Review Article
Biomarkers of head and neck cancer stem cells and targeted therapeutic strategies

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Abstract: Increasing evidence indicates that the spread and growth of cancers are driven by a small subpopulation of cells-"cancer stem cells" (CSCs)-capable of self-renewal and then generating a heterogeneous, diverse tumor cell population. So far, researchers have identified and isolated CSCs from various human cancers including head and neck squamous cell carcinoma (HNSCC); however, challenges exist in the identification and isolation of these CSCs. Many putative markers have been identified, but some controversial results have also resulted, and to date, no consensus exists on which marker(s) might provide the best specificity for HNSCC CSCs. Further understanding of their unique phenotype may help in discovering potential molecular targets and improving therapeutic outcomes.

Introduction

Head and neck cancer, the sixth most common cancer worldwide, causes the deaths of more than 200,000 people annually [1], and HNSCC comprises 90% of all head and neck cancers. In the past three decades, the use of innovative surgical techniques aimed at the preservation of organ function in the treatment of head and neck cancers, especially laryngeal tumors, has improved the quality of life of patients [2]. However, HNSCC remains a major cause of morbidity and mortality, and 5-year survival rates for HNSCC have not improved much in more than 30 years [3]. Thus, to provide better treatment options and improve patient outcomes, clarifying the mechanisms that drive the disease process is important.

Over the last 15 years, advances in tumor biology have resulted in the discovery that many cancers, including HNSCC, appear to be supported by cells with stem-like properties, and studies in a wide variety of malignancies have demonstrated such a distinct subpopulation of tumor cells called "cancer stem cells". In this review, we summarize current knowledge on the biomarkers of CSCs in HNSCC and describe the target therapies against these molecules and future directions in this field.

Theory of cancer stem cells

Recently the CSCs theory of tumorigenesis has become popular because of identification of a rare subpopulation of cells with the ability for self-renewal, regeneration of a heterogeneous cancer cell population, and the ability to initiate cancers in vivo. CSCs represent in itself one of the most topical in the field of cancer research. The American Association for Cancer Research Workshop on Cancer Stem Cells defined CSCs as cells within a tumor that are capable of self-renewal and generating heterogeneous lineages of cancer cells comprising the tumor bulk [4]. Chen et al. reported that CSCs were characterized by (1) differentiation, which gives rise to heterogeneous cancer cells; (2) self-renewal, which maintains a stem cell pool for expansion; and (3) homeostatic control, which ensures regulation between differentiation and self-renewal according to environmental stimuli and genetic constraints of each organ tissue, which account for the tissue specificity of CSCs [5].
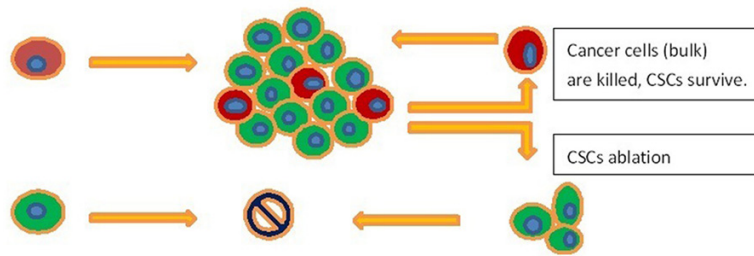
Figure 1. CSC Theory. Only CSCs are able to give rise to new tumors (red: CSC, green: differentiated cell population). Cancer cells (bulk) are killed, but CSCs survive, CSCs will give rise to new tumors, so the tumors recur. When the CSCs are targeted, the CSCs are ablated, the differentiated cells survive, but differentiated cells cannot give rise to new tumors, so the tumors regress.

The CSC theory indicates that this subpopulation of cells is of responsibility for tumors growth and spread, while non-CSCs have limited capacity for regeneration of progeny [6]. CSCs has been identified in some solid tumors, including head and neck cancer, and it shows certain characteristics that it has the capacity to maintain the tumor population, metastasize, and be resistant to chemoradiotherapy [2]. Further studies are needed generally to demonstrate the role(s) of CSCs in cancer (**Figure 1**).

Biomarkers of cancer stem cells in HNSCC and the role of therapeutic targets

Because CSCs are responsible for the maintenance of all tumor cells, a treatment targeting CSCs would seem to be the most effective way to eradicate a tumor. However, challenges persist in the identification and isolation of these CSCs. Although many putative markers have been identified, to date, no consensus exists on which marker(s) might provide the best specificity for HNSCC CSCs. Here, we summarize the biomarkers of CSCs in HNSCC and describe the target therapies against these molecules.

CD44

CD44 is a type I transmembrane glycoprotein that functions as a receptor for hyaluronic acid. The interaction between hyaluronic acid and CD44 influences adhesion to extracellular matrix, and it is involved in the stimulation of aggregation, cell proliferation, migration, and angiogenesis [7]. CD44 includes "standard" and "variant" isoforms, depending on the exons expressed. The former is expressed in mesen-

chymal cells and hematopoietic cells and the latter in epithelial cells.

The study of CD44+ tumor expression and its role as a marker of cancer stem cells in head and neck is interesting. Its correlation with the outcome and prognosis in HNSCC seems to be a good attempt in this direction and the results obtained are promising (**Table 1**). Prince et al. first identified a subset of cells in head and neck tumors expressing the surface biomarker CD44 with stem-like characteristics in 2007, and these cells were able to regeneration of progeny when implanted into immunosuppressed mice [8]. Research has indicated that CD44 may be a CSC marker in some cases of HNSCC [8-10]. Some studies have shown that CD44+ cells had a stronger proliferative capacity [11]; moreover they were related to metastasis [12] and correlated with poor prognostic factors such as advanced T classification and recurrence [13, 14]. Atsushi Okamoto found that HNSCC-CD44+ cells showed a high expression levels of chemoresistance genes, including ATP-binding cassette, subfamily G, member 2(ABCG2), ABCB1, CYP2C8, and TERT, and indicated that CD44+ cells were more resistant to chemotherapeutic agents compared to CD44‾ cells [15]. Some authors found that the high expression of CD44 was correlated with a greater tendency for locoregional or distant metastasis and resistance to radiochemotherapy [16-18]. Shi et al. knocked down CD44 expression with small interfering RNA (siRNA) in a nasopharyngeal carcinoma cell line, with results indicating that tumor growth was inhibited *in vivo* and *in vitro* [19]. According to the results described, CD44, an important molecule in maintaining stem cell properties and therapies targeting CD44, or its relevant signaling pathways, may allow the development of new treatment strategies.

The literature about CD44 expression patterns and its role as a CSC marker in HNSCC is unclear, there are conflicting findings about the clinical significance of CD44 expression. The overexpression of CD44 showed a statistically significant association with decreased 5-year survival rates in oropharynx, hypopharynx, and

Table 1. Studies performed about the role of CD44 as a biomarker of HNSCC CSCs

Study	Subsite	Examined markers	Main results
Prince et al. [8]	Head and neck cancer	CD44	CD44+ cells were able to regeneration of progeny when implanted into nude mice.
Perez et al. [9], Han et al. [10]	HNSCC	CD44	CD44 may be a CSC marker.
Shen et al. [11]	Hypopharynx cancer	CD44	CD44+ cells had a stronger proliferative capacity.
Pries et al. [12]	Head and neck cancer	CD44	CD44+ cells were related to metastasis.
Joshua et al. [13] and Kokko et al. [14]	HNSCC	CD44	CD44+ cells correlated with poor prognostic factors. Overexpression of CD44 are associated with decreased 5 year survival rates in oropharynx, hypopharynx, and larynx squamous cancers, but not in oral cavity squamous cancers.
Okamoto et al. [15]	HNSCC	CD44	CD44+ cells showed a high expression of chemoresistance genes and were more resistant to chemotherapeutic agents.
Yuce et al. [16] Uwa et al. [17] Jong et al. [18]	Hypopharynx cancer, Larynx cancer.	CD44	Overexpression of CD44 was correlated with a greater tendency for locoregional or distant metastasis, resistance to radiochemotherapy.
Shi et al. [19]	Nasopharyngeal carcinoma cell line	CD44	Tumor growth was inhibited *in vivo* and *in vitro* after knocking down CD44 expression by siRNA.
Stoll et al. [20]	Oral squamous cell cancers	CD44	No relationship existed between CD44 expression and T or N stage, decreased CD44 expression was a independent predictor for shorter survival time and shorter recurrence free interval.
Mack et al. [21]	Head and neck normal tissues and carcinomas	CD44	CD44 was consistently highly expressed in all head and neck tissues.
Oh et al. [22]	HNSCC	CD44	CD44- cells are resistant to cisplatin and show increased levels of ABCG2, upregulation of SOX2, OCT4 and nestin.As few as 1,000 CD44- cells were able to give rise to cancers in nude mice.
Wang et al. [23]	HNSCC	CD44v3	CD44v3 was associated with tumor growth, clonal formation, migration, lymph node metastasis, and matrix metalloproteinase activity.
Bourguignon et al. [24]	HNSCC	CD44v3 and CD44v6	CD44v3 and CD44v6 seemed to correlate with lymph-node metastasis, systemic diffusion, and failure of radiotherapy.
Wang et al. [25]	HNSCC	CD44v3, CD44v6, CD44v10	CD44v3, CD44v6 and CD44v10 were expressed in a greater proportion of metastatic lymph nodes. CD44 variant isoforms were associated with T stage (v3 and v6), radiation failure (v10), regional (v3) and distant (v10) metastasis, perineural invasion (v6), and shorter disease-free survival (v6 and v10).
Spiegelberg et al. [26]	HNSCC	CD44v4	CD44v4+ subpopulation displayed increased proliferation and radioresistance
Kunishi et al. [27]	Oral squamous cancer cells	CD44v6	CD44v6 was downregulated in OSCC, cancers with lower levels of CD44v6 showed more frequent regional lymph node metastasis.
Gonzalez-Moles et al. [28]	Tongue cancer	CD44s	Low expression of CD44s was related to tumor cell invasiveness and may be a prognostic factor.

larynx squamous cancers, but not in oral cavity squamous cancers [14]. Also, Stoll et al. indicated that no relationship existed between CD44 expression and T or N stage, and that decreased CD44 expression was a statistically significant independent predictor for shorter survival time and shorter recurrence free interval in oral squamous cell cancers [20], while Mack et al. found that CD44 was consistently highly expressed in all head and neck tissues (from normal tissues to carcinomas) [21]. Moreover, Oh et al. found CD44- cells to be chemoresistant to cisplatin and to show increased levels of ABCG2, upregulation of sex-determining region Y box2 (SOX2), and octamer-binding transcription factor 4 (OCT4) and nestin, and as few as 1,000 CD44- cells were able to give rise to cancers in nude mice [22]. These variable results are likely due to the different experimental methods and the use of different antibodies by different investigators.

Wang et al. found CD44v3 was associated with HNSCC tumor growth, clonal formation, migration, and matrix metalloproteinase activity, and CD44v3 was identified in lymph node metastasis [23]. Bourguignon indicated that CD44v3 and CD44v6 seemed to correlate with lymph-node metastasis, systemic diffusion, and failure of radiotherapy [24]. Steven J. Wang et al. [25] indicated that CD44v3, CD44v6 and CD44v10 were expressed in a greater proportion of metastatic lymph nodes when compared with primary tumors, however, expression of CD44s was not significantly different in metastatic lymph nodes and primary tumors, mean-

while they also found that expression of CD44 variant isoforms were associated with T stage (v3 and v6), radiation failure (v10), regional (v3) and distant (v10) metastasis, perineural invasion (v6), and shorter disease-free survival (v6 and v10). Thus, CD44v3 isoforms may be tumor markers and therapeutic targets in HNSCC. A CD44v4+ subpopulation displayed increased proliferation and radioresistance [26]. Whereas Mack found that CD44v6 expression did not differentiate normal and benign from malignant tissues of the head and neck; CD44s and CD44v6 were present abundantly in most cells of the head and neck, including malignant tissues [21]. Kunishi demonstrated that the expression of CD44v6 was obviously downregulated in oral squamous cancer cells (OSCCs), but not in normal oral mucosa; cancers expressing lower levels of CD44v6 showed more frequent regional lymph node metastasis [27]. Some authors concluded that the low expression of CD44s was related to tumor cell invasiveness and may be a prognostic factor in tongue cancer [28]. However, note that these results are based on a limited selection of cancer cell lines. The value of CD44, CD44v3, CD44v4, and CD44v6 as markers for the identification of HNSCC CSCs needs further investigation.

The anti-CD44v6 antibody 'U36' has been studied widely in patients with primary HNSCC and lymph node metastasis [29]. Tijink et al. killed CD44v6+ cells with bivatuzumab, a humanized monoclonal antibody directed against CD44v6, and their study showed some effects in the stabilization of advanced HNSCC; the main toxicity of bivatuzumab mertansine was skin reactions, but most of these were reversible [30]. The therapeutic approaches targeting CD44 need to be further studied.

CD133

CD133 is a transmembrane glycoprotein characterized by its tendency to localize to cellular protrusions, and it is a protein usually expressed in hematopoietic stem cells and normal tissue stem cells. CD133 is one of the markers of HNSCC CSCs. Some researchers discovered that CD133+ cancer stem-like cells in HNSCC possessed higher clonogenicity, increased invasiveness and tumorigenicity *in vivo* and *in vitro* compared to CD133- cells, and were associated with poor cancer prognosis in patients

with HNSCC[31, 32]. Canis et al. first reported a gradual relationship between increased numbers of CD133+ cells and decreased overall survival (OS) [33]. Zhang et al. found that after treatment with paclitaxel, xenograft tumors showed threefold enrichment of a CD133+ subpopulation versus untreated tumors [31], which suggests that the subpopulation of CD133+ cells in head and neck cancer exhibited increased resistance to chemotherapeutic agents compared to its CD133- counterpart, and that CD133+ OSCC cells expressed higher mRNA levels of hTERT, Oct-4, Nanog, and ABCG-2 genes, showing higher protein levels of hTERT, Oct-4, and β-catenin than CD133- counterparts. Chiou et al. concluded that patients who were Oct-4, Nanog, and CD133 triple-positive in oral cancer had the worst survival prognosis [34].

However, a study also reported that although CD133- cells had poor reproductive activity in the Hep-2 cell line, they did not completely lose the ability to reproduce. CSCs also existed within CD133- cells. These findings indicate that CD133 is not an exclusive marker of Hep-2 cell line stem cells. Co-expression of other particular molecular markers may exist [35]. Showed that CD133 negative glioma cells were tumorigenic in nude rats, and CD133 positive cells could be obtained from these tumors. CD133 expression is upregulated when passaging of the tumors in vivo. So they suggested that CD133 expression was not necessary to initiate brain tumor, but it might be involved during brain tumor progression [36].

Recently, an evaluation of anti-CD133+ CSC subpopulation therapy in HNSCC has yielded promising results, which could be useful as a guide for the development of future clinical antitumor therapies. Waldron's study showed that dCD133KDEL, a novel anticancer agent, could inhibit cell proliferation and tumor initiation effectively, and eliminate xenotransplant tumors impressively by targeting the CD133 subpopulation; this agent shows significant promise for the development of a useful antitumor therapy [37]. Additionally, another group found that after cytolethal distending toxin (Cdt) was conjugated to an antihuman CD133 monoclonal antibody (mAb); the Cdt-mAb complex preferentially inhibited the proliferation of CD133+ cells in HNSCC cell lines, but did not affect normal primary gingival epithelial cells

[38]. In a previous study, we demonstrated that CD133^{+} cells showed higher proliferation [39], Glut-1 (glucose transporter-1) mRNA and protein levels in CD133^{+} cells were higher than those in CD133^{−} cells. Our results suggested that Glut-1 was important in the energy supply of laryngeal CD133^{+} Hep-2 cells and may represent a potential therapeutic target for the inhibition of laryngeal CSC proliferation.

Aldehyde dehydrogenase

Aldehyde dehydrogenase (ALDH) is a cytosolic enzyme responsible for catalyzing the pyridine nucleotide-dependent oxidation of aldehydes to carboxylic acids. To date, 17 different isoforms of ALDH have been identified in a variety of human tissues, including liver, kidney, erythrocytes, skeletal muscle, lung, breast, brain, prostate, pancreas, and others [40]. ALDH has been shown to be an HNSCC stem cell marker. ALDH^{+} cells represented 1-7.8% of tumor cells, and as few as 500 cells could initiate xenograft tumors [41].

ALDH1 is a member of the aldehyde dehydrogenase family. Recent studies have shown that ALDH1 is a specific marker for the identification of head and neck CSCs and plays an important role in maintaining the self-renewal and tumorigenic properties in HNSCC CSCs [42]. Also, ALDH1 has prognostic value for HNSCC survival [43], and the ALDH1 enzyme had been identified as a factor responsible for the chemoresistance of progenitor cells [44], Moreover, studies have demonstrated the role of ALDH1^{+} cells in metastasis in HNSCC [42]. Studying 226 patients with HNSCC, Yu-Chih Chen showed that increased expression of ALDH1 was positively related with cancer staging in the patients and HNSCC-ALDH1^{+}-lineage cells were demonstrated to be involved in tumor invasiveness and to exhibit refractory properties in radiotherapy [42]. A multivariate analysis revealed that expression of ALDH1 resulted in a 4.2-fold increased risk of oral leukoplakia transformation [45].

ALDH1A1 as an ALDH1 family member is a cytosolic ALDH isoform expressed in human cells, which is important in regulating epithelial cell growth and differentiation. To date, ALDH1A1 has been regarded as a specific marker of HNSCC CSCs that plays an important role in maintaining CSC properties [46]. Some studies have indicated that in patients with HNSCC or OSCC, the higher the expression of ALDH1A1, the shorter the OS. Also, ALDH1A1 has been shown to be an independent prognostic factor for survival in patients with HNSCC or OSCC [47]. However, others have questioned whether total ALDH1A1 expression is a biomarker of HNSCC stem cells because its percentage was high (>25%) in most HNSCC tissues, especially in metastasis tissues [48].

Visus reported that ALDH1A1 was an essential source for developing ALDH1A1-based vaccination [49]. ALDH1A1-specific cytotoxic T cells could eliminate bright ALDH cells present in HLA-A2+ HNSCC in vitro [50]. These findings explained ALDH1A1 to be a potentially valuable marker and promising target for therapy.

CD98

Amino-acid transporters are crucial for the cancer cells survival and growth, and have an important role in the development and invasiveness of cancer cells. L-type amino acid transporter 1 (LAT1) transports large neutral amino acids, such as leucine, valine, tyrosine, phenylalanine, methionine, and requires a covalent association with the heavy chain of 4F2 cell surface antigen (CD98) for its functional expression in the plasma membrane [51, 52]. The expression of LAT1 is closely associated with cell proliferation, angiogenesis, and also with the expression of CD98, cooperative overexpression of LAT1 and CD98 is essential for the progression and metastasis of human neoplasms [53]. So CD98 is a crucial factor for cancers. Martens-de Kemp et al. demonstrated that CD98 (high) cells, in contrast to CD98 (low) cells, had the ability to generate tumors in immunodeficient mice, indicating that CD98 (high) cells had stem cell characteristics. Moreover, the CD98 (high) fraction indicated high levels of cell cycle control and DNA repair genes, while the CD98 (low) subpopulation had expression patterns that corresponded with the more differentiated cells in the bulk of the tumor. Thus, they concluded that CD98 was a promising CSC marker in HNSCC [54]. Rietbergen et al. [55] found that because CD98 expression was restricted to cells in the basal layer and was expressed in a more restricted cell population than CD44, CD98 was more distinctive than CD44. Furthermore, in patients

with HPV+ oropharyngeal cancer, OS and progression-free survival (PFS) were significantly worse for patients with a higher percentage of CD98+ tumor cells.

Glucose-regulated protein 78

Recently, glucose-regulated protein 78 (GRP78) was used to identify CSCs from a HNSCC cell line, and GRP78 was found to be overexpressed in several cancers, including HNSCC; moreover, co-expression of the stem cell marker Nanog with GRP78 was associated with decreased survival of patients with HNSCC. In HNSCC, GRP78 was required for tumorigenicity, invasion, and metastasis. Specifically, knockdown of GRP78 reduced self-renewal and tumorigenicity in nude mice, suggesting that GRP78 was not merely a marker for HNSCC CSCs but also involved in their stemness [56]. GRP78 may be a potential therapeutic target.

CD200

CD200 is a highly conserved type 1 transmembrane glycoprotein with two extracellular immunoglobulin superfamily domains, a single transmembrane domain, and a short cytoplasmic tail with no defined function [57]. Significant overexpression of CD200 in various human cancer tissues have been reported, including head and neck carcinoma, and CD200 was found to be a potential therapeutic target and prognostic factor in cancers [58]. Jung et al. showed that CD200 was diversely expressed in wild-type HNSCC lines and correlated with the expression of sonic hedgehog and B-cell-specific Moloney murine leukemia virus integration site 1 (Bmi-1), and that it modulated the response to chemotherapy and radiotherapy *in vivo,* High CD200 expression significantly decreased the survival rate compared with the control, suggesting that targeting CD200 together with conventional treatment may be a potential approach to improve treatment outcomes [59]. Further studies on the correlation between these molecules and mechanisms and CD200's oncological implications are needed.

Bmi-1

Bmi-1 is a member of the polycomb family of transcriptional repressors, it influenced the cell cycle and the self-renewal of tissue stem cells by regulating the chromatin and histone structures, it impacts the tumor suppressors p53 and Rb by suppressing the INK4a locus, which encodes the tumor suppressors p16 and p14ARF [60, 61]. Bmi-1 was considered as a stem cell related protein, and was implicated in the tumorigenesis of head and neck cancers [8, 62]. Some studies have demonstrated that the mRNA and protein levels of Bmi-1 increased in HNSCC CSCs that possess the property of self-renewal and the ability to form tumors [8, 63, 64]. Allegra et al. [65] found that in laryngeal SCC the expression of Bmi-1 seemed to be a potential marker of more aggressive behavior of the tumors, also in the absence of P16 expression, Bmi-1 expression seemed to identify a subset of patients who were at higher risk of lymph node metastasis. In Allegra's another study, the results appeared that nuclear expression of Bmi-1 in laryngeal carcinoma played a significant role in the lymph node-metastasizing capacity, whereas cytoplasmic expression of Bmi-1 played a role in the ability to metastasize to distant sites, so a high expression of Bmi-1 may indicate lymph node metastasis at diagnosis and play a important role in a subset of patients to decide on neck treatment [66].

To explore the function of Bmi-1, some studies found that knocking down Bmi-1 expression, silencing Bmi-1, could significantly enhance the sensitivity of HNSCC-ALDH1+ cells to chemotherapy or radiotherapy and increase the level of chemoradiation-mediated apoptosis, improve the effectiveness of radiotherapy, and cause the inhibition of tumor growth in xenograft tumors of HNSCC-ALDH1+ cells [63, 67]. Moreover, in xenograft tumors, the overexpression of Bmi-1 in HNSCC-ALDH1− cells increased tumor volume and the number of pulmonary metastatic lesions; knocking down Bmi-1 in HNSCC-ALDH1+ cells decreased metastases to the lungs significantly [67].

Further examples of the mechanisms by which factors regulate Bmi-1 expression in cancer cells and a better understanding of how Bmi-1 mediates radioresistance or chemoresistance in HNSCC are needed. Targeting Bmi-1 in HNSCC cells may be a promising anticancer therapy.

Alternative therapeutic approach

In addition to CSC biomarkers, microenvironmental factors, such as niche-specific proper-

ties, also represent potential therapeutic targets to allow the eradication of HNSCC cells.

A niche is a microenvironment that supports CSC survival and growth, and niches may also represent potential therapeutic targets. This is an area that need further research in HNSCC [68]. Recent evidence that HNSCC CSCs reside in perivascular niches represents a potential target, and that therapeutic strategies exploiting the interdependence of CSCs and vascular endothelial cells may decrease the rate of HNSCC recurrence and distant metastasis [69]. Krishnamurthy observed that selective resection of tumor cell-related blood vessels was sufficient to decrease the proportion of head and neck CSCs within 4 days, while no change in tumor volume was observed in the same time period [64]. Selective ablation of tumor cell-associated blood vessels using a caspase-based artificial death switch reduced the proportion of HNSCC CSCs *in vivo*, demonstrating that endothelial cells initiate signals that enhance HNSCC CSC self-renewal and survival [70]. Bevacizumab, as an antiangiogenic drug, mediated CSC depletion in gliomas [70] and proved to be useful in reducing the proportion of HNSCC CSCs [69]. The data demonstrated that therapeutic targeting of the tumor endothelium could reduce the rate of head and neck tumor relapse and metastasis through decreasing the proportion of CSCs [64] and could weaken the source of nutrition and change the crucial signals needed by CSCs to proliferate [71].

CSCs possess transmembrane transporters which can transport the chemotherapy agents out of the cell and prevent the therapeutic action to resist chemotherapy, and they have developed repair mechanisms for oxidative stress, inducing radioresistance [72]. Gaining better insight into the mechanisms of CSC resistance to chemotherapy may lead to new therapeutic targets and better anticancer approaches. ABCG2 is a member of the ATP-binding cassette (ABC) transporter family, which was first cloned from doxorubicin-resistant human MCF-7 breast cancer cells and was then named the "breast cancer resistance protein" [73]. ABC transporters might represent important markers defining CSCs [74]. ABCG2 was overexpressed in many cell lines and tumor types, where it pumped a variety of endogenous and exogenous compounds out of the cells [75], and ABCG2 was found to be associated with CSCs and multidrug resistance [76, 77]. Shen et al. found a significant relationship between ABCG2 expression and clinical stage and lymph node metastasis; positive expression of ABCG2 may accelerate progression and induce metastasis in HNSCC, moreover, no significant correlation, only an increasing trend, between ABCG2 expression and histological grade was discovered in the tumor tissues of laryngeal and nasopharyngeal cancers. These results revealed that the chemosensitivity of HNSCC cells was a multifactorial phenomenon, influenced not only by ABCG2, but also by other ABC transporters [78].

Currently, therapeutic strategies targeting CSCs have been developed, and more strategies have been shown to be effective in experiments and clinical trials. Beyond the therapies and targets mentioned above, other potential targets still exist.

MicroRNA (miRNA) is a class of highly conserved small RNA molecules that can act both as oncogenes and as tumor suppressors [79]. miRNAs are involved in the regulation of cellular processes, such as cell differentiation, proliferation, and apoptosis in OSCC [80]. Lo et al. found that microRNA-200c (miR200c) reduced the abilities of self-renewal, tumorigenicity, and invasion in HNSCC CSCs, and further facilitated the differentiation of ALDH$^+$/CD44$^+$ cells into ALDH$^-$/CD44$^-$ cells, overexpression of miR200c in ALDH1$^+$/CD44$^+$ cells significantly suppressed tumorigenicity, radioresistance, chemoresistance, and metastatic properties of HNSCC CSCs *in vivo*. Moreover, miR200c could suppress lung metastasis in ALDH1$^+$/CD44$^+$ cell xenograft tumors. Thus, miR200c may be a potential tumor suppressor in HNSCC [81]. Restoration of miR200c in HNSCC CSCs may be a promising therapeutic strategy.

"Signal transducer and activator of transcription 3" (STAT3) had been suggested to be a prognostic indicator for tumor growth and progression [82]. Recent reports had suggested that inhibition of STAT3 in cancer cell lines could significantly increase radiosensitivity and radiation-induced apoptosis [83]. Chen et al. demonstrated that cucurbitacin I, a selective inhibitor of the JAK/STAT3 signaling pathway, was a potential antitumor agent in HNSCC CSCs *in vivo* and *in vitro.* They found that with increasing concentrations of cucurbitacin I, the

viability of HNSCC-CD44+ ALDH1+ cells decreased significantly, and that colony formation ability was blocked. Cucurbitacin I promoted differentiation and resulted in apoptosis; the numbers of ALDH1 and CD44 cells decreased markedly. Moreover, cucurbitacin I improved radiosensitivity and showed synergistic effects with ionizing radiation on tumorigenicity inhibition and metastatic ability suppression (suppressing metastasis to the lung) in HNSCC-ALDH1+ transplanted immunodeficient mice [43]. However, whether STAT3 plays a definite role in maintaining self-renewal and radioresistance in HNSCC CSCs is still an open question.

Grandis et al. demonstrated that epidermal growth factor receptor (EGFR) played a key role in the function, survival, and maintenance of CSCs, and that overexpression of EGFR was an independent prognostic factor for local control and survival in patients with HNSCC [84]. Thus, agents that target EGFR administered in combination with "conventional" chemotherapy may be an efficient treatment for HNSCC. Abhold et al. [85] demonstrated that EGF-treated cells showed 1.5- to 4-fold increases in the expression of Bmi-1, CD44, Oct-4, Nanog, CXCR4, and SDF-1 mRNAs. In contrast, cells treated with gefitinib, a small molecule EGFR inhibitor, showed 2- to 5-fold decreases in the same genes. Additionally, the study showed that gefitinib may be an effective agent to promote drug sensitization and inhibit invasion when combined with conventional chemotherapy. Their observations suggested that EGFR inhibition may be an effective approach to target the CSC subpopulation in HNSCC. Additionally, these findings demonstrated that EGFR in HNSCC was not only a promoter of cell growth, but also could regulate the key properties of CSCs that were essential for cancer initiation and progression.

UCN-01, a checkpoint kinase inhibitor, and all-trans retinoic acid (ATRA) as two pharmacological approaches were administered separately or in combination and permitted us to demonstrate that pharmacological adjuvant treatments targeting the inhibition of survival and self-renewal pathways or the triggering of apoptosis strongly sensitized CSCs exposed to photon or carbon ion radiation therapy [86].

Histone deacetylase inhibitors (HDACis) have multiple biologic effects as a consequence of alterations in the patterns of acetylation of histones and are a promising potential group of anticancer agents. Chikamatsu et al. investigated the effects of two HDACis, suberoylanilide hydroxamic acid (SAHA) and trichostatin A (TSA), in two CD44+ HNSCC CSC lines and found that the HDACis resulted in cell cycle arrest and apoptosis in these cell lines. Expression of the cancer stem cell markers CD44 and ABCG2 on these cell lines was diminished by treatment with HDACis. Additionally, HDACis decreased mRNA expression levels of stemness-associated genes and suppressed the epithelial-mesenchymal transition (EMT) phenotype of CSCs. As expected, the combination of HDACis and chemotherapeutic agents, including cisplatin and docetaxel, had a synergistic effect on head and neck cancer cell lines. Thus, they concluded that HDACis may have therapeutic value against CSCs of head and neck cancers [87]. To date, several HDACis have been exploited and used in clinical trials for cancer treatments. HDACis were shown to have the ability to cause growth arrest, induce differentiation, and cause apoptosis in tumor cells [88, 89].

To overcome the treatment resistance of CSCs, various strategies such as molecularly targeted drugs, immunotherapy, and gene therapy are being developed. Further understanding of epigenetic mechanisms as well as interaction among epigenetic factors may provide new insights in developing new therapeutic strategies.

Conclusions

CSCs play important roles in tumorigenesis, metastasis, and recurrence of HNSCC. Identification and a better understanding of reliable molecular markers are needed to characterize CSCs in HNSCC. Targeting CSC-specific markers and molecular pathways may help in developing novel CSC diagnostics and therapeutic approaches.

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Disclosure of conflict of interest

None.

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