Cancer Stem Cells in Head and Neck Squamous Cell Carcinoma- Treatment Modalities

SUMMARY

Head and neck squamous cell carcinoma (HNSCC) belongs to the most frequent cancer subtypes in the world. Mutations due to genetic and chromosomal instability, syndromes such as Fanconi anemia and the Bloom syndrome, environmental risk factors such as tobacco smoking, alcohol and human papillomavirus infection (HPV) subtypes 16,18,31,33,35,52,58 are implicated in its pathogenesis. The HNSCC belongs to the solid tumors of epithelial origin and consists of stromal, inflammatory, cancer cells and most importantly a fraction of them, the cancer stem cells (CSCs). The identification of the CSCs through their biomarkers such as CD44, CD10, CD166, CD133, CD271, ALDH, Oct4, Sox2 and Bmi1, the maintenance of their subpopulation through epithelial to mesenchymal transition, the role of HPV infection regarding their prognosis and of their microenvironment regarding their resistance to therapy, all constitute key elements that must be taken thoroughly into consideration in order to develop an effective targeted therapy. There are already therapies in place targeting specific related biomarkers, important biochemical pathways and growth factors. The aim of this literature review is to illustrate the treatment modalities available against the cancer stem cells of head and neck squamous cell carcinoma.

Key Words: Cancer Stem Cells, Oral Cancer, Oral Cancer Stem Cells, Head and Neck Cancer, HPV and Cancer, Cancer Stem Cells Treatment

Introduction

The prevalence of head and neck squamous cell carcinoma (HNSCC) classifies it as the 5th more frequent cancer in the world, most probably due to its inherent invasiveness and proximity to structures of critical importance, with an overall incidence of over 300,000-600,000 cases per year1-8. It consists an epithelial tumor, affecting the oral cavity, pharynx and larynx. The pathogenesis involves both environmental factors and mutations due to genetic instability. The environmental risk factors include tobacco smoking, alcohol and human papillomavirus infection (HPV) subtypes 16,18,31,33,35,52,582,3,4,6,9. The genetic risk factors include chromosomal instability and syndromes such as Fanconi anemia and the Bloom syndrome4.

The HNSCCs are solid tumors with heterogeneous content, including cancer cells, stromal cells, and inflammatory cells2,3. The cancer cells originate from stem cells (characterized by self-renewal ability), from progenitor cells (which are abundant in adult tissue and have partial ability to self-renew) and from de-differentiated cells (they de-differentiate in order to convert to stem cells, this process is mediated by the phenomenon of Epithelial–Mesenchymal Transition (EMT))10. As a result, there are only 2 possible models for further cancer development: either random oncogenic mutations leading to the formation of neoplasia through cancer cells2,11 or a distinct subpopulation of cancer stem cells (CSCs) on top of the cancer cell hierarchy (characterized by symmetrical and asymmetrical division following the pattern of normal stem cells) initiates them. The first model is the Stochastic whereas the second
one is the Hierarchical. In the case of HNSCC, research findings support the cancer stem cell theory. The key features of CSCs\textsuperscript{12} are the following:

1. Only a small fraction of the cancer cells within a tumor have tumorigenic potential when transplanted into immunodeficient mice.
2. The CSC subpopulation can be separated from the other cancer cells by distinct surface markers.
3. Tumors resulting from the CSCs contain the mixed tumorigenic and nontumorigenic cells of the original tumor.
4. The CSC subpopulation can be serially transplanted through multiple generations, indicating that it is a self-renewing population.

A pluripotent cell subpopulation featuring stemness, self-renewal and oncogenic properties, has the ability to generate, maintain and drive the whole volume of the heterogeneous tumor\textsuperscript{11,13}. On some level, tumor cells persist on normal differentiation, leading to disturbed maturation and heterogeneous histological sections. As a result, certain products are present in HNSCC creating the keratinization. Regarding the theory of “field cancerization” which is associated with cancer recurrences and development of secondary tumor, molecular alterations which appear to the cells of the mucous membrane adjacent to the tumor are driven by the presence of CSCs either in a polyclonal or monoclonal pattern\textsuperscript{14}. On that note, apart from molecular and genetic changes in normal mucosa, putative CSCs are also found in seemingly tumor free surgical margins of oral cancer\textsuperscript{15}.

The role of CSCs in oral squamous cell carcinoma (OSCC) is well established. They may originate from the local basal layer, vessel walls, blood, muscle tissue, adipose tissue, the cell fusion between hematopoietic stem cells and oral keratinocytes, and finally EMT de-differentiated cells\textsuperscript{10}.

Treatment modalities have consistently improved. Most patients receive primary radio chemotherapy as this appears to be the most effective treatment modality in relation with the overall survival rates and the control of loco-regional recurrences and distant metastasis. The prognosis remains poor. The 5-year overall survival (OS) rate is 50% \textsuperscript{6}, when regarding the frequency of cancer induced mortality HNCSS is the 7th most common malignancy\textsuperscript{5}. The low survival rate is attributed to late diagnosis\textsuperscript{16}, local-regional relapses (10–50% of the patients)\textsuperscript{5,6,17}, development of second primary tumor (2-30% of the patients)\textsuperscript{5}, lymph node and distant metastasis (25%)\textsuperscript{14,16,18} and radio- and chemoresistance\textsuperscript{14,16}.

Suboptimal control of loco- regional recurrences has been associated with multiple factors, including the volume of the tumor and hypoxia-induced alterations: A more voluminous tumor is considered to have a larger number of CSCs while hypoxia causes through multiple mechanisms a reduction in tumor radiosensitivity\textsuperscript{19}, enhancing the survival of malignant cells\textsuperscript{5}. Especially OSCC relies on hypoxia cellular response\textsuperscript{10}. This means that hypoxia leads not only to direct resistance to conventional treatment but also to cellular phenotypic alterations creating an ever more resilient CSCs’ subpopulation\textsuperscript{5}.

### Material and Methods

This review aims to investigate the role of HNSCC-CSCs regarding tumor pathogenesis as well as current and experimental treatment modalities. An electronic search of the literature was performed between July 2019 and February 2020 to identify articles investigating HNSCC, CSCs and treatment options. The literature search was conducted using MEDLINE (National Library of Medicine)-PubMed without restrictions concerning the date of publication. The following keywords were used (connecting different keywords with AND, OR): head and neck squamous cell carcinoma, oral squamous cell carcinoma, cancer stem cells and targeted therapies. This was followed by a manual search and references were used to identify relevant articles. The articles identified from the electronic and manual search were screened to eliminate those that failed to meet the respective inclusion and exclusion criteria as listed below.

Inclusion criteria for analysis of human and animal studies:

- Randomized controlled clinical trials, prospective and retrospective clinical studies, Case reports or case studies, Animal studies that evaluated the pathogenesis of HNSCCs, the HNSCSCs’ implication and relevant treatment regimes.

Exclusion criteria for analysis of human and animal studies:

- Studies in a language other than English or without an English abstract.

After this screening 41 studies remained for full text analysis.

### Results

Identification of CSCs’ subpopulations and clinical importance of the respective CSCs’ biomarkers. The identification of the subpopulations of CSCs is immensely important since it could lead to improved treatment modalities. Every CSC subpopulation expresses different markers in accordance with the tissue of origin. Flow cytometry/fluorescence-activated cell sorting (FACS) is the best technique so far for the identification and isolation of CSCs\textsuperscript{20} although MACS has proven itself as a different and in some experiments, more convenient
method for cell isolation\textsuperscript{21}. The most reliable marker so far is CD44 and its isoforms. CD44v3 is associated with advanced T stage and regional metastasis. CD44v6 is associated with advanced T stage, perineural invasion and shorter disease-free survival. CD44v10 is associated with distant metastasis, radiation failure and shorter disease-free survival\textsuperscript{22}. CD44v9 specifically enhances the defense mechanism against ROS. CD44 in general correlates with tumor-sphere formation and resistance to chemotherapeutics. The combination of CD44 with the marker CD24 proves to be even better at isolating CSCs\textsuperscript{20}. The CD10 marker enhances the stem cell proliferation and tumor-sphere formation. Its level increases after RT or CT treatment. Its expression associates with recurrence and metastases. The CD166 marker associates with larger tumors and localization at the tumor invasive front. The CD133 marker correlates with increased proliferation, EMT phenotype, tumor-sphere formation, self-renewal and in-vivo tumorigenicity. The combination of both positive CD133 and CD44 showed more malignant features compared to only CD133 or CD44 expression\textsuperscript{23}. The CD271 marker correlates with high tumorigenicity and localization at the tumor invasive front. The ALDH marker is involved in EMT, self-renewal, tumor formation, and resistance to chemotherapeutics. Its ALDH1A1 isoform correlates strongly with enhanced ALDH activity\textsuperscript{20}. The markers Oct4, Nanog and Sox2 are associated with EMT and metastasis. Finally, the Bmi1 marker is associated with HNSCC and especially the oral squamous cell carcinoma\textsuperscript{24} (Table 1).

### Table 1. Biomarkers of cancer stem cells (CSCs)

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Associations</th>
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<tbody>
<tr>
<td>CD44</td>
<td>tumor-sphere formation and resistance to chemotherapeutics</td>
</tr>
<tr>
<td>CD44v3</td>
<td>advanced T stage, regional metastasis</td>
</tr>
<tr>
<td>CD44v6</td>
<td>advanced T stage, perineural invasion, shorter disease-free survival</td>
</tr>
<tr>
<td>CD44v9</td>
<td>ROS defense mechanism</td>
</tr>
<tr>
<td>CD44v10</td>
<td>shorter disease-free survival, radiation failure, distant metastasis</td>
</tr>
<tr>
<td>CD10</td>
<td>stem cell proliferation and tumor-sphere formation</td>
</tr>
<tr>
<td>CD166</td>
<td>larger tumors and localization at the tumor invasive front</td>
</tr>
<tr>
<td>CD133</td>
<td>increased proliferation, EMT phenotype, tumor-sphere formation, self-renewal and in-vivo tumorigenicity</td>
</tr>
<tr>
<td>CD271</td>
<td>high tumorigenicity and localization at the tumor invasive front</td>
</tr>
<tr>
<td>ALDH</td>
<td>EMT, self-renewal abilities, tumor formation, and resistance to chemotherapeutics</td>
</tr>
<tr>
<td>Oct4</td>
<td>EMT and cancer metastasis</td>
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<tr>
<td>Nanog</td>
<td>EMT and cancer metastasis</td>
</tr>
<tr>
<td>Sox2</td>
<td>EMT and cancer metastasis</td>
</tr>
<tr>
<td>Bmi1</td>
<td>head and neck tumorigenesis</td>
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</table>

### Maintenance of CSCs’ subpopulation through EMT

Epithelial-to-mesenchymal transition (EMT), a phenomenon that takes place mostly during development, epithelial cells acquire a mesenchymal phenotype associated with tumor growth and metastasis due to enhanced motility and invasiveness. This invasive phenotype allows them to gain easier access to the lymphatic, venous and/or arterial circulation\textsuperscript{3}. Phenotypic plasticity seems to be a key component of EMT, as CSCs with this characteristic are considered more resistant to chemotherapeutic agents\textsuperscript{25}. Thus, the transformed epithelial cells obtain mesenchymal traits, enabling them to cross endothelial barriers and enter blood and lymphatic circulations, thus contributing to metastasis. When the respective tissue is reached, the stimuli that led to the initial transition cease and the metastasized cells invert to their original epithelial phenotype via mesenchymal–epithelial transition\textsuperscript{26}. Multiple signal pathways are considered to be implicated in EMT such as Notch, TGF-β, Hedgehog and the Wnt/β-catenin signaling\textsuperscript{26}. Transcription factors involved in the CSCs’ gene expression, directly and indirectly leading to the aforementioned EMT seem to sustain the survival of CSCs subpopulations\textsuperscript{26}.

### Resistance to therapy and CSCs microenvironment

The characteristics of CSCs lead to resistance to any conventional treatment protocols. The radioresistance is related to self-renewal capacity, DNA repair capacity, free-radical scavenging, upregulation of cell cycle control mechanisms and specific interactions with the stromal microenvironment. The resistance to chemotherapy is attributed to accelerated drug removal e.g. due to upregulated efflux pumps and drug metabolism\textsuperscript{3}.

Hypoxia is a major micro-environmental influence. Initially, as the HNSCC grows, blood supply does not suffice and areas of hypoxia develop\textsuperscript{5}. Hypoxia-inducible factor 1-alpha (HIF-1α) decreases the response to radiotherapy through suppression of the production of ROS, a prerequisite for an effective radiation treatment\textsuperscript{5}. HIF-1α also supports the migration and the invasive growth of cancer as well as the EMT process (possibly through the loss of cell adhesion, increased cell motility and production of proteinases)\textsuperscript{5}. HIF-1α may also assist the cell adaptation to hypoxic stress through the upregulation of genes such as the glucose transporter 1 (GLUT1) and the vascular endothelial growth factor (VEGF). Thus, the maintenance of the stem cells niche stabilizes\textsuperscript{3}. Regarding current therapeutic regimens, HIF-1α supports the resistance to drug-induced senescence in cancer cell lines\textsuperscript{27}. Unfortunately most of the therapy regimens focus on the bulk tumor mass\textsuperscript{27}, meaning that they focus on successfully inducing hypoxia to prevent further growth, accidentally increasing the CSC fraction.
of tumors through the role of HIF-1α. This fraction further aids the post-treatment repopulation of cancer cells, leaving behind the assassin CSCs that serve as a reservoir for tumor repopulation post-therapy.

Regarding possible correlation to CSCs’ biomarkers, increased CD44 levels are associated with tumor hypoxia through HIF-1α induced upregulation of the CD44 gene expression. Another pathway of critical importance is the MET. The transcription of the MET proto-oncogene is partially triggered by hypoxia. The MET pathway promotes the self-renewal and tumorigenicity in HNSCC stem cells. The pharmacologic selective inhibition of MET leads to the elimination of CSCs. Therefore, the MET inhibition prevents hypoxia-induced cell growth and a link between hypoxic areas and MET overexpression is established.

The CSCs depend on their microenvironment for subsistence and self-maintenance. Most of the stem cells are located close to blood vessels since the endothelial cells secrete factors that promote the self-renewal and survival and provide nutrients and oxygen. The components (stromal cells, inflammatory cells and vessels) of the niche in general provide the signals that maintain CSCs in their undifferentiated state. Therefore, a key factor regarding therapeutic resistance is the complex interaction between the neoplasia and various niche components. Thus, targeting the stem cell niche or tissue compounds diminishes the nutrition and negates the signaling needed by CSCs to proliferate.

The resistance to therapy is further reinforced by the general immunosuppression induced by cancer. Increased apoptosis of tumor-specific CD8+ T-cells and increased tumor infiltrating T regulatory cells have been demonstrated. Elevated IL-6 levels could independently predict tumor recurrence, poor survival, and tumor metastasis. Initially, CSCs can be destroyed by the immune system but after a certain point equilibrium is reached where cells with non-immunogenic phenotype survive the initial termination process, escape and prevail through constant growth. This evasion of the immune mediated destruction is manifold. To name but a few, lack of expression of known differentiation antigens or MHC molecules, lack of co-stimulatory ligands, expression of co-inhibitory ligands as well as expression of inhibitory cytokines, all play a critical role in surviving the initial phase of extermination.

**HPV and HNSCC**

Human papilloma virus is a well-established causative factor of HNSCC and more specifically oropharyngeal cancer with distinct epidemiologic and prognostic characteristics. Over 160 genotypes of HPV have been found, including the high- risk oncogenic subtypes (α- HPV genus). The most important HPV-related cancer subtypes appear to be subtypes 16 and 18. More specifically, HPV interacts with cell surface receptors, abundant in basal cells and epithelial stem cells, and in malignant tumor the virus integrated into the genome of the host. From the 8 viral proteins, encoded by the viral genome, E6 and E7 oncoproteins seem to be responsible for changes related to cell proliferation. The presence of HPV causes chromosomal anomalies and cellular immortalization. Linge et al. point out that the presence of HPV consists a positive prognostic factor for the control of loco-regional recurrence. Moreover, the investigators refer that the expression of stemness biomarkers has been found to be decreased in HPV positive tumors and HPV16 positive tumors appear to be more radiosensitive at the same time. Nevertheless, treatment remains suboptimal for patients with HPV positive cancer, the exact reasons remain unclear. Smoking seems to be associated with increased EGFR expression that leads to a poor prognosis in comparison with other HPV positive tumors. However, the unanswered question is why HPV+ tumors are considered to generally have a better prognosis when treated with chemoradiation compared to non-HPV. In HPV+ HNSCC, although E6 and E7 genes immortalize infected cells, field cancerization is not present. This lack of a genetically unstable area provides genomically speaking a much more homogenous cellular genotype, thus facilitating a better prognosis. The immune mediated destruction of HPV infected cells and the fact that P53 and pRb are rendered dormant but not silenced via E6 and E7 genes are also regarded as positive prognostic markers. On the other hand, the immune response (defined in this case as antibody production against the highly expressed p16 in HPV+ HNSCC) is not significantly strong.

Regarding the role of EGFR, its activity is essential for maintaining cancer stem-like state while also interacting with CD44 to manifest chemoresistance. This interaction is not documented in HPV+ tumors, partially explaining why they respond better to chemotherapy. According to Tang et al., the recurrences of HPV positive tumors have a relationship with CSCs and a probable poor prognosis can be attributed to the presence of increased EGFR expression and ALDH. In literature, studies that investigate the relationship between CSCs and HPV present equivocal findings. Thus, the effect of E6 on CSCs remains poorly understood. It has been speculated that HPV positive cancers contain fewer CSCs, conferring a better prognosis and less treatment resistance. Finally, two closing remarks regarding independent but nonetheless noteworthy findings: there are no clear results about how resistant HPV- positive tumors are to cisplatin therapy. The immunostaining for p16 protein is a surrogate marker for HPV-related oropharyngeal carcinomas due to the positive correlation observed between HPV detection and p16 overexpression.
**Conclusions**

A multitude of targeted therapies are already in the phase of clinical trials. The future looks promising as stem cell research works diligently at identifying more diagnostically and prognostically significant biomarkers and unraveling the still elusive pathomechanisms behind epithelial to mesenchymal transition, the biochemical pathways which allow cancer stem cells to metastasize, propagate and prevail as well as the exact nature and characteristics of their niche. In the not so distant future treatment protocols are bound to become even more selective, thus achieving the ideal combination of patient-selective therapy whilst keeping major and minor adverse effects at the bare minimum. Whether these protocols will consist of monoclonal antibodies, small molecule inhibitors, more exotic entities such as antisense oligonucleotides or a combination of them all remains to be seen.

**References**


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**Table 2. Targeting specific molecules for oral cancer management**

<table>
<thead>
<tr>
<th>Target</th>
<th>Therapy</th>
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<tbody>
<tr>
<td>Bmi1⁺</td>
<td>PTC-209</td>
</tr>
<tr>
<td>5T4</td>
<td>MED10641</td>
</tr>
<tr>
<td>FGF</td>
<td>BGJ398</td>
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<tr>
<td>Porcupine (PORCN)</td>
<td>LGK974</td>
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<tr>
<td>(Wnt signaling)</td>
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<tr>
<td>ALDH1</td>
<td>Aldi-6</td>
</tr>
<tr>
<td>cMET/FZD8</td>
<td>PF-2341066</td>
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<tr>
<td>CD44</td>
<td>Radionuclide ¹⁸⁶Rε-cmAb (U36)</td>
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<tr>
<td>CD44v6</td>
<td>Anti-CD44v6 antibody BIWA</td>
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**HNSCCs’ targeted therapy**

An ideal treatment would preferentially target specific CSCs’ subpopulations which are responsible for the tumor expansion, thus establishing a patient-specific therapy with minimal adverse effects. The combination of Cisplatin and PTC-209 dramatically reduced Bmi1⁺ tumor cells after 5 days, thus successfully targeting Bmi1⁺ tumors. 5T4 shows a high expression pattern in head and neck CSCs (CD44⁺/ALDH⁺). The Anti-5T4 antibody–drug MED10641 reduces the fraction of those CSCs and prevents local recurrence. The therapeutic agent BGJ398 dramatically reduced cisplatin-resistant CD44⁺/ALDH⁺ cells by inhibiting FGFR receptors (suggesting that FGFR confers at least partially a resistance to cisplatin). Loss of function mutations of Notch lead to upregulation of the Wnt signaling (since Notch suppresses Wnt signaling). HNSCC lines, responsive to treatment by LGK974, are enriched by LoF Notch1 mutations. LGK974 exhibits after a mere 2 weeks post treatment strong inhibition of tumor growth. ALDH1 is upregulated in HNSCC tumors following exposure to cisplatin (suggesting that ALDH1 also mediates a partial cisplatin resistance). ALDH1 induction’s inhibition through Aldi-6 leads to a reduction of cell survival. Selective inhibitor PF-2341066 of the c-MET downregulates the Wnt/β-catenin signaling through the FZD8 receptor, thus eliminating the HNSCCs. Both CD44 and its CD44v6 subtype constitute main important targets. The radionuclide ¹⁸⁶Re-cmAb (U36) targets CD44 in general while the anti-CD44v6 antibody BIWA enables the intraoperative imaging of tumor borders and remnants, invasion zone as well as the microscopic mapping of the tumor lesions. Nivolumab and pembrolizumab, both anti-PD1 antibodies, improve the overall survival of HNSCC compared to traditional chemotherapy as a second line therapy (Table 2).
Conflict of Interests: Nothing to declare.
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Animal Rights Statement: None required.