosis and altered expression of regulatory proteins [108]. Molecular agents responsible for these mechanisms are upregulated in cisplatin-resistant cells, suggesting that the corresponding transcription factors play an important role in drug resistance [110]. Besides the tumour-suppressor proteins p53, p73, and the c-Myc oncoprotein, that are known to dictate sensitivity to cisplatin, studies have identified several transcription factors involved in the genomic response to cisplatin which are suggested to interfere with processes related to DNA repair [110]. It is also shown that CSCs are responsible for treatment failure after chemo-radiotherapy and resistance to chemotherapy remains a major challenge in oncology that requires an effective solution.

Given traditional chemotherapy preferentially acts upon proliferating rather than quiescent cells, it is critical that targeted cells are actively cycling. CSCs however are shown to be long-lived and quiescent [111], an asset that renders them effectively more resistant to chemotherapy. Recent studies investigating mechanisms behind CSC chemotherapy resistance report a growing body of evidence supporting the role of protein glycosylation in drug resistance, suggesting alteration in glycosylation is the possible trigger of chemoresistance [112,113]. An association was found between CSC proteins and the protein-specific cancer-associated glycan, CD72-4, as the latter have often been found overexpressed in many advanced cancers, stimulating metastatic spread of CSCs. Selective inhibition of glycosylation pathways may represent a therapeutic approach to overcome drug resistance [112].

The chromatin regulator Bmi-1, a stem cell-related gene and CSC marker has been evaluated for its association with drug resistance [114,115]. Bmi-1 expression is upregulated in several cancers, including HNSCC, where cisplatin-resistant cells express higher levels of Bmi-1, and this protein is identified as prognostic for overall survival [114]. Using a HNSCC mouse model, Chen, Felix [115] showed Bmi-1 CSCs to mediate invasive growth and spread, whereas the inhibition of the oncoprotein sensitised the tumour to cisplatin and eradicated lymph node metastases.

Studies on the expression of CD133 in CSCs and its role in chemoresistance to cisplatin and fluorouracil in HPV positive cell lines revealed that CD133 overexpression fosters chemoresistance via cell cycle transition arrest and reduced apoptosis, significantly promoting stemness in HNSCC [113]. CD44 and EGFR interactions have also been investigated with EGFR signalling pathways promoting CD44-mediated chemoresistance to multiple drugs used to treat HNSCC including cisplatin, doxorubicin, etoposide and methotrexate. Complex, multiple downstream signalling pathways that involve CD44/EGFR interactions, facilitated by hyaluronic acid,

Table 3
CSC markers in HNSCC.

Marker	Reference	Actions	Identification in HPV status
ALDH1	Keysar 2017 [83]	Positive correlation between ALDH expression and EMT transcription factor SNAI1	HPV+ & HPV–
Bmi-1	Xu 2014 [89] Allegra 2016 [84]	Promotes radioresistance and cell cycling via transcriptional suppression of Ink4a/Arf. Coordinates with E6 to immortalise keratinocytes	HPV+ & HPV–
CD10	Fukusumi 2014 [90]	Membrane protein associated with significantly greater expression of stemness marker Oct-3/4	HPV–
CD29 (β1 Integrin)	Koukourakis 2012 [91]	Transmembrane protein with direct relationship to Oct-4 expression. Significant link with distant metastasis	NS
CD44	Keysar 2017 [83] Naor 2002 [92]	Surface antigen and adhesion molecule. Functional role in cell migration and proliferation	HPV+ & HPV–
CD56	Desidero 2015 [93]	Surface adhesion glycoprotein	HPV–
CD98	Rietbergen 2014 [86] Cantor 2012 [94]	Cell membrane glycoprotein. Promotes amplification of integrin signalling, cell proliferation and invasion.	HPV+ & HPV–
CD133	Bano 2018 [95]	Surface antigen and glycoprotein. Overexpression associated with features of stemness	HPV+ & HPV–
c-Met	Rothenberger 2017 [87] Baschnagel 2013 [96]	Receptor for hepatocyte growth factor. Promotes proliferation, angiogenesis, invasiveness & metastasis	HPV+ & HPV–
JARID 1B	Lin 2015 [97]	Demethylase enzyme, expression correlates with stemness markers Oct-4, Nanog, KLF4 and CD133	NS
Nanog	Lee 2015 [98] Pozzi 2015 [99]	Transcription factor controlling self-renewal. Association with stemness, metastasis, poor differentiation, treatment resistance	HPV–
Oct-4	Koo 2015 [100] Baillie 2017 [101] Zeineddine 2014 [102]	Transcription factor. Carcinogenic reprogramming of cells. Role in EMT, metastasis, dedifferentiation and invasiveness	HPV–
SOX2	Chou 2015 [103] Liu 2013 [104] Baillie 2017 [101]	Transcription factor, has role in self-renewal and pluripotency. Associated with anti-apoptotic function, radioresistance and anchorage independent growth	HPV–
STAT3	Baillie 2016 [105] Gkouveris 2014 [106]	Transcription factor. Oncogenic role in cell cycle progression and anti-apoptosis	NS
ZsGreen-cODC+	Vlashi 2009 [107] Vlashi 2016 [88]	Identifies cells with low 26S proteasome activity, indicative of capacity for self-renewal and tumourigenesis	HPV+ & HPV–

NS = not specified.

and further promote tumour progression and inhibition of these molecular pathways are considered plausible therapeutic strategies that require more attention [116,117].

Radiotherapy and cancer stem cells

Surgery can achieve lasting tumour control in HNSCC but is limited to early stage HNSCC with clear resectable margins [118,119]. For locally advanced HNSCC requiring radiotherapy, measures of CSC density and radiosensitivity potentially provide critical parameters in addition to normal cancer staging that can guide treatment planning and prognosis. Both intrinsic and extrinsic mechanisms of radioresistance are demonstrated to favour CSCs. Post irradiation, a greater ability to initiate checkpoint activation and induce cell cycle arrest is demonstrated where regulating kinases Chk1 & Chk2 halt cell cycle progression, allowing time for DNA repair prior to the onset of mitosis and evading potential apoptosis or mitotic catastrophe [120–122]. Compared to differentiated cancer cells, CSCs more efficiently repair DNA damage due to elevated expression of RAD51 in CSC phenotypes, enhancing capacity for homologous recombination of DSBs [123–125]. Significant overexpression of genes for glutathione (GSH) synthesis and regulation have also been found to support CSC radioresistance. GSH is an intracellular antioxidant, and elevated production provides CSC with superior reductive defence against radiation damage [126]. Additionally, a supportive microenvironment provides conditions allowing CSCs to be maintained in a comparatively hypoxic state, rendering them extrinsically radioresistant as the efficiency of cell killing by X-rays and gamma rays, is dependent on tissue oxygen tension [67,124].

Despite the development of many high throughput assays for treatment sensitivity, clonogenic assays remain the gold standard for determining intrinsic radiosensitivity of cell populations. By virtue of counting only clonogenic colonies among surviving cells, it remains the measure most consistent with potential CSC survival and repopulation, albeit this test would likely count some surviving progenitors. The assay has limitations however, requiring

culturing of cells at low population densities, often problematic with tissue cultures [127,128]. Additionally, lengthy culturing times may limit the application of this test and make it impractical for clinical use.

Given the plasticity demonstrated by CSCs and non-CSCs, determination of CSC numbers cannot be absolute, but inherently responsive to environment and treatment. Differences in CSC density, radiosensitivity and response to treatment, between HPV positive and negative statuses, seen in the same phenotype, need to be reconciled for their use as biomarkers. Importantly, the combined use of CSCs with other biomarkers can more specifically stratify patients into treatment groups [7] gaining specificity in predicting response to treatment when sub-stratified by other relevant biomarkers such as HPV involvement. The question arises however, what is the effect of the HPV aetiology on CSCs in HNSCC? Oncogenic transformation requires an accumulation of genetic alterations and stem cells are viewed as a likely point of origin given their long functional lifespan over which mutations may accrue [129]. Whether HNSCC oncogenesis commences by generation of CSCs is unclear [118,130,131], and the role of aetiology needs to be evaluated in the inherent and resulting CSC population density and treatment responsiveness over the course of therapy.

Putting it together: the case for using multiple biomarkers

CSCs have been called the "beating heart of the tumour" [132] and progressively viewed as the critical cellular factor in tumour control [7]. Significant differences in treatment outcomes for HNSCC patients between the HPV groups raises the question of the influence of this aetiology on the malignancy of the tumour's CSCs. Given inter-tumoural heterogeneity of CSCs, accounting for their contribution to treatment outcome requires determinations of their radiosensitivity and responsiveness in terms of their HPV status.

Concurrent contributors to treatment outcomes like CSCs and HPV, may be accounted for by sub-stratification of primary patient groups. In a clinical study of OPSCCs that aimed to evaluate the contributions of HPV, smoking history, tumour size and node involvement to treatment outcomes [29], patients were first stratified by HPV status then sub-stratified by smoking history. HPV positive patients with a smoking history greater than 10 pack years were further stratified by node involvement and HPV negative patients with a less than 10 pack year smoking history were stratified by tumour size. This study demonstrated an ordering of a panel of significant contributors to treatment outcome which then allocated 266 patients into 3 prognostic groups of low, intermediate and high risk by correlating treatment outcomes with biomarker expression as represented by the schematic in Fig. 1. This stratification however, did not account for relative measures of CSC densities or treatment sensitivity in the groupings.

Clinical studies of HNSCC, and more specifically OPSCC, investigated CSC influence in both HPV statuses by measure of CSC expression using CD44 and CD98 phenotypes, and compared the treatment outcomes of LRC, PFS and OS. Analysis of pre-treatment HNSCC tissue samples for HPV/p16+ and CSC expression by CD44 and CD98 facilitated stratification of patients into 4 groups, HPV+/CSC^{low}, HPV+/CSC^{high}, HPV−/CSC^{low}, HPV−/CSC^{high} [39,85,133,134]. The HPV+/CSC^{low} classified patients showed consistently better treatment outcomes over the HPV−/CSC^{high} group illustrating the utility of tumour profiling by multiple biomarkers to specify patients into good, intermediate and poor prognostic groups for treatment planning and possible dose de-escalation.

Among biomarkers identified for prognostic contribution to clinical outcomes in HNSCC, factors such as EGFR, TP53 gene mutation, smoking history, hypoxia, tumour size, and node involvement have all shown significant prognostic value [5,29,39,135]. Stratifying cohorts into more specific groupings by prediction of response to therapy alters the dose response curve for that group. When plotting the tumour control probability (TCP) as a function of dose for all HNSCCs, the dose–response relationship schematically forms a shallow sigmoid curve, where the start of the curve reflects response by the most sensitive tumours, and the top of the curve

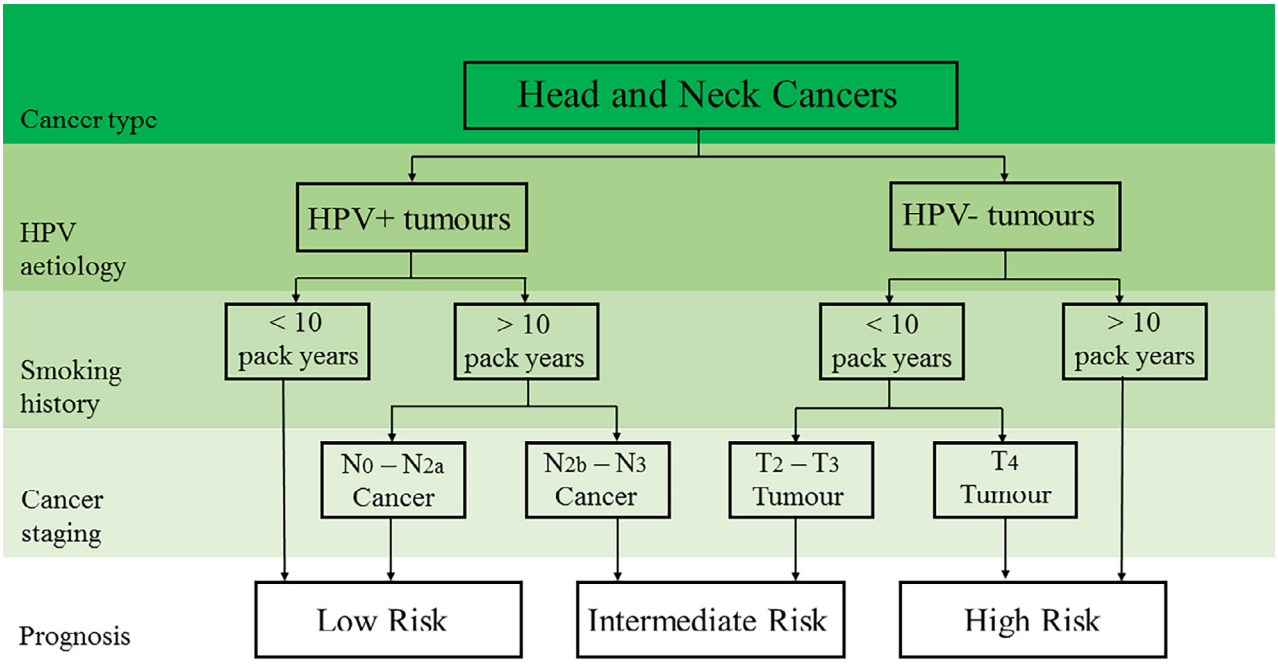

Fig. 1. Stratification of contributors to prognosis in HNSCC. Adapted from [29].

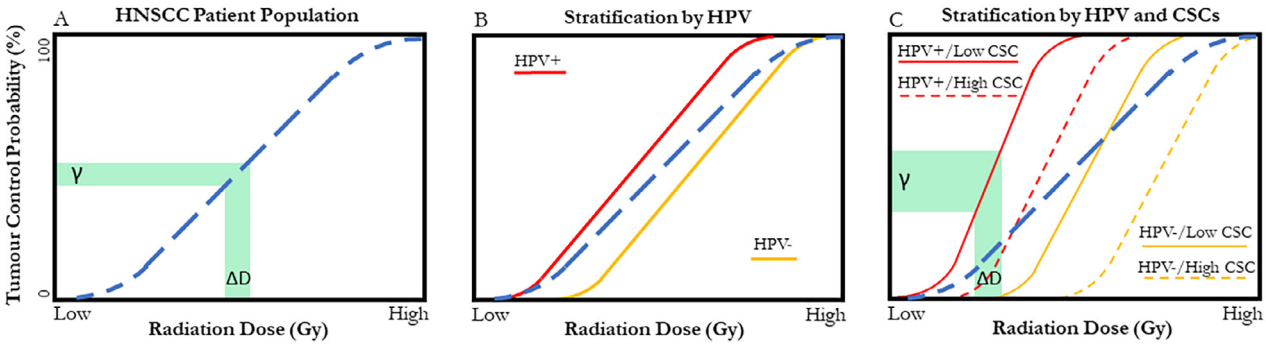

Fig. 2. Schematic of dose to tumour control probability relationships. (A) The dose–response curve for all HNSCCs receiving the same treatment reflects the response of the most sensitive tumours at the low dose and most resistant at the high dose, resulting in a shallow sigmoid relationship. (B) As biomarkers (HPV) are introduced, heterogeneity in treatment sensitivity is reduced by group and the response curves become steeper. (C) Sub-stratification by the relationship of additional biomarkers (CSCs) steepens the curve again and can further open a therapeutic window against normal tissue complication probability for the more sensitive groups. Adapted from [7].

the most resistant. This dose–response curve can be expected to become progressively steeper with TCP increasing over a narrower dose range as groupings are more specific by biomarker contribution (Fig. 2) [7]. A measure of therapeutic advantage (γ) is the change in response for the change in dose (ΔD) [136].

Correlations of CSC expression and HPV status to therapeutic endpoints illustrate the influence these biomarkers have on treatment outcomes and could be used to identify the most suitable candidates for de-escalation trials. The selection of candidates with HPV positive OPSCC for de-escalation of treatment in clinical trials has been approached by various means. As mentioned previously, the replacement of cisplatin with cetuximab has been trialled with a total radiation dose of 70 Gy in different treatment regimens in the RTOG 1016 [50], TROG 12.01 [52] and De-ESCALaTE HPV trials [51,137]. Based on their response to induction chemotherapy, participants in the Quarterback trial were randomised to receive standard dose radiotherapy group or low dose radiation, both supported with carboplatin weekly [6]. Stratification of participants in the PATHOS, NCT02215265 [138] and ECOG 3311, NCT01898494 [139] trials following trans-oral robotic surgery (TORS) into low, intermediate and high-risk groups where low risk participants receive no adjuvant treatment and the intermediate and high-risk groups receive reduced irradiation doses [137,140].

Given the consistent clinical outcomes in favour of HPV positive HNSCC, it has been understandable to expect greater CSC densities in HPV negative tumours, but results have been varied. For example, Vlashi et al. [87] looked at 6 HNSCC cell lines (2 HPV+, 4 HPV−) to examine CSC response as a function of HPV status where CSCs were identified by low proteasome activity using ZsGreen-cODC. A significantly higher CSC population was measured in the HPV negative cell lines and HPV positive cell lines were found to be significantly more radiosensitive by clonogenic assay. After eliminating the ZsGreen-cODC-positive CSCs by fluorescence activated cell sorting (FACS), cultures absent for CSCs were observed for regeneration of CSCs from non-CSCs. HPV negative cell lines were found to repopulate CSCs, putatively by de-differentiation, with significantly greater efficiency than the HPV positive cell lines. But contrasting with this are studies that found no difference in CSC expression between the HPV groups or observed greater CSC populations in the HPV positive status [141]. A study using 6 HNC cell lines, 3 of each HPV status, and pre-treatment patient tissue samples, reported an unexpected finding of higher CSC densities in the HPV positive cultures of both tissue sample and cell lines [142]. Mean percentage population of CSCs in HPV positive tissue and cell line cultures was 2.4 and 2.9 times greater than the HPV negative cultures respectively. Identifying CSCs by ALDH expression, they also observed a significantly greater tumoursphere growth (2-fold higher) in HPV positive cell lines and tissue samples, demonstrating higher CSC densities and their more vigorous influence on growth. This evidence is counterintuitive with results from clinical studies and illustrates that CSC treatment responsiveness may be more critical than inherent CSC density. A study by Reid et al. [142] has corroborated the finding of greater CSC proportions in the HPV positive status. Using the ALDH marker as a refinement on elevated CD44 expression (CD44+/ALDH+) for CSC identification, HPV positive cells showed a CSC population density 5-fold that of the HPV negative. Additionally, greater plasticity was demonstrated in the HPV positive cell line by a capacity to repopulate the CSC phenotype (CD44+/ALDH+) following cell sorting to be absent of CSCs. HPV negative cells however, demonstrated a persisting elevation in CSC density following repeated irradiations whereas HPV positive cells were found to have returned to the pre-treatment CSC density after 10 days [142]. *In vitro* evidence of CSC densities and treatment responsiveness however, are typically limited in power due to low cell line numbers and tissue samples. These studies nevertheless, are capable of identifying intrinsic cellular responses that may contribute to the treatment outcomes observed.

Future directions

Development of a model profiling the radiosensitivity of individual tumours, with the aim of personalising treatment has been reported [143]. The genomic-adjusted radiation dose (GARD) model is designed to predict tumour response to radiotherapy, allocating scores of 0 to 100, the higher score predicting greater radiotherapeutic effect. Genomic data were derived from 8271 tissue samples from the Total Cancer Care (TCC) cohort for 20 different cancer types. Expression values of 10 radiosensitivity index genes were ranked to determine an individual genomic radiosensitivity index. GARD scores were then calculated from the radiosensitivity index, the standard schedule of dose and fractionation for cancer type, and the linear quadratic model of cell response to irradiation [143]. This model of scoring tumour response to radiotherapy was found to be an independent predictor of response to treatment for glioblastoma, breast, lung and pancreatic cancers. For HNSCC, higher scores for radiosensitivity were reported in OPSCC than non-OPSCC with median GARD scores of 39.71 and 32.56 respectively. This is consistent with the greater incidence of HPV positive tumours in the oropharyngeal anatomy. Ranges of GARD scores for cancer types are indicative of the heterogeneity of radiotherapeutic benefit observed clinically. OPSCC patients demonstrated less inter-tumoural heterogeneity in radiosensitivity

where GARD scores ranged from around 26 to 55. Non-OPSCC ranged from 14 to 72, being a much wider scope of response to treatment [143]. This model however, does not account for contributors such as hypoxia or the proliferative influence of CSCs, but given the predictive value in clinical outcomes of this model, offers alternative evidence to support patient stratification [144].

Changes in the presentation and treatment responsiveness of tumours and their biomarker expression have been identified as an under-researched area [127]. Adaptive radiotherapy is a strategy that seeks to modulate treatment in response to changing parameters during the course of treatment. A planning study using adaptive radiotherapy, was guided by hypoxic imaging at pre-treatment, after 7 and 17 fractions, and used to target hypoxic sub-volumes in HNSCC tumours with dose escalation or dose painting [145]. The extra dose to tumours in the study was found to be achieved without substantial dose increases to normal tissue.

In vitro work investigating responsive changes in CSC density to 4 Gy irradiation of HNSCC cell lines has used the development of "generational" HNSCC cell cultures. These are cells that have survived re-irradiation at fractionated dose level and are re-investigated for CSC populations [142]. Future studies with "generational" cell line cultures are planned to investigate changes in the radiosensitivity of clonogens with subsequent irradiation and any differences in those responses observed between HPV statuses.

Targeting the microenvironment of CSCs, as a strategy to overcome heterogeneity between tumours and de-differentiation within tumours, has been proposed [146]. CSCs are dependent on the microenvironmental support of niche cells such as cancer associated fibroblasts (CAFs). Blocking of signalling pathways between CSCs and CAFs is proposed to induce differentiation among CSCs or even apoptosis.

Conclusions

The potential of HPV status and CSCs as biomarkers is yet to be realised in terms of individualising treatment. The evidence reviewed, points to the elimination of CSC being vital to achieve tumour control and prevention of recurrence. The complexity of CSC heterogeneity, plasticity and response to treatment is an obstacle to the realisation of their potential promise as a biomarker in treatment planning. This difficulty notwithstanding, determining the radiosensitivity and responsiveness of a tumour's CSCs will provide vital parameters to individualise treatment for tumour control. Efforts to characterise the radiosensitivity of a tumour's CSC by its biomarker profile may to translate the prognostic values of associated indicators such as, HPV, hypoxia, tumour volume, to predictive measures of treatment outcome. This would inform patient stratification and in terms of HPV status and possible treatment escalation or de-escalation. Furthering the predictive values of biomarkers in HNSCC will also inform the strategic use of mixed modalities to target CSC sensitivity with least effect on normal tissue. Bringing together the chemo-radio responsiveness of CSCs as a function of their HPV status, along with other significant contributors to prognosis, may develop biomarker profiles that offer predictive outcomes towards individual treatment planning in HNSCC.

Conflicts of interest

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