

HPV^{+ve/-ve} oral-tongue cancer stem cells: A potential target for relapse-free therapy

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ABSTRACT

The tongue squamous cell carcinoma (TSCC) is a highly prevalent head and neck cancer often associated with tobacco and/or alcohol abuse or high-risk human papillomavirus (HR-HPV) infection. HPV positive TSCCs present a unique mechanism of tumorigenesis as compared to tobacco and alcohol-induced TSCCs and show a better prognosis when treated. The poor prognosis and/or recurrence of TSCC is due to presence of a small subpopulation of tumor-initiating tongue cancer stem cells (TCSCs) that are intrinsically resistant to conventional chemoradiotherapies enabling cancer to relapse. Therefore, targeting TCSCs may provide efficient therapeutic strategy for relapse-free survival of TSCC patients. Indeed, the development of new TCSC targeting therapeutic approaches for the successful elimination of HPV^{+ve/-ve} TCSCs could be achieved either by targeting the self-renewal pathways, epithelial mesenchymal transition, vascular niche, nanoparticles-based therapy, induction of differentiation, chemoradio-sensitization of TCSCs or TCSC-derived exosome-based drug delivery and inhibition of HPV oncogenes or by regulating epigenetic pathways. In this review, we have discussed all these potential approaches and highlighted several important signaling pathways/networks involved in the formation and maintenance of TCSCs, which are targetable as novel therapeutic targets to sensitize/eliminate TCSCs and to improve survival of TSCC patients.

Background

Head and neck cancer (HNC) is the 6th most predominant cancer globally and a major public health problem in India and South-East Asia [1,2]. In India, HNC is positioned first in males and third in females, accounting for over 205,325 new cancer cases and 122,834 deaths in 2018 [3]. HNCs are highly heterogeneous group of malignancies with variable rates of incidence, mortality and prognosis. These cancers originate mainly from the epithelium of the lip, cheek, floor of mouth, oropharynx, pharynx, or nasopharynx, larynx and tongue (the most common location) [4,5].

Among HNCs, human tongue squamous cell carcinoma (TSCC) has highest incidence and it is the most feared and predominant sub-type of HNCs, which accounts for $\geq 40\%$ of all oral cancers. Majority of TSCCs

(90%) are histologically originated from squamous cells and show different levels of cell differentiation. The most common anatomical sub-site of TSCC is the lateral border of the tongue and the left-side of the tongue is more frequently affected than the right-side because of majority of right-handed tobacco smokers inhaling smoke stream to their left-side [6]. It is often mis-interpreted as oropharyngeal carcinomas particularly those arise at the base of the tongue.

TSCCs are characterized by aggressive behavior, loco-regional recurrence and a higher rate of occult metastasis with poor prognosis [7-9]. Recently, number of studies have showed an increased incidence of TSCCs afflicting not only low-middle income countries (LMICs) but also in several high-income developed countries including US and UK [10-13]. The incidence of oral-tongue cancers is highest in India compared to other nations of the world [14]. This is mainly because of constant use

* **Rationale:** Tongue squamous cell carcinoma (TSCC) is a predominant subtype of head and neck cancer with overall five year survival of only 50% and characterized by highly aggressive biological behavior, distant metastasis, tumor relapse and drug resistant. Majority of patients with tobacco habit are HPV^{-ve} and have bad prognosis while HPV^{+ve} TSCCs are mostly non-tobacco users who show well-differentiated tumors with much better treatment outcome. A small sub-population of tongue cancer stem cells (TCSCs) are resistant to chemo-radiotherapy, escape immune surveillance and responsible for cancer relapse. Selective targeting of TCSCs may provide efficient therapeutic strategy for relapse-free survival leading to improved quality of life and reduced mortality.

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of a variety of tobacco products (Cigarette, Bidi, Khaini, Gutkha and Pan Masala) areca nut and various ingredients contained in betel leaf (Pan, a special chewable preparation most commonly used in India) etc. together with alcohol (including indigenous raw alcohol preparations) are the classical major etiological factors associated with the development of TSCCs [9,15-18]. A concerted efforts and public awareness campaigns to educate the low or middle income group people regarding harmful health effects of tobacco and alcohol could lead to significant decrease in the incidence of HNC including TSCC. Furthermore, most alarming is the recent findings that incidence and proportion of TSCC specifically at the base of the tongue (BOT) is rising mainly in younger age population and none of the typical risk-factors (tobacco and alcohol) are found to be associated with the disease indicating other biological factors might be playing an important role in the development and progression of TSCCs [9,19-22]. BOTSCCs seem to originate from lingual tonsillar tissue, since majority of BOTSCCs display tumor cell growth, proliferation and metastasis pattern as that of tonsil tumors, particularly in HPV⁺ cases [23,24]. In recent decades, infection with specific types of high-risk human papillomaviruses (HR-HPVs) have been implicated as causative agents in the development and progression of a sub-set of TSCCs [9,20,25-27].

Thus, TSCCs are now divided into two distinct categories; i) HPV negative TSCCs and ii) oncogenic-HPV positive TSCCs. HR-HPV-positive TSCC patients show distinct clinical presentation, socioeconomic profiles as well as molecular and metabolic characteristics as compared to HPV negative TSCCs [9,25,28]. The prevalence of HR-HPV-induced TSCCs showed significant variability across populations and geographic regions including within the Indian subcontinent where the use of tobacco products is a part of the social-status and culture in certain sections of the society. It has been observed that HPV⁺ TSCCs are mostly prevalent among non-tobacco users, who have distinct genetic, molecular and clinical characteristics and show better response to therapy [9,20,25,29]. It indicates that HPV associated TSCCs constitute an unique mechanism(s) of tumorigenesis, biological behavior and treatment outcomes [9,15,25,27,30-32].

Disappointingly enough, despite great advancement in diagnostic and surgical techniques, radiation therapy, targeted and combinatorial therapeutic approaches (chemo-radiotherapy and immuno-therapy), the overall 5-year survival rate of TSCC patients is only 50% and it has not increased significantly over the last several decades [11,29,33]. However, these traditional therapies are effective for few subsites of HNCs, hence a better understanding of the underlying mechanism for TSCC development will only help in identifying more reliable markers and therapeutic targets for early detection and effective treatment of this aggressive cancer [34,35]. The poor survival and the treatment failure of TSCC patients is due to high recurrence rate, aggressive metastasis, tumor heterogeneity, failure of immunological surveillance and resistant to traditional therapies. One of the main reasons for TSCC treatment failure is supposed to be related to the presence of a heterogeneous small subpopulation of cancer stem cells. The growing cancer research has revealed existence of this small subpopulation (~0.05–2%) of “cancer initiating” or “cancer stem-like cells” (CSCs) in almost all cancers [36]. Similar to other cancers, TSCCs also have heterogeneous cancer stem-like cell population identified as tongue cancer stem cells (TCSCs) [37-39]. CSCs were first isolated and recognized in 1990 in leukemia using two stemness markers CD34+ and CD38 [40]. Subsequently, CSCs expressing various cell surface markers including CD133, CD44, ALDH, BMI-1, SOX2, OCT-4, nestin and NANOG, have been identified in several solid and non-solid tumors including TSCCs [39,41-44]. These rare population (1–2%) of CSCs or progenitor cancer cells manifest specific stemness properties with a capability to form new tumor following self-renewal show multi-drug resistance and progressive metastasis and recurrence of tumor (Fig. 1). Therapeutic resistance of these cells has been attributed to their cytotoxic drug refluxing and self-renewal ability, stem cell plasticity, efficient DNA repair capacity, free-radical scavenging, alteration in cell cycle control mechanism and modulation of tumor microenvi-

ronment. These characteristics of CSCs play a critical role in provoking aggressive metastasis and poor prognosis of the disease which in turn is allusive of the fact that detection and targeted inhibition and/or sensitization of CSCs can be a new conceptual framework for TSCC treatment [45-48].

Therefore, it has become imperative to understand the complex functional role of CSCs in the patho-physiology of the disease and deregulated self-renewal signaling pathways elucidating novel biomarkers for early diagnosis and designing novel treatment strategies for targeting CSCs.

CSCs have an active process of cytotoxic drug exclusion property because of expression of an ATP binding cassette (ABC) transporter transmembrane protein ABCG2 (a multidrug resistance protein) [49]. CSCs have been isolated from variety of tumours including TSCCs and explored to design novel therapeutic approaches that could target CSCs. However, despite concerted efforts, CSC-targeting strategies have not been efficiently translated to the clinic. This is partly due to partial understanding the molecular biology and signaling pathways underlying CSC treatment-resistance. The intra-tumoral heterogeneity and functional heterogeneity of CSCs depend on their nature of plasticity that modulates tumor invasiveness, metastasis and treatment response. The CSC population of various cancers including oral-tongue cancers are plastic in nature (bidirectional conversion of CSCs from non-CSC-state) that can gain stem cell features depending upon the tumor microenvironment, epigenetic and genomic modifications [50,51]. Further, the CSC plasticity can cause dynamic alterations in CSC markers expression and interactions with their niche [14, 52]. Certainly, CSC plasticity is an additional hurdle to overcome for an effective treatment of TCSC patients [52]. The CSC plasticity modulated by tumor microenvironment and cellular interactions that may participate in cellular transformation of cancer cells and provide protection to CSCs from chemo-radio therapeutic agents [52].

Since the survival of TSCC patients is poor as the current radio-chemotherapy treatments are not fully curative, isolation, identification and characterization of TCSCs and their efficient targeting must be taken into account when developing novel anti-cancer therapeutics. Deeper understanding of molecular mechanisms that contribute in tumor heterogeneity and CSC plasticity represent one of the greatest interests and challenges in translational cancer and cancer stem research.

Methods for identification, isolation and characterization of CSCs

The main purpose to study CSC biology is to design CSC-based remedies to treat chemo-radio resistant cancer cells which are presently untreatable by conventional therapies. But, the mechanistic understanding of CSCs to make them sensitive to chemo-radio therapy and identification of CSCs from the pool of cancer cells constitute a major experimental challenge in molecular oncology research. To date, there are at least four main methods are available for the isolation, identification and characterization of CSCs from cultured cancer cells or solid tumors based on the CSC properties [53,54]. These methods include the (i) dye efflux by multidrug resistant transporter proteins, (ii) tumorosphere-formation assays, (iii) the expression of CSC specific cell surface CD markers and (iv) single-cell omics technologies for analysis of individual CSCs from a mix population.

SP (Side population) analysis by dye efflux method

It is an important method for identification of SP cells from a subset of cancer cells population. SP analysis from cultured cancer cells using Hoechst 33,342 dye (a fluorescent DNA-binding dye that facilitates the efflux of this dye and other drugs by members of the ABC transporters) or dye cycle violet (DCV) or Rhodamine 123 staining and followed by fluorescence-activated cell sorting (FACS) analysis can provide a distinct cell population on the left quadrant of the dual-color emission spectra [55-58]. These SP cells can be distinguished by their depletion

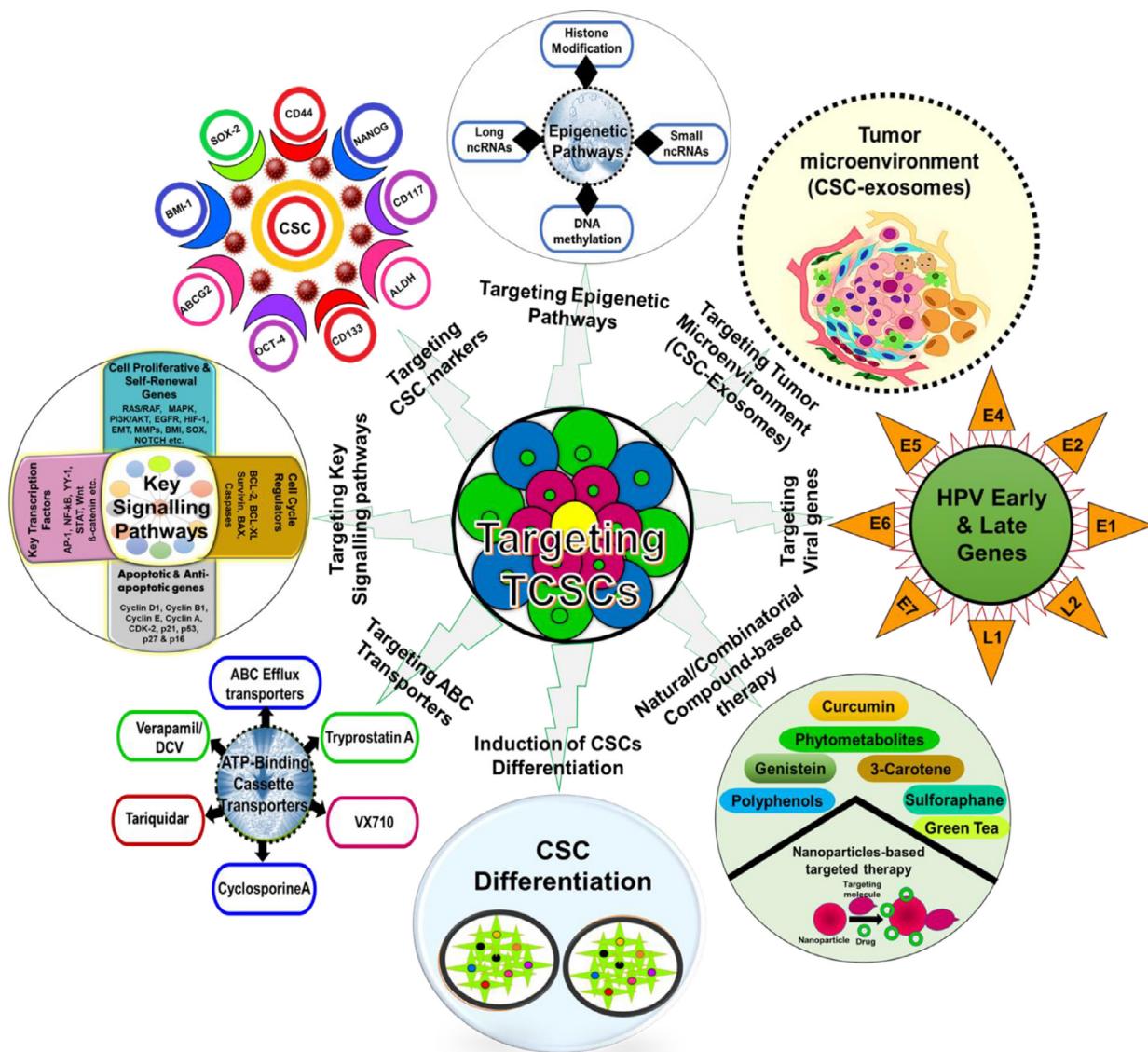


Fig. 1. Different therapeutic strategies for tongue cancer stem cell targeting. Strategies for CSC targeting based on specific molecular characteristics including targeting the signaling network, targeting the stemness markers, inhibiting the tumor microenvironment, targeting with nanoparticle-based therapy, sensitizing cancer stem cells with natural and synthetic compounds or induction of CSC differentiation including targeting epigenomic pathways and ABC transporter cassette proteins.

using Hoechst transporter inhibitors Verapamil or FTC (Fumitremorgin C). SP cells have been identified and isolated from various cancers including TSCCs and this method does not need any cell specific stemness marker for isolation and sorting of CSCs from various tumor tissues and cells. Recently, several studies have identified SP cells in TSCC and characterized them as most aggressive and tumorigenic stem cell-like population [43,59–61]. However, SP analysis technique has some limitations such as low specificity, low purity of CSCs, dye concentration and toxicity etc. However, successful isolation or sorting of CSCs by FACS is mainly depend greatly on the flow cytometry and expertise in the technology.

Identification of CSCs by self-renewal and/or tongueosphere formation assays

The ability of CSCs to create spheres or tongueospheres after they were cultured under-low attachment surface and defined-conditioned medium (DCM) containing differential growth factors enhance CSC growth and maintenance [56,57,62]. Sphere-forming capacity is a “hall-

mark” and unique characteristic of CSCs and has been widely used to identify and characterize CSCs based on their self-renewal and differentiation ability at a single cell level more efficiently *in-vitro* non-adherent nutritionally deficient culture conditions [63]. Though, the identification and characterization of CSCs by functional sphere formation assays has been widely used in cancer stem cell research, numerous issues may limit the utility of this assay [64].

The disadvantages of this assay are to prove sphere clonality, inherently slower cell cycle kinetics, selection of single-cell derived spheres, differentiation potential of cultured cells may bias/affect due to use of high concentrations of exogenous growth factors and cell aggregation [65]. Further, other challenges are that the assay may not be able to identify quiescent cells, a read-out of *in-vivo* stem cell frequency and efficient assessment of the cultured sphere's size and number. Recently, three-dimensional (3D) culturing technique has emerged as a very powerful tool that provides a proper microenvironment for development of clonal mini-organs, functional reporter of stem cells and progenitor cell activity. 3D cultures are ideal system and can be used as an alternative approach for the assessment of quiescent stem cells.

CSCs identification using potential stemness markers

This is the most common method for identification and characterization of CSCs based on the specific cell surface protein markers that nurture cells with CSC properties [66-68]. These markers are highly tumor specific and contain distinct cell-specific membrane proteins that distinguish them from non-stem cell population (NSP). Growing number of studies have identified the essential role of putative CSC markers in a variety of solid tumors but very few of them have been studied in TSCCs. ALDH, CD44 and CD133 cellular markers are very common surface markers used for the identification and sorting of CSCs in oral-tongue cancers.

i. ALDEFLUOR assay for ALDH activity: Recently, aldehyde dehydrogenase (ALDH), a cytosolic enzyme that is responsible for the oxidation of aldehydes to carboxylic acids. ALDH has been used for the identification and isolation of CSCs in variety of human tumors including TSCCs and it is now become an universal stemness marker for epithelial malignancies [69,70]. An elevated activity of detoxification enzyme ALDH in TCSCs have established and its overexpression is associated with multidrug resistance transport proteins [71]. The technique used to identify and sort high ALDH^{+ve} (ALDH1A1) cell populations is called ALDEFLUOR assay [72]. With the emergence of this assay, identification and sorting of live cells from patient's samples have been possible [72,73] with high ALDH expression (ALDH^{high}). It helps in functionally characterize the role of ALDH^{high} cells in oral-tongue cancer progression. ALDEFLUOR is a non-immunological fluorescent reagent that measures the activity of ALDH enzyme through the cleavage of a BODIPY-Aminoacetaldehyde (BAAA) fluorescent substrate to its corresponding BODIPY-aminoacetate (BAA) carboxylic acid [74]. Diethylaminobenzaldehyde (DEAB), an ALDH inhibitor can be used as a control to measure the exact percentage of CSCs with ALDH^{high} activity [72]. High ALDH^{+ve} subpopulations in TSCCs cause more tumorigenic phenotype, aggressive metastasis and chemo-radiotherapy resistance [61,75]. Further, ALDH1^{+ve} cells showed higher CSC-like characteristics than ALDH1^{-ve} oral cancer cells [38,71]. Thus, specific targeting ALDH in TSCC significantly inhibits several CSC properties in cancer cells and may serve as a unique intracellular marker for identification of TCSCs.

ii. Identification of cellular marker CD44: CD44 is a well-known transmembrane glycoprotein marker that play a significant role in intercellular interactions, cell migration and cell adhesion [76]. It has been suggested that CD44 play a key role in cell growth, survival, differentiation, tumor progression and metastasis [77]. In a variety of cancers, interaction of CD44 with hyaluronic acid (HA), heparan sulphate and chondroitin sulphate leads to the binding of CD44 with growth factors and metalloproteinases and can activate tyrosine kinase receptors and promote cell growth, proliferation and survival [78]. Further, CD44 enable release of cancer cells to blood vessels during cancer metastasis. Prince et al. (2007) first identified CD44^{+ve} subpopulation in HNC and showed higher tumor-initiating capability compared with CD44^{-ve} subpopulation in xenograft mouse model suggesting the importance of CD44^{+ve} CSCs in HNCs [47]. Following this, a large number of studies have shown that CD44^{+ve} cell populations display higher potential for cell growth, proliferation, migration, differentiation, tumor sphere formation, and chemo-radio resistance in primary oral-tongue tissues and cell lines [47,79-81]. Thus, these studies indicate that CD44 is an important prognostic factor and can be used as a potential stemness marker to identify and isolate CSCs from TSCCs.

iii. Identification of CD133: Transmembrane glycoprotein CD133 (120 kDa in size), also known as prominin-1 or AC133 was one of the first stemness marker used for identification of neuroepithelial stem cells in 1997 and later defined in human hematopoietic stem cells. High CD133 cells display tumor initiation, progression, strong tumorigenicity, and CSCs maintenance. Highly strong expression of CD133 is associated with poorer survival and identified as a putative CSC marker in variety of cancers including TSCCs [82,83]. Several studies have revealed that CD133⁺ subpopulations increased proliferation, clonogenicity, tu-

mor sphere formation, self-renewal and tumorigenicity in oral-tongue cancer cell lines [84,85]. The upregulation of CD133 is often identified in oral-tongue cancer stem cells, but its functional role as a CSC marker in TSCC is still not well-understood and requires further investigation.

Although, the accumulation of experimental data supporting the potential role of specific CSC markers in aggressive TSCCs [47,83] yet to date, no single full-proof TSCC specific stemness marker have been identified which might provide the best specificity for identifying/targeting TCSCs.

Single-cell omics technology for analysis of individual CSCs

Despite huge advancement in oral cancer treatment, the dynamics and complex tumor cell biology, extensive cell-to-cell variation and intra-tumoural heterogeneity are intrinsic and fundamental features of cancer and cancer stem cell populations which affect treatment efficacy and patient's survival, hence posing a significant challenge to developing targeted cancer therapies. However, these variations are masked when bulk of cells are employed for high-throughput single-cell omics technologies that provide precision to analyze cellular heterogeneity and detect distinct cell phenotypes within a 'homogeneous' stem cell population. Single cell omics are advanced technologies with high sensitivity and high-throughput with multiplexity that allow unbiased analysis of the individual cell type out of a complex subpopulation of cells at the level of genomics, transcriptomics, proteomics, epigenomics and metabolomics [86-88]. It provides a new opportunity to profile specific cells within heterogeneous tumours or CSCs and may help to discover new therapeutic targets for personalized medicine approaches for effective treatment [89,90]. The single cell isolation technique is useful for efficiency, purity and recovery of single cells and all these parameters provide huge advantages in the field of biomedical research [91].

TSCC being an extremely aggressive cancer, understanding potentials of TCSCs as prospective biomarkers and therapeutic targets, isolation and characterization of CSCs using a battery of techniques are of paramount importance to enrich effective diagnosis, prognosis and treatment of TSCC patients.

Various molecular approaches/signaling pathways for targeting TCSC

Targeting TCSCs by cell surface markers

In recent years, studies have been aimed at the application of stem cell biology in clinical medicine, particularly in regenerative medicine and its role in tumor recurrence and metastasis. Several CSC stemness markers have been identified mainly in the basal layers of oral-tongue mucosa which are essential for tissue homeostasis. The TCSCs overexpress specific surface markers as compared to NSP, therefore characterization of TCSC-specific stemness markers provide differential profiling of CSC populations which helps in the development of new drugs against those TSCC patients who develop resistance to conventional therapy.

Numerous studies suggest that TSCC harbours CSCs and specific stemness markers including BMP-4, CD44, OCT-4, NANOG, ALDH, CD133 and BMI-1 which have been identified and characterized in TSCC [47,92-96]. An earlier study by Kang et al. (2010) showed that CD133 may be used as a potential CSC marker for TSCC [93] while, Zou et al. (2011) revealed that TSCC cells (Tca8113) harbor 1.3% of ALDH1^{+ve} cells, which show an increased proliferative, self-renewal capacity as compared to ALDH^{-ve} cells. Therefore, ALDH has been considered as a potential therapeutic marker for TSCC [61]. Targeting ALDH with Aldi-6 and DEAB inhibited ALDH3A1 expression and blocked enzymatic activity of ALDH in TSCCs and TCSCs (see Table 2) [61,97].

Earlier findings suggest overexpression of ALDH1, CD44, OCT4 and SOX2 which can be used as independent prognostic stem cell markers for addiction habits, clinical staging and overall survival of TSCC [93]. Positive expression of OCT4 and SOX2 markers in TSCC cells

[98] indicates that these markers help in reprogramming and development of tongue tumorigenesis [99]. Immunomagnetic microbead sorting method for isolation of high-purity CSCs revealed that CD133^{+ve} and CD44^{+ve} sorted subpopulation has capacity of elevated proliferation, invasion, self-renewal and tumor-formation which are indicative of their essential role in TSCC initiation, progression, metastasis and recurrence and hence they might serve as potential targets for TSCC treatment [59,61,93]. Recently, Baillie et al. (2016) identified subpopulation of CSCs along-with cytoplasmic localization of stemness markers (CD44, OCT4, SALL4, SOX2, NANOG and phosphorylated STAT3) in moderately differentiated TSCC (MDTSCC) which is indicative of a novel therapeutic target(s) in TSCC [59].

Numerous studies have revealed that altered overexpression of SOX2 is associated with cell proliferation, self-renewal, cancer cells plasticity, anti-apoptosis, tumorigenesis, tumor relapse and chemo-radio resistant leading to bad prognosis in TSCC patients [100-103]. Interestingly, specific silencing of SOX2 in TSCC significantly inhibits self-renewal and invasion capacities, in-vivo tumorigenicity and increases chemo-radio sensitization [104]. Further, TSCC patients with SOX2^{high} and MTA3^{Low} (metastasis-associated protein 3) showed poorest prognosis suggesting SOX and MTA3 may serve as a potential prognostic markers for these patients [105].

Further, stem cell marker CD44 played significant role during aggressive metastasis of TSCC leading to increased cell proliferation, migration, invasion of TSCC cells [106,107]. The expression of CD44v6 and ABCG2 has also been correlated with lymph node metastasis, tumor progression, recurrence and poor survival of TSCC patients [108]. In addition, CD44⁺ TCSCs injection in nude mice formed larger tumor size and aggressive metastasis compared to CD44⁻ TSCCs [107]. The expression of CD44 in TSCC and oropharynx is associated with a poor clinical outcome [109] and combination therapy with SN-38 and gefitinib inhibits the expression of CD44 by promoting its lysosomal degradation in TSCC cells (Table 2) [110].

Some studies have also reported altered regulation of BMI-1 and c-MYC which is correlated with progression, proliferation and invasion of tongue cancer and cancer stem cells [92,111].

BMI-1 (B lymphoma Mo-MLV insertion region 1 homolog) serves as an essential key factor of the polycomb repressive complex-1 (PRC1) and its overexpression often associates with advanced tumor stages, aggressive behavior, self-renewal, treatment resistance and poor prognosis in TSCC. Further, specific targeting and pharmacological inhibition of BMI-1 can inhibit tumor sphere formation and reduced tumorigenesis in TSCC [47,112-114]. Toshihiro Tanaka and co-workers (2016) used multicolor lineage tracing method and revealed that BMI1-positive tumorigenic cells display TCSC-like characteristics in a chemically induced tongue cancer mouse model [115]. Further, inhibition of BMI-1 through PTC-209, PTC596 inhibitors reduced BMI-1 expression, tumorsphere formation, the percentage of ALDH1^{+ve} subpopulation and increased chemosensitivity against 5-FU and cisplatin in TSCC cells (Table 2) [51,112,114,116].

Certainly, these studies strongly suggest that all these stemness markers are indispensable and essential for identifying and characterizing CSCs including TCSCs for basic research and screening of anti-CSC drugs. However, these markers may not be sufficiently specific or may be poor therapeutic target(s). Therefore, it is reliable and most useful when the identified and isolated TCSCs using specific stemness marker(s) have the ability to develop tumorspheres *in-vitro* system and subsequently on the injection of these spheres can produce tumors *in-vivo* athymic mice.

Targeting ABC efflux transporters

ABC membrane efflux transporter superfamily proteins (ABCB1-MDR1, ABCC1-MRP1 and ABCG2-BCRP) play potential roles in drug metabolism and toxicity and its overexpression leads to higher efflux of cytotoxic drugs and hence chemo-radio resistance of cancer and CSCs (see Table 1). Side population fraction of cancer cells display increased

ABCG2 expression, which facilitate drug resistance [60,117]. It has been shown that 90.9% of oral-tongue precancer lesions have higher ABCG2 expression which may help in early prediction of oral-tongue cancer progression [118]. Overexpression of efflux transporter proteins; ABCG2, ABCA3 and ABCG5 have been found in oral cancer SP cells [119-121]. Overexpression of solute carrier transporter SLC2A13 also correlated with tumorsphere formation ability and may serve as a putative molecular marker for TCSCs (Table 2) [122]. Recently, it has been shown that specific inhibition of ABCG2 via Fumitremorgin C (FTC) & tariquidar (XR9576) inhibitors (Table 2) reduced the expression and function of ABCG2 [17,123]. Therefore, targeting the two major superfamilies of efflux transporters; i) ATP-binding cassette transporters, and ii) the solute carrier (SLC) transporters will possibly significantly enhance the efficacy of cancer therapy.

Targeting CSC signaling pathways

TSCC including CSC generation involves various dysregulated cellular and molecular signaling pathways, some of them have been implicated in TCSC drug-resistance. Numerous altered signaling pathways such as PI3K/Akt, Notch, EMT, Wnt/β-catenin, STAT, AP-1 and NF-κB are associated with regulation of CSC properties and its self-renewal pathways involve in the development of various cancers including TSCCs [9,25,75,124,125]. Recently, studies have reported that dysregulation of PI3K/AKT and JAK/STAT pathways have been noted in the pathogenesis of head and neck cancers (HNCs) [126,127] and acts as potential therapeutic CSC-target(s). The altered activation of PI3K/AKT/mTOR signaling pathways play a critical role for cellular growth, transformation and in chemo-radio resistance in TSCC [128,129]. Further, NVP-AEE788, a tyrosine kinase inhibitor inhibited EGF and VEGF signaling, cell growth and proliferation, induced apoptosis, and reduced the phosphorylation of EGFR/VEGFR-2/AKT in TSCC cells (Table 2) [130]. It indicates that these signaling pathways have potential of therapeutic targets which may serve as promising network for the development of CSC-targeted chemo-radio sensitizing drugs for TSCC patients [129,131,132]. Abnormal/overactivation of Notch signaling pathway plays a key role in the CSC maintenance during TSCC and therapeutic targeting of this pathway prevents Notch receptor cleavage on the cell surface leading to inhibition of self-renewal and tumorigenicity of oral-tongue cancer stem-like cells thus acting as a potential chemotherapeutic target for TSCC patients [133-136] (Table 1 & 2). In a recent study, aberrant expression of SOX8 correlated with activation of the Wnt/β-catenin and epithelial-mesenchymal transition (EMT) signaling leading to maintenance of stemness properties and treatment resistance in TSCC cells [137]. β-catenin also directly controls OCT4 expression and increases tumor growth in oral cancer stem-like cells [104]. Further, Liping Zhang et al. (2019) examined the expression of R-spondins proteins 2 (Rspo2) and leucine-rich repeat-containing G protein-coupled receptor 4 (LGR4) in TSCC cases and cell lines found that aberrant expression of Rspo2-LGR4 promotes cell proliferation, invasion, migration, self-renewal and EMT through the activation of Wnt/β-catenin signaling pathway [138].

Also, bone morphogenic protein 4 (BMP-4) expression promoted acquisition of TCSCs and EMT activation in TSCC cells [139]. Further, forced expression of EMT markers (Snail and Slug) enhanced cell invasion, migration and stemness features in TSCC cells. Altered expression of BMI-1 has been found to be correlated with self-renewal of CSC in HNC [47,140]. BMI-1^{+ve} CSCs in TSCC cells have been associated with drug resistance in a mouse model [115,141]. Recently, Chen and co-workers (2017) investigated that BMI-1^{+ve} CSCs can mediate invasive growth and lymph node metastasis in a mouse model and the primary oral tumors associated with high tumorigenicity, invasiveness and cisplatin-resistance leading to overexpression of transcription factor activator protein-1 (AP-1) [142]. Recently, it has been shown that AP-1 signaling involved in radio-sensitization of cervical and oral cancer stem cells and treatment of curcumin with conventional anti-cancer drugs

Table 1

Various targeted agents and therapeutic approaches against CSCs.

| I. Therapeutic strategies targeting cancer stem cell surface markers | | |
|---|--|----------------|
| Therapeutic approaches against Anti-CSC markers | Targeted CSC markers | References |
| H4C4, H90, P245, RO5429083 | Targeting CD44 | [202,203] |
| GV5 | Targeting CD44R1 | [202,203] |
| BsAb | Targeting CD3 or CD133 | [202,203] |
| OMP-21M18 | Targeting DLL4 (Delta-like ligand) | [202,203] |
| Vismodegib+ gemcitabine | Targeting CD44+/CD24+ | [202-204] |
| Catumaxomab | Targeting EpCAM | [202,203] |
| SL-401, SGN-123A, talacotuzumab, MGD006, KHK2823, CAR-T | Targeting CD123 | [202,203] |
| TTI-621 | Targeting CD47 | [202,203] |
| Amcasertib (BBI503), napabucasin (BBI608) | NANOG inhibitors | [203] |
| II. Therapeutic strategies targeting cancer signaling pathways | | |
| MK-0752 (γ -secretase inhibitors), RO4929097, Demcizumab (DLL inhibitors) | Targeting Notch pathway | [202,203] |
| GDC-0449 (Vismodegib), LDE225 (Sonidegib), Glasdegib, Cyclopamine, IPI26909 | Targeting Hedgehog pathways | [202,203,205] |
| OMP-54F28 (Ligand sequestration), PRI-724 (Inhibitors of β -catenin), CWP232291, Galunisertib (LY2157299), Fresolimumab (GC-1008), Trabedersen (AP 12009), DKK1 | Targeting Wnt/ β -catenin pathway | [202,203,206] |
| III. Therapeutic strategies targeting CSC microenvironment | | |
| Plerixafor (CXCR4 inhibitor), Reparixin (CXCR1/2 inhibitor), Defactinib (FAK inhibitor), Sorafenib, Sunitinib, PX-478, Bevacizumab | Targeting CSC niches | [202,203] |
| IV. Therapeutic strategies targeting metabolic pathways | | |
| Venetoclax (Bcl2 inhibitors) | Targeting CSC metabolism | [203] |
| V. Therapeutic strategies targeting ATP binding cassette transporters/multiple drug resistance | | |
| ABC transporters, Verapamil, CDF (Difluorinated Curcumin), ALDH, Dofetilide (MS-209) | drug efflux transporters, MDR inhibitors | [202,203] |
| VI. Inducing CSC differentiation therapy | | |
| Smad inhibitors, retinoic acid (RA)-induced differentiation, BMPs, OSM (oncostatin) | Potential of differentiation therapy | [207,208] |
| VII. Targeting with anti-cancer and CSC-sensitizing phytocompounds | | |
| Curcumin | P53 \uparrow , PTEN \uparrow , c-Myc \downarrow , k-Ras \downarrow , Bcl-2 \downarrow , EGFR \downarrow , SMO \downarrow , GLI1 \downarrow , DNMT1 \downarrow , DNMT1, 3A and 3B[pan-HDAC inhibitor, Angiogenesis \downarrow , Cancer-associated fibroblasts \downarrow , Cytotoxic effect of NK cells \downarrow , CD8(+) \uparrow T cells \uparrow , Anti-CSC effect | [36,56,57,209] |
| Genistein | CD 163 \downarrow , p-STAT3 \downarrow , IL-10 \downarrow , IL-12 \uparrow , CD133 \downarrow , CD44 \downarrow , Twist1 \downarrow , N-cadherin \downarrow , E-cadherin \uparrow , CD133 \downarrow , CD44 \downarrow , ALDH1 \downarrow | [209] |
| Berberine | P53 \uparrow , PTEN \uparrow , c-Myc \downarrow , Bcl-2 \downarrow , EGFR \downarrow , DNMT1, DNMT3B \downarrow , CREBBP, EP300, SIRT3, KDM6A, SETD7 \uparrow , HDAC8, H3K4me3, H3K27me3, H3K36me3 \downarrow , pan-HDAC inhibitor, Angiogenesis \downarrow , Anti-CSC effect | [74] |
| Resveratrol | p53 phosphorylation \uparrow , mutant p53 \downarrow , PTEN \uparrow ; c-Myc \downarrow , Bcl-2 \downarrow , k-Ras \downarrow , EGFR phosphorylation \downarrow , β -catenin \downarrow , Twist1 \downarrow , Snail \downarrow , DNMT1, DNMT3A, DNMT3B \downarrow , CD8(+) \uparrow T cells \uparrow , Angiogenesis \downarrow , Cancer-associated fibroblasts \downarrow , HDAC1, HDAC2 \downarrow , CSC \downarrow | [209] |
| Dioscin | p53 \uparrow ; Bcl-2 \downarrow ; VEGF-A \downarrow ; TET1 \downarrow , TET2 \uparrow , TET3 \uparrow , DNMT3A \uparrow , Macrophage sensitivity \uparrow | [210] |
| Celastrol | p53 phosphorylation \uparrow , c-Myc \downarrow , Bcl-2 \uparrow , VEGF \uparrow , VEGF \downarrow , EGFR \downarrow , Remodelling fibrotic and immunosuppressive tumor | [210] |
| Silibinin | Microenvironment, Inflammatory microenvironment \downarrow , p53 acetylation \uparrow , PTEN \downarrow , c-Myc \downarrow , Bcl-2 \downarrow , VEGF, EGFR \downarrow DNMT1 \downarrow , H3K27me3 \uparrow , SIRT1 \downarrow , acH3, acH4 \uparrow , HDAC1-3 \downarrow , Cancer-associated fibroblasts \downarrow , Tumor-associated myeloid-derived suppressor cells, Anti-CSC effect | [210] |
| Sulforaphane | SMO \downarrow , GLI1 \downarrow , GLI2 \downarrow , Nanog \downarrow , Oct-4 \downarrow , Bcl-2 \downarrow , Zeb-1 \downarrow , E-cadherin \downarrow , VEGF \downarrow , CR1 \downarrow , CR3 \downarrow , Nanog \downarrow , ALDH1A1 \downarrow , Wnt3 \downarrow , Notch4 \downarrow | [209,211] |

significantly help for effective cancer treatment [9,56,134]. Further, we have shown that HR-HPV16 plays an essential role in maintaining stemness in cervical cancer [57] but in oral cancer [124] through functional interaction with NF- κ B pathways, it lead to well differentiation of tumours and good prognosis.

Recently, Upadhyay et al. (2016), demonstrated that activation of NOTCH pathway levels is associated with spheroid formation, stemness markers expression and lymph node metastasis in TCSCs and may serve as therapeutic target for tongue cancer patients [135]. Further, upregulation of NOTCH1 enhances cells growth, proliferation and migration during tongue tumorigenesis [143] while inhibition of NOTCH1 with FLI-06 inhibitor could reduce tongue cells proliferation, migration, invasion and induce apoptosis both *in-vivo* and *in-vitro* [143] (Table 2). In addition, the treatment of ASCs (adipose-derived

stem cells)-conditioned medium inhibited cisplatin-induced cell death and enhanced anti-apoptotic effects through activation of IGF-1R and ERK/AKT pathways in TSCC cells. Furthermore, p75 neurotrophin receptor (p75^{NTR}) is a cell-surface receptor glycoprotein was significantly expressed in TSCC cells and showed CSC-like characteristics such as proliferation, self-renewal, multidirectional differentiation and tumorigenesis and may be considered as a potential stem cell marker for TSCC [144]. Moreover, upregulation of BCL-2 expression is associated with increased cell growth, proliferation and cisplatin treatment resistance in TSCC cell lines. Targeting Bcl-2 through ABT-199 inhibitor (Table 2) effectively suppresses BCL-2 expression and impairs mitochondrial functions in TSCC cells [145].

It is therefore suggested that current cancer research should focus towards novel treatment approaches either singly or combinato-

Table 2

Potential therapeutic agents that target TCSCs.

| S.N. | Inhibitors | Targeted gene/marker/pathway | Cancer cell type | References |
|------|---|--|------------------|------------------|
| 1. | Fumitremorgin C (FTC) & tariquidar (XR9576) | Inhibition of ABCG2 expression and function | TCSC | [123,212] |
| 2. | ABT-199 | Inhibit Bcl-2 and impairs mitochondrial functions in TSCC cells | TSCC | [210] |
| 3. | PTC-209, PTC596 | Reduced tumorsphere formation, the percentage of ALDH1 ⁺ subpopulation, BMI-1 expression and increased chemosensitivity against 5-FU and cisplatin in CAL27 TSCC cells | TSCC and TCSC | [51,112,114,116] |
| 4. | Gefitinib (EGFR tyrosine kinase inhibitor) | Inhibited EGFR signaling and reduced SOX2 expression in TSCC cells | TSCC | [213,214] |
| 5. | Combination therapy of SN-38 and gefitinib | inhibits the expression of CD44 stemness marker by promoting its lysosomal degradation in TSCC cells | TSCC | [110] |
| 6. | GSK1120212 | MEK1/2 inhibitor in TSCC | TSCC | [215] |
| 7. | Simvastatin & AR-42 (TAZ inhibitors) | TAZ (PDZ-binding motif) inhibited tumor growth in-vivo, reduced cell proliferation, migration and tumorsphere formation in TSCC cells | TSCC and TCSC | [216,217] |
| 8. | FLI-06 (NOTCH inhibitor) | Inhibited tumor growth and increased cell apoptosis, suppressed both the mRNA and protein expression of Notch receptor and block the proliferation and self-renewal of tongue cancer stem cells. | TSCC and TCSC | [143] |
| 9. | Aldi-6 and DEAB (ALDH inhibitor) | Aldi-6 inhibited ALDH3A1 and DEAB blocked enzymatic activity of ALDH in TSCC. [61,97] | TSCC and TCSC | [61,97] |
| 10. | T-5224 (AP-1 inhibitor) | Inhibited the invasion, migration, MMP activity and prevented lymph node metastasis of TSCC cells | TSCC | [218] |
| 11. | NVP-AEE788 (tyrosine kinase inhibitor) | Inhibited EGF and VEGF signaling, cell growth and proliferation, induced apoptosis, and reduced the phosphorylation of EGFR/VEGFR-2/AKT in TSCC cells | TSCC | [130] |

rial approaches against these signaling pathways that regulate CSC self-renewal, growth and differentiation. This will facilitate better understanding of TSCC pathobiology and provide cues to cure highly aggressive and metastatic tumors without relapse.

Targeting HPV-specific CSCs in TSCC

A large number of studies have now well established that infection of high-risk HPV (HR-HPV) specifically type 16 is an important carcinogenic agent responsible for a significant percentage of TSCCs. These HPV positive TSCCs possess a unique signaling mechanism for tumorigenesis as the patients show better prognosis with improved responsiveness to chemo-radiotherapy leading to favorable survival rates as compared to tobacco or alcohol-induced HPV-ve TSCCs. Therefore, understanding the role played by HPV in TCSCs may improve treatment strategies as well as patient outcome [9,20,146,147]. Highly interesting is the findings that unlike cancer of cervix; in which HR-HPV is the major causative factor for tumorigenic transformation and invasive cancer, HPV infected TSCCs in contrast, show well differentiation of tumours with koilocytes (indicative of HPV) and better prognosis [20]. It indicates an inherent difference between HPV+ve and HPV-ve TCSCs [25], which also highlights the difference in tumor aggressiveness and response to treatment. CSCs share much of the genetic machinery with non-tumorigenic stem cells to maintain pluripotency and allow self-renewal and tumor formation. Therefore, it is tempting to speculate that HPV infection leads to establishment of the viral circular double-stranded DNA genome as a stable episome within the basal layer of epithelial cells and may alter pluripotency of several genes [148,149]. HR-HPVs that produce two principal transforming oncoproteins (E6/E7) play essential roles in viral integration, tumorigenic transformation and immune evasion by targeting cytokine expression including monocyte chemoattractant protein-1 (MCP-1) [150] to alter cell proliferation and interferon responses. HR-HPV E6 and E7 oncoproteins dysregulate two major tumor suppressor gene products p53 and pRb which also constitute important cellular regulatory pathways in maintaining stemness. Dysregulation of these genes leads to increased CSC production and aggressive carcinogenesis. It has been reported that HPV+ve oropharyngeal cancers show higher intrinsic CSC population than that in HPV-ve oral cancers and it is shown to be associated with HPVE6-mediated p53 inactivation [54]. p53 protein is deregulated by HPVE6 protein which is responsible for maintaining genomic integrity and stemness by altering expression of NANOG and

NOTCH. Generally, p53 inhibits NOTCH expression but following HPV infection HPV-E6 binds to p53 and the inhibition to NOTCH is relieved leading to increased CSC production and tumorigenesis. HPV E7 protein on the other hand, binds to pRb which normally remains bound with E2F protein, the E2F becomes now free to bind to promoters of cell cycle regulatory genes allowing cells to produce cell cycle proteins and to progress from G1 to the S phase leading to increased cell division and oncogenesis. E2F is also bound to promoter of stemness factors such as NANOG and alters stem cell genes expression. Earlier, Lee et al. (2015) reported that four HPV+ve oral cancer cell lines show relatively a higher tumor growth and self-renewal capacity [151]. Interestingly, CSC marker CD44 expression is lower in patients with HPV-positive oral cancer as compared with HPV-negative oral cancer patients [152]. Further, Zhang et al. (2014) revealed that HPV+ve oropharyngeal cancers (OPSCCs) had a higher intrinsic CSC population as compared to HPV-ve tumors and elevated ALDH1 staining suggesting that CSC phenotype might be more important than CSC population density for disease aggressiveness and clinical outcome in OPSCCs [51]. Moreover, low percentage of CSCs in tumor bulk and high expression of CSC markers are associated with cisplatin drug resistant in HPV-ve OPSCCs implying CSC properties play a key role in treatment resistant cases [41]. Furthermore, *in-vivo* data suggests that CSCs from the bulk of HPV-positive oral cancer may respond better to platinum-based therapy [153,154]. On the other hand, it has also been demonstrated that HPV+ve oral cancer cell lines have a lower proportion of CSC than HPV-ve cells and radiation therapy stimulated tumor cell dedifferentiation proposing tumor CSC density in HPV+ve oral cancers have a better prognosis and treatment outcome [155]. These contradictory observations strongly suggest that additional biological or molecular features of CSC is responsible for the treatment response of oral-tongue cancer [51].

Recently, we have shown that a significantly higher subpopulation of CSCs in cervical cancer has been shown to overexpress HR-HPV E6 oncogene and found to regulate and maintain stemness properties via Hes1 signaling pathway [57]. We have further shown that HPV16 plays a crucial role through its functional interaction with NF- κ B leading to well differentiation of tumours and better prognosis when treated [124]. Additionally, we have recently observed that higher expression and selective participation of NF- κ B/c-Rel with p50 that in cross-talk with AP-1/Fra-2 in tongue cancer and cancer stem cells induced poor differentiation and aggressive tumorigenesis only in HPV-ve TSCC patients while HPV infection induced increased expression of p65 and p27 leading

to well differentiation and better prognosis preferably in non-smoking TSCC patients [25,156]. These studies have established that CSCs with stemness markers along-with HPV infection could play an important role in the pathobiology and treatment outcome of oral-tongue cancer.

Targeting CSC-specific altered epigenetic pathways

Alterations in epigenetic pathways are commonly linked with tumor initiation, progression, differentiation and drug resistance. These alterations are also involved in cellular plasticity during cancer progression, and further contribute to the formation of CSCs. Increasing evidence suggests that CSCs expression can be influenced by epigenetic alterations in differential epigenetic signaling network such as histone modifications, involving DNA methylation, acetylation, post translational modifications and non-coding RNAs (ncRNAs) that dynamically control gene expression and are frequently occur in cancers including HNCs but the role of epigenetic alterations in TSCC certainly warrants further investigation [157-160]. The epigenetic alterations are emerging as key markers for early diagnosis and prognosis of TSCCs. Altered DNA methylation patterns and histone modifications in cancer stem cells leading to aberrant expression of miRNAs, indicated distinct DNA methylation profiles in CSCs [161]. Histone protein modifications can also play an important epigenetic role in maintaining and controlling CSC properties in HNC [25,158,162,163]. Recently, several long non-coding RNAs (lncRNAs) are shown to play an important role in development, stemness, differentiation and cancer development. Recently, lncRNAs have been categorized on the basis of their association with various subtypes of HNCs and overexpression of metastasis-associated lung adenocarcinoma transcript1 (MALAT1) was found to be a prognostic biomarker for oral cancer patients. MALAT1 can also play a significant oncogenic marker for TSCC [164]. LncRNA HOTAIR was also found to be an essential marker for advanced grades of HNCs [165]. Emerging evidences demonstrate that significant role of P-element-induced wimpy testis (PIWI)-interacting RNAs (piRNAs) in cancer and other diseases, but it remains to be identified its role in CSCs formation. While, its role in germline stem cell maintenance and genome stability is well established and overexpression of PIWIL1 and PIWIL2 is associated with OCT4 and SOX2 overexpression in colon cancer, it would be interesting to look for the potential role of piRNAs in TSCC-specific CSCs.

Small ncRNAs the miRNAs, are also play key roles in tumorigenesis via increasing oncogene expression, inhibiting tumor suppressor gene function and contribute to drug resistance, initiate cell reprogramming, promote epithelial-mesenchymal transition (EMT) and stemness properties [158,166]. It has been demonstrated that in majority of oral cancer cases, miR-34b, miR-137, miR-193a, and miR-203 are located in close proximity to the CpG islands and are found silenced through hypermethylation [167]. Earlier, miR-145 has been found to be downregulated in ALDH+ and CD44+ oral cancer stem cells and modulation of miRNAs in CSCs will improve better understanding of the disease biology [168]. Reduced expression of miR-200b, miR-203 and let-7a and upregulation of miR-138, miR-183, miRNA-145 and miR-205 have been analysed in oral-tongue cancers, which suggest that altered expression of these miRNAs are associated with stem cell regulation in oral-tongue cancer [169]. Very recently, it has been reported that upregulation of miR-21 and miR-155 and reduced expression of miR-34a in HPV16-E6-positive CSCs is responsible for stemness maintenance in oral cancer cells [124]. These reports highlight the promising role of miRNAs in characterizing tongue cancer stem cells during oral-tongue carcinogenesis and targeting of these miRNAs may help in developing novel therapeutic strategies for effective management of TSCCs.

Targeting stem cell niches, tumor microenvironment and cancer stem cell-exosomes

The niche or heterogeneous tumor microenvironment plays a very significant role in the maintenance of CSCs which are responsible for

chemo-radio resistance exist in a highly specialized niche. The main role of CSC niches to maintain and protect the CSCs, allowing them to resist standard anti-cancer therapies. The CSC niches allow the cell to remain dormant for a long time before initiating recurrent/distance metastasis [170]. Therefore, targeting whole tumor will not produce good results while targeting CSCs [171]. Furthermore, the cancer stem cells derived-exosomes are also crucial for tumor relapse, metastasis and maintenance. It can mediate cell to cell communication within the niches created in the tumor microenvironment to promote tumorigenesis. So it is possible that CSCs produce exosomes with unique characteristics and functions. Therefore, this is one of the best exosome cancer therapies to have chemo-radio therapeutic drug-loaded exosomes for targeting CSCs. There are also several studies now reported CSC derived exosomes containing specific type of miRNAs in renal, prostate, gastric and glioma cancers [172,173]. The exploitation of CSC-derived exosomes as drug delivery vehicles offers significant advantages as compared to other nanoparticulate drug delivery systems [174]. Exosomes are small extracellular membrane-based with different compositions that are involved in several biological and pathological processes of cancer and function as intercellular communicators by delivering their content to a target cell via membrane fusion or endocytosis [175,176]. Exosomes can be isolated from various body fluids including amniotic fluid, nasal lavage, saliva, serum, plasma, urine and CSCs etc. and used as microvesicles for therapeutic drug and gene delivery. As they are biocompatible, nontoxic, easy to produce, stable, easy to store, have a long life span and high cargo loading capacity [114,177,178], these characteristics making exosome a potential drug-delivery tool for the treatment of TSCCs [154,175,179]. Recent evidences have shown that exosome-based drug delivery has higher anti-tumor effects when compared to free drug delivery in mouse model [114]. Synthetic nanoparticles-based drug delivery have been employed to deliver drugs to bulk of the CSCs [180]. Further, it has been reported that exosomes derived from docetaxel- or adriamycin-resistant breast cancer cells contain distinct miRNAs which can decrease the chemo-sensitivity in targeted tumor cells [112]. Mutschelknaus and co-workers (2016) demonstrated a functional role of exosomes that transmitted pro-survival signals leading to tumorigenesis and radioresistant phenotype in TSCCs indicative of the fact that exosomes are potential targets to improved therapeutic strategies for better understanding of drug resistance TSCCs [175].

Chronic inflammation, secretion of inflammatory cytokines/chemokines and they have capacity to regulate proliferation, hypoxia and perivascular niches. Due to these characteristics, CSCs interact with heterogeneous tumor microenvironment leading to tumor progression and relapse [181-183]. Therefore, identification of CSC niches, CSC-derived exosomes and specific miRNAs there in and oral tumor microenvironment and understanding their interactions in TSCC will only allow us to gain the knowledge needed to develop targeted therapies against tumor progression and relapse (see Table 1 and Fig. 1).

Targeting CSCs by phytocompounds and nanoparticle-based therapy

CSCs are responsible for tumor heterogeneities and drug resistance to conventional therapies in many aggressive cancer types including TSCCs. These conventional treatment strategies such as surgery, radiotherapy, chemotherapy and immunotherapy are strategized depending on the tumor type, site, size, grade, stage and expression of specific biomarkers in TSCC patients. But these therapies are not much successful due to several limitations. These limitations are associated with systemic and local drug toxicity, cancer relapse, metastasis and self-renewal of CSCs or acquisition of CSC traits by non-CSCs. Hence, CSCs are becoming novel target for cancer therapy. Therefore, it is important to develop effective treatment/drugs that can target CSCs to prevent tumor relapse and reduce systemic toxicity. This will improve survival and quality of life of the TSCC patients. Emerging evidences suggest that herbal and nanoparticle-based therapy of CSCs

can be achieved by several targets such as genetic or epigenetic signaling pathways, stemness markers, efflux transporters, HPV oncoproteins, CSC niches and CSC-derived exosomes containing specific miRNAs which are critically responsible for self-renewal and maintenance of CSC phenotype and tumorigenicity. Use of nanoparticles with different sizes, configurations, chemical properties, and bio-functional compositions, may enhance both target selectivity and drug internalization in cancer cells [184,185]. Various nanomaterial including DNA (origami and tetrahedron), carbon (graphene oxide, carbon nanotubes and nano-diamond), noble metals (sphere AuNPs, gold nanorods and silver), organic nanoparticles (liposome and polymer) are emerging approaches in cancer stem cell therapy [186,187]. Nanoparticle-based delivery have high drug carrier capabilities, higher drug solubility and efficacy and site specific drug accumulation in cancer cells than conventional free drug delivery. These molecules also serve as suitable carriers of chemopreventive herbal compounds for variety of cancer diagnosis and therapeutic outcome. NV-1 was a liposomal formulation having higher anticancer drug effect to breast cancer stem cells [180,186]. Recent studies also suggest that nano-formulation of herbal compound, curcumin (diferuloylmethane) can be used effectively for cancer and cancer stem therapy [180,188,189]. Nano-formulation of curcumin (NP-Cur) can induce cell necrosis and decrease the expression of several signaling pathways such as NF- κ B, AP-1, VEGF, MMP-9, and TNF- α . It has been shown that delivery of Curcumin-difluorinated liposomes is useful for cisplatin resistant HNC therapy [190,191]. Cur-microemulsion formulation carry toxic effects and show damaged and ruptured tongue cancer cells after treatment than free Cur formulation [192]. Valproic acid, a histone deacetylase inhibitor (VPA) has been shown to downregulate the expression of stemness markers (OCT4, SOX2, and CD44) and can suppress self-renewal abilities of oral cancer stem cells [193]. Further, Nano-Cur can induce apoptotic death of TSCC and TCSCs with a low cytotoxic effect, curcumin being a strong antioxidant which is known to sensitize cancer and cancer stem cells [194]. These studies together indicate that development of anti-CSC drugs using nano-formulation may target growth and functionality of tongue cancer and cancer stem cells making them ideal approaches for effective TSCC/TCSC treatment outcome.

Inducing differentiation of CSC

Poor or undifferentiation is an essential hallmark of all cancer stem cells. While 99% of cells in a tumor are differentiated cells which are often killed by standard therapy, 1–2% cells are un-differentiated cancer stem-like cells which are responsible for tumor relapse and metastasis. Therefore, apart from targeting different genes or pathways, one of the best strategies for CSC-targeted therapy is though differentiation of CSCs that holds great promise for cancer treatment. Usually, differentiation therapy tend to have no toxic effect than conventional cancer treatment. In 1980, differentiation therapy had been adapted to leukemia CSCs to induce retinoic acid (RA)-induced differentiation in certain hematopoietic cells [195,196]. As CSCs have the ability to differentiate into several types of cells, including lineage-differentiated and non-cancerous-differentiated tumor cells after exposure to specific differentiation agents/signals, differentiation therapy might induce CSCs into terminally differentiated cancer cells, and then, these cells could be easily treated with alkylating agents or a combination of the two or more conventional therapies (Table 1). Treating CSCs through the induction of differentiation has been an attractive concept, but clinical development of differentiation-inducing agents to treat CSCs, especially CSC from solid tumours has been limited till date [196]. Few studies have shown that RA, a small lipophilic derivative of vitamin A, is a well-known inducer of differentiation and proliferation in normal stem cells [197]. All-trans retinoic acid (ATRA) is an inducer of differentiation, which can enhance sensitivity to anticancer therapies and decrease CSC motility, invasion and tumorigenicity in glioblastomas [198] and breast cancer [199]. BMP signaling is the most powerful inducer of differentiation and a variant type of BMP reduces tumorigenicity by in-

ducing differentiation in glioblastomas [200,201]. Further, induction of mesenchymal-to-epithelial transition (MET) has been proposed as a new alternative as it has potential to reduce aggressive tumorigenicity of CSCs.

We have recently demonstrated that higher DNA binding and over-expression of AP-1/c-JUN with FRA-2 and NF- κ B/c-REL with p50 promoted poor differentiation, aggressive tumorigenesis and worst prognosis only in HPV-ve TSCC patients, while HPV infection promoted selective activation of AP-1/JUND and NF- κ B/p65 and upregulation of p27 leading to well differentiation and better prognosis mainly in non-smoking TSCC patients [9,25]. Thus, to eradicate TCSCs and prevent TSCC recurrence, induction of virus-based differentiation strategies may provide new insights for the treatment of solid tumors.

Concluding remarks

TSCCs are the most predominant and aggressive HNC subtype and despite numerous therapies have come into existence in the last few decades, late diagnosis, locoregional recurrence, aggressive metastasis and drug resistance have been the major confounders for the poor 5 years survival of TSCC patients. The treatment failures are mainly attributed to presence of a small subpopulation of 'tongue cancer stem-like cells (TCSCs)' within the tumor microenvironment. These TCSCs show hallmark abilities of self-renewal, cancer growth, metastasis, drug resistance, quiescence, apoptotic and immune evasion, DNA damage resistance and expression of drug efflux transporter proteins. Despite several attempts in studying the role of CSCs in tongue cancer and development of combinatorial therapeutic approaches that focus on targeting the stemness signaling pathways, stemness markers, blocking epithelial mesenchymal transition, CSC niche, stem cell plasticity, nanoparticles based therapy, sensitizing of stem cells with pharmaceutical agents, modulation of epigenetic pathways and induction of differentiation may offer useful platform to eradicating high-risk TCSCs. Further understanding including single cell omics technology in CSC biology developing personalized and targeted therapies could provide recurrence-free treatment and improved quality of life for the TSCC patients. Targeting of TCSCs remains an attractive yet elusive therapeutic option, with the goal of increasing specificity and effectiveness in tumor eradication and prevention of recurrence as well as decreasing off-targeting and systemic toxicity. Further research for characterization and targeted delivery of therapies separately for HPV+ve/-ve TCSCs would be important because of their differential biology and prognostic characteristics.

Key Future directions

- Therapy resistance and tumor a recurrence following standard treatment is quite common in TSCCs as TCSCs, a subpopulation of cancer cells are difficult to eliminate with conventional treatments. Therefore, strategic therapeutic interventions can be designed to target CSCs and/or CSC-exosomes to control aggressive metastasis and tumor relapse.
- Identification of site-specific CSC markers for TSCC and development of alternative treatment strategies in combination with existing therapies are the critical steps in right direction for TCSC eradication with better understanding of disease pathogenesis and treatment outcome.
- Furthermore, the use of emerging technologies including single cell omics, high-throughput DNA and RNRA sequencing and analysis of CSC-derived exosomal ncRNAs will help to discover new therapeutic avenues for personalized therapy and improved TSCC patient's survival outcome.

Declaration of Competing Interest

No conflicts of interest.

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