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Molecular signalling in cancer stem cells of tongue squamous cell carcinoma: Therapeutic implications and challenges

Joshi P *et al*. Cell signaling in CSCs of tongue carcinoma

Priyanka Joshi, Sanjeev Waghmare

Abstract

Head and neck squamous cells carcinoma is the seventh most common cancer worldwide with high mortality rates. Amongst oral cavity cancers, tongue carcinoma is a very common and aggressive oral cavity carcinoma. Despite the implementation of a multimodality treatment regime including surgical intervention, chemo radiation as well as targeted therapy, showed an overall 5-year survival pattern, which is attributed to therapy resistance and recurrence of disease. The presence of a rare population i.e. cancer stem cells (CSCs) within tumor, which are involved in therapy resistance, recurrence and distant metastasis that results in poor survival patterns. Therapeutic agents targeting CSCs have been in clinical trials, although they are unable to reach into therapy stage which is due to their failure in trials. A more detailed understanding of CSCs is essential for identifying efficient targets. Molecular signaling pathways which are differentially regulated in CSCs are one of the promising targets to manipulate CSCs that would provide an improved outcome. In this review, we summarize the current understanding of molecular signaling associated with the maintenance and regulation of CSCs in tongue squamous cell carcinoma in order to emphasize the need of the hour to get a deeper understanding to unravel novel targets.

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Core Tip: Tongue squamous cell carcinoma is one of the most common and aggressive oral cavity carcinomas, particularly among the Indian population. Despite of various treatment strategies employed, the survival rates of the patients remain poor. A rare population *i.e.,* cancer stem cells (CSCs) play an important role in resistance, recurrence as well as metastasis which are factors responsible for the poor survival outcome. In this review, we discuss recent findings regarding cell signaling pathways and markers associated with CSCs and the need to gain a deeper understanding on the properties of CSCs.

INTRODUCTION

Global cancer statistics by GLOBOCAN in 2020, showed that 2.0% of new cancer cases reported worldwide were lip and oral cavity cancers, while 1.8% of the total cancer related deaths were of lip and oral cavity cancers. Lip and oral cavity cancers are the most commonly diagnosed cancers that are responsible for most cancer related deaths in India[1]. Most cases of oral squamous cell carcinomas (OSCCs) are presented at advanced stages *i.e.*, stages III or IV [tumor-node-metastasis (TNM) staging] where the 5-year survival of the patients is less than 50%. Further 40% of the oral carcinomas are presented as tongue carcinomas[2].

Head and neck squamous cell carcinomas (HNSCCs) are carcinomas of the oral cavity, nasopharynx, oropharynx, larynx and hypopharynx[3]. The oral cavity carcinomas comprises of the anterior $2^{nd}/3^{rd}$ of the tongue, buccal mucosa, retromolar trigone, lower and upper alveolar ridge, hard palate and floor of mouth[3].

The poor survival observed in HNSCCs is primarily attributed to loco-regional/ distant metastasis and therapy resistance. Therefore, understanding the molecular mechanism underlying these properties of tumors becomes very crucial. Current treatments for HNSCCs include surgery, chemo and/or radiotherapy and targeted therapy.

First evidence of cancer stem cells (CSCs) was observed in acute myeloid leukemia[4] where it was reported that only 10000 expressing $CD34^+/CD38^-$ cells could give rise to leukemia in non-obese diabetic-severe combined immunodeficient (NOD-SCID) mice. These cells possess high tumorigenic potential which are termed as CSCs. CSCs exhibit stem cell-like properties such as self-renewal, slow cycling and ability to divide and differentiate into various sub-populations[5]. Further, CSCs were isolated from solid tumors such as breast cancer, HNSCCs, colorectal cancer, ovarian cancer, lung cancer *etc.*[5].

Owing to their unique properties, these CSCs escape the current treatment regimes, thereby adversely affecting patient survival. Therefore, to design an effective treatment regime to achieve better efficiency and treatment outcome, it is crucial to understand the

molecular mechanism involved in maintaining these CSCs within the tumor. In this review, we focus on the tongue squamous cell carcinoma (TSCC) and have summarized the known molecular markers of CSCs, molecular signaling involved in the regulation of CSCs, and the inhibitors used in clinics for treatment, and the ones that are in clinical trials.

CSC MARKERS FOR TSCC

CD44 and variants

CD44 is a single chain proteoglycan transmembrane glycoprotein expressed on human embryonic stem cells at the developmental stages of cell types such as bone marrow and connective tissue. CD44 interacts with molecules such as hyaluronic acid (HA), collagen, osteopontin, fibronectin, chondroitin and serglycin/sulfated proteoglycan. CD44 has variant isoforms such as CD44, CD44s, CD44v3, CD44v6, CD44v8-10[6] with HA as the mostspecific ligand for CD44 and its isoforms[6].

The first report of isolation and characterization of CSCs from HNSCC showed that 5000 CD44+/Lin- cells gave rise to *in vivo* tumors in NOD-SCID mice[7]. CD44 expression is correlated with the expression of known stem cell marker, BMI-1 in HNSCC cells.

Recently, CD44v3 is reported to be overexpressed in HNSCC tumors than the cut margin. Transfection of CD44v3 in HNSCC cells increases cell migration[8]. Tumor cell growth, migration, matrix metalloproteinase activity and lymph node metastasis in patients are associated with CD44v3 overexpression in HNSCC cells[9]. Decreased expression of CD44v9 correlates with poor overall survival (OS) in TSCC[10]. Overexpression of CD44 correlates with tumor invasiveness and epithelial-mesenchymal transition (EMT). Expression of CD44 in invasive margins of OSCC was associated with samples showing poor histopathological differentiation, high tumor budding activity and single cell invasion[11]. Further, increased expression of CD44 together with increased expression of NANOG was associated with poor survival in HNSCC patients as compared to those showing low expression of CD44 and NANOG[12].

Cells overexpressing CD44 (CD44^{+}) showed a self-renewal property with high tumorigenic potential, metastasis, and chemo-resistance. Therefore, CD44^{+} cells in HNSCC tumors are considered as CSC-rich population. CD44 paired with the overexpression of other stem cell markers such as ALDH and CD133 are being used for the isolation of CSCs from HNSCC tumors[13]. In TSCC, CD44^{+}/CD133^{+} cells showed stem cell-like properties such as high proliferation, invasion & migration with high tumorigenicity[13].

There have been recent reports linking CD44^{+} cells in HNSCC tumors to early angiogenesis[14], lymph node metastasis[15] and occult metastasis[16]. Moreover, overexpression of CD44 in adjacent normal epithelium of TSCC correlated with clinical stage and nodal metastasis in patients[17]. CD44 mRNA expression showed to have no correlation with age, sex, smoking history, size of the tumor and 5-year survival rate[18].

ALDH

ALDH is an enzyme superfamily, which converts aldehydes to carboxylic acids that are involved in drug resistance and detoxification. The human ALDH1A subfamily is involved in the retinoic acid pathway, which regulates gene expression and cell development in both the normal and cancer cells. The enzymes belonging to ALDH1A subfamily viz., ALDH1A1, ALDH1A2 and ALDH1A3 are located in the cell cytosol that catalyze the irreversible conversion of retinaldehyde to retinoic acid.

Amongst the ALDH1A subfamily, ALDH1A1 is overexpressed in the CSCs of HNSCC. Overexpression of ALDH is shown to correlate with overexpression of other stem cell markers such as OCT-3/4, NANOG, STELLA, SNAIL and BMI-1 in HNSCC. ALDH^{high} cells are also shown to have increased *in vitro* sphere formation ability and *in vivo* tumorigenesis ability[19,20]. Higher expression of ALDH also correlates with poor patient survival. Importantly, 500 ALDH^{high} cells isolated from HNSCC tumor showed a higher tumorigenic potential upon *in vivo* serial transplantations as compared to ALDH^{low} cells[21]. High expression of ALDH1A1 in oropharyngeal carcinoma co-related with poor differentiation in tumors and poor OS patterns in patients[22]. ALDH[high] TSCC

cells showed a serum independency and high ability to form tumorospheres than ALDH^{low} cells. ALDH^{high} cells also exhibited overexpression of stem cell-related genes such as NOTCH2[23]. ALDH1A1 expression directly co-relates with OS and lymph node metastasis in HNSCC[24]. The study showed a correlation of ALDH1A1 expression with TWIST1 expression in primary tumor tissues and lymph node metastases. Recent reports have demonstrated the involvement of ALDH isoforms in cisplatin resistance in HNSCC. Treating cells with ALDH inhibitors showed a decreased cell viability and reduced tumor burden *in vivo* when given in combination with cisplatin as compared to only cisplatin treatment. This study also showed that treating cells with the ALDH3A1 activator along with cisplatin increased cell survival[25]. Overexpression of ALDH1A1 in HNSSC tissues correlates with poor survival as compared to low ALDH1A1 expression[26]. Additionally, the expression of ALDH1 increased from epithelial dysplasia to oral carcinoma that was co-related with poor survival rates in OSCC patients[27]. In addition, low ALDH1A1 in the HNSSC patients showed significantly better OS as compared to high ALDH1A1 expression[25].

CD133

CD133/AC133/prominin-1 is a 97 kDa pentaspan transmembrane glycoprotein encoded by the prominin 1 (*PROM1*) gene. CD133 protein has an intracellular C-terminal domain, an extracellular N-terminal domain, and five transmembrane segments[28].

Spheroids obtained from HNSCC patient cells showed higher CD133 expression than normal epithelial cells[29]. High expression of CD133 with high expression of CD44 and CD117 was observed as marker of CSCs in OSCC cells[30]. TSCC cells overexpressing CD133 showed a higher *in vitro* and *in vivo* tumorigenicity as compared to low expression of CD133[31]. Recent reports have showed that CD133$^+$ OSCC cells exhibit properties such as self-renewal, drug resistance, higher tumorigenic potential and higher growth rate as compared to CD133$^-$ cells. Further, increased expression of stem cell markers such as NANOG, OCT4, SOX2 and ALDH1A1 in CD133$^+$ cells suggests that CD133 is a potential

CSC marker for OSCC CSCs[32]. TSCC-derived spheroids have been reported to overexpress CD133[33].

Other markers

Stemness markers such as OCT-3/4, NANOG, SOX2, KLF-4 and BMI-1 which have been associated with characteristics such as self-renewal, pluripotency and development of embryonic stem cells are overexpressed in CSCs in TSCCs.

Invasive TSCC cells overexpressing CD44 and SOX9 showed a higher expressions of SOX2 and OCT-4[34]. SOX2 overexpression in TSCC tissues correlated with poor OS in patients[35]. In addition, SOX2 overexpression was correlated with tumor size, cell differentiation, nodal metastasis and clinical TNM stage. In TSCC cells, knockdown of SOX2 showed a decrease cell proliferation, cell migration and invasion, and colony forming, which was reversed with overexpression of SOX2[36]. Moreover, an increased SOX2 expression was associated with poor OS, disease-specific survival and disease-free survival (DFS) in TSCC[37]. Downregulation of SOX2 by MTA-3 was reported to repress CSC properties and tumor growth in TSCCs and patients exhibiting MTA3^{low}/SOX2^{high} showed the worst prognosis[38]. Additionally, SOX2 regulate HEY1, which in turn regulates NOTCH4 expression followed by increased EMT in HNSCC cells[39].

The expression of both OCT-3/4 and NANOG was high in side population cells that correlated with distant metastasis[40]. Also, high OCT-4 expression in TSCC samples and NANOG in adjacent cut margin tissues have been reported as indicators of lymph node metastasis and worst prognosis[12]. Furthermore, TSCC showed an association between BMI-1 overexpression and increased proliferation, nodal metastasis and decreased OS in patients. Further, knocking down BMI-1 in TSCC cells showed a reduction in cell proliferation and migration, increased cell apoptosis, senescence and cisplatin sensitivity[41]. Ectopic overexpression of BMI-1 increased susceptibility of tongue carcinogenesis after exposure to 4-nitroquinoline-1-oxide in mice. The ectopic expression of BMI-1 regulates the pathways such as mTOR signaling, EIF2 signaling and p70S6K

signaling[42]. Additionally, high expression of OCT4 and BMI-1 along with ALDH1 co-related with poor survival in OSCC patients[27].

The *TRIM* (tripartite motif) gene family have ubiquitin ligase function that plays an important roles in various human diseases such as muscular dystrophies and atrophies and HIV infections *etc.*[43]. A recent report showed that overexpression of TRIM14 induces CSC-like properties with an increased sphere formation ability and cisplatin resistance in TSCC cells. Further, inhibition of TRIM14 by miR-15b, these characteristics were reversed implying that TRIM14 might play an important role in the maintenance of these properties[44].

MOLECULAR SIGNALING

The Wnt pathway

The Wnt signaling consists of the Canonical (involving β-catenin) and non-canonical pathways. Canonical Wnt signaling initiates when Wnt ligand binds to FRZ receptor and low-density LRP5/6 co-receptors. In the absence of the Wnt ligand, a complex of Axin, APC, GSK3 and CK1 phosphorylates β-catenin, leading to ubiquitination and subsequent proteasomal degradation of β-catenin. When present, Wnt ligand binds to FRZ receptor leading to FRZ-induced LRP5/6 phosphorylation followed by activation of the scaffold protein DVL. Activated DVL recruits Axin to receptors, which then inhibits the phosphorylation of β-catenin. Subsequently, β-catenin translocates into the nucleus promoting the transcription of Wnt target genes by interacting with T cell-specific factor/lymphoid enhancer-binding factor. Proper functioning of the Wnt signaling pathway is important for embryonic development and self-renewal of normal stem cells[45]. Deregulated Wnt signaling is involved in the development of various cancers such as colorectal cancer, epidermal cancer[46], hepatocellular carcinoma, breast cancer, glioma, *etc.*[47].

Recent studies showed that the suppression of the Wnt signaling inhibits the progression of OSCC. Micro RNAs such as miR-29a[48], miR-638[49], miR-106a*[50], *etc.* have been shown to suppress tumor progression by regulating the Wnt signaling. In addition,

chemical compounds such as quercetin (bioactive flavonoid) and niclosamide (antihelmenthic) have been reported to inhibit tumor progression by affecting the Wnt signaling in OSCC. Quercetin induces miR-22 expression thereby inhibiting the Wnt1/β catenin axis[51] while Niclosamide downregulates the expression of β-catenin, DVL2, phosphorylated GSK3β and Cyclin D1[52]. SOX8 has been shown to regulate chemoresistance and EMT in TSCC cells by activating the Wnt pathway suggesting that it might play a crucial role in maintainence of tongue CSCs[53] Furthermore, the spheroid forming ability and expression of CSC markers (CD44 and ALDH) that negatively impacted in the presence of the Wnt antagonist sFRP4 in tongue carcinoma cells. In presence of the Wnt ligand, WNT3a, these properties reverted[54]. The Wnt on and off pathway and their role in TSCC are shown in Figure 1.

The Hedgehog pathway
The Hedgehog pathway has three different types of ligand proteins in mammals such as Sonic-Hedgehog (SHH), Indian-Hedgehog, and Desert-Hedgehog. The ligand binds to the receptor PTCH1, and removes the inhibition on the transmembrane protein Smoothened (SMO). This further lead to the SMO accumulation in the cytoplasm. Subsequently, translocation of glioma-associated oncogene (GLI) proteins into the nucleus initiates the transcription of target genes that are involved in intercellular communication, organogenesis, regeneration and homeostasis[55].

Hedgehog pathway activation promotes angiogenesis in OSCC. Overexpression of SHH ligand in human TSCC and expression of PTCH1, GLI1 and GLI2 proteins in microvascular cells have been observed in the tumor invasive front[56]. Involvement of Hedgehog pathway has been shown in angiogenesis by macrophages and endothelial cells[57]. Hedgehog and TGFβ signaling are involved in bone invasion and destruction. GLI2 expression is associated with bone invasion. Silencing of GLI2 showed a reduction in invasiveness in orthotopic *in vivo* mice models[58]. GLI3 knockdown in TSCC cells have resulted in the downregulation of the CSC markers such as CD44, OCT-4 and BMI-1 genes and reduction in CSCs[59]. Further, increased expression of GLI1 has been shown

in spheroid forming cells in TSCC cell line[60]. The Hedgehog pathway and their role in TSCC have been shown along with inhibitors in clinical trials for various cancers except HNSCC (Figure 2).

The Notch pathway

The Notch pathway has four receptors such as NOTCH 1, NOTCH 2, NOTCH 3, NOTCH 4. The ligands are of two types viz. Delta-like ligands (DLL1, DLL3 and DLL4) and Jagged ligands (JAG1 and JAG2). Notch pathway also involves proteolysis by metalloprotease, tumor necrosis factor-α-converting enzyme (TACE) and γ-secretase. Binding of the ligand to the receptor releases the extracellular domain by TACE activity, which then binds to the receptor on adjacent cell, while the intracellular domain is cleaved by γ-secretase activity that further gets translocated into the nucleus, which acts like a transcriptional factor for the activation of the target genes (HES family, HEY, NF-κB, VEGF and c-MYC)[61].

Notch signaling has been shown to induce EMT in OSCC cells. Activation of Notch signaling directly co-related with the expression of markers such as E-cadherin and Vimentin and increased invasiveness of OSCC cells[62,63]. Decreasing NOTCH1 in the TSCC cells showed a reduction in invasiveness of the cells and decreased expression of MMP-2 and MMP-9 (associated with metastasis and invasion) in TSCC[64]. Additionally, activation of notch intracellular domain in tongue cell lines correlates with stemness characteristics such as spheroid formation and expression of stemness markers viz. OCT4, SOX2 and CD44. The knock down of NOTCH1 correlated with chemosensitization and loss of spheroid-forming ability. Further, high expression of NOTCH1 showed a significantly poor OS as well as DFS in HNSCC patients[65]. Further, NOTCH4 expression promoted cell-cycle dysregulation, cell proliferation, drug resistance and inhibition apoptosis. Elevated expression of NOTCH4 along with HEY1 co-related with OCT4, SOX2 and CD44 that showed increased migration and invasion in tongue carcinoma cells[66]. Recently, it was reported that a STAT3-activated long non-coding RNA, hepatocyte nuclear factor 1 homeobox A antisense RNA 1 promoted tumorigenesis by

activating Notch pathway in OSCC cells[67]. Further, high expression of NOTCH1 and JAGGED1 have been shown to be predictors of poor OS as well as DFS in oral carcinoma patients[68]. Notch signaling pathway and their role in TSCC are shown in Figure 3.

The HGF/c-MET pathway

HGF/c-MET pathway is involved in tumorigenesis in various cancers such as head and neck cancer, non-small cell lung cancer, hepatocellular carcinoma, ovarian cancer, bladder cancer, cervical cancer *etc.*[69]. The binding of ligand HGF to the kinase receptor c-MET leads to dimerization of two subunits. The dimerization results in the tyrosine residues autophosphorylation in cytoplasmic domain of the receptor, which then creates a docking site for various adaptor proteins that regulate pathways such as PI3K/AKT pathway and Wnt pathway[69].

HGF treatment has been shown to increase the expression of CSC markers and sphere forming ability of HNSCC cells, which were decreased upon c-MET knockdown. Transcriptional level of c-met was higher in cells with high ALDH activity (one of HNSCC CSC markers). Moreover c-MET knockdown in the HNSCC stem-like cells resulted in better survival in *in vivo* orthotopic tongue xenograft models[70].

Significant correlation has been observed in TSCC between the c-met expression and tumor stage, nodal status, clinical stage, locoregional recurrence, and distant metastasis. In addition, high expression of c-MET and autocrine motility factor receptor (AMFR) is associated with worse DFS. The study suggests that c-MET and AMFR expression can be potent prognosis marker that targets to decrease metastasis in OSCC[71]. Immunostaining for the c-MET showed a significant correlation with lymph node metastasis, recurrence and pathological stage of TSCC[72]. High c-MET expression is correlated with lymph node metastasis, greater depth invasion, decreased patient survival, increased invasion & migration *in vitro* and subcutaneous *in vivo* mice model injected with TSCC cells[73]. Further, knockdown of c-MET has shown to reduced cervical lymph node metastasis and improve survival patterns in *in vivo* models[74]. Overexpression of c-met was shown to correlates with occult metastasis in TSCC[75]. The HGF/c-MET pathway and its role in

TSCC is shown along with inhibitor in clinical trials for various cancers except HNSCC (Figure 4).

Other pathways

The transcription factor Nrf2 has been shown to induce expression of genes involved in cellular antioxidant and anti-inflammatory response. Normally, Nrf2 is located in the cytoplasm. Upon activation, it translocates to the nucleus, forming heterodimers with proteins such as c-JUN and small musculoaponeurotic fibrosarcoma protein that bind to antioxidant response element, which regulate expression of around 200 genes regulate anti-inflammatory and antioxidant response. Nrf2 is involved in the regulation of mitochondrial biogenesis pathways[76]. A compound named Plumbagin is shown to suppress EMT and stemness characteristics by regulating redox homeostasis and inducing reactive oxygen species (ROS) generation within the cell by suppressing the Nrf2 regulated pathways[77].

The Hippo/transcriptional coactivator with PDZ-binding (TAZ) signaling pathway is also involved in the regulation of properties such as cell proliferation, apoptosis, invasion, migration *etc.* in TSCC. When the pathway is off, the Yes-associated protein (YAP) and TAZ motif translocate to the nucleus thereby inducing the transcription of various genes by binding to the TEA domain family proteins and other transcription factors. LATS1 is activated by MST1 with Salvador through phosphorylation, which then phosphorylates the YAP/TAZ, retaining it in the cytoplasm, which then binds to 14-3-3 and gets degraded[78]. Factors such as HIF-1α[79] and epigallocatechin-3-gallate[80] affect the Hippo pathway to modulate proliferation, apoptosis, invasion and migration in TSCC cells.

Approximately, 90% HNSCCs overexpress EGFR pathway[81]. The EGFR is a receptor tyrosine kinase of ErbB family. The ligands of the EGFR are EGF, heparin binding EGF and TGFα. Upon receptor-ligand binding, the inactive monomer of the receptor dimerizes, either with another monomer of EGFR forming a homodimer or with another ErbB family receptor forming a heterodimer. This active dimer then

autophosphorylates the C-terminal domain of the receptor providing a docking site for the phosphotyrosine binding domain and Src homology 2 domain resulting in the activation of several signaling pathways such as MAPK, PI3K/Akt pathway, and phospholipase Cγ pathways[82]. Stimulation of OSCC cells by EGF showed an induction of EMT in the cells, which revealed morphological changes with downregulation of E-cadherin and upregulation of vimentin in the cells. In addition, stimulated cells showed an enrichment of stem-like population (CD44+/CD24-) with an increase in CSC markers such as ALDH1 and BMI-1 suggesting that EGF may be responsible to induce CSC properties in OSCCs[83].

Ephrin (EPH) receptors and their ligands play important roles in controlling the actin cytoskeleton and cellular responses including attraction/repulsion, migration and cell positioning during developmental stages[84]. Recent report have shown regulation of the expression of stemness markers by EPHA10. EPHA10 and its ligand EFNA4 showed to increase cell migration, sphere formation and expression of markers such as SNAIL, NANOG and OCT4 in OSCC cells. It was also reported that high m-RNA levels of EFNA4 with NANOG or OCT4 co-related with poor survival patterns in the OSCC patients[85].

CURRENT TREATMENT REGIME

The current treatment regime for HNSCCs is dependent on the TNM staging of the carcinoma based on T (tumor size considering depth of invasion), N (nodal metastasis considering extranodal extension or ENE) and M (presence of distant metastasis)[86]. For early stage of cancer, single modality treatment, in which mostly surgery, is preferred. With the progression of disease manifested either through larger tumor dimensions or nodal metastasis, multimodality in treatment is employed wherein surgery is followed by adjuvant chemotherapy or radiotherapy. In case of distant metastasis, where surgical intervention is difficult, chemotherapy is the preferred choice. Further, administration of adjuvant chemo or radiation therapy following surgery is shown to exhibit better patient survival[87]. Chemotherapeutic agents commonly administered are cisplatin, carboplatin, docetaxel, 5-fluorouracil, methotrexate and paclitaxel. Radiotherapy are also employed

in cycles of 1.8-2 Gy/day with a total dose of 66-72 Gy[88]. Targeted therapies specifically acting on certain upregulated pathways such as EGFR. For example, cetuximab targets the EGFR pathway in the HNSCC patients or, anti-PD1 agents for immunotherapy in HNSCC. Agents such as pembrolizumab and nivolumab, which target PD-1 have shown promising results in clinical trials[87]. Although, recent reports highlighted that owing to heterogeneity in PD-L1 expression throughout tumors and utilization of different methods and antibodies, there might arise errors in immunohistochemical assessment of PD-L1 prior to therapy decisions[89-92]. In course of currently existing assessment methods across various cancers, Marletta *et al*[91] observed that in HNSCC, the registration trial utilized the 22C3 clone (Dako) on Agilent autostainer link 48 while the European Medicines Agency granted administration of immunotherapy regardless of antibody, and the instrument used. The reports emphasized on the establishment of a standardized uniform protocol considering the heterogeneity of expression as well as the antibodies and platforms used for the assessment of PD-L1 before deciding whether immunotherapy should be administered to the patients[89-92].

Recently, a clinical trial consisting of 13 TSCC patients showed that immunotherapy of cytokine-induced killer cells after chemotherapy helped in reversion of immunosuppression caused during chemotherapy and surgery. Twelve out of thirteen patients survived for more than 90 mo post-therapy. No immune system related toxicities were reported in the surviving patients. No other side effects of the treatment were observed except for complains of aphthous ulcers by the two patients[93]. This showed that multiple modality treatments might improve survival and quality of life of the patients. However, for further improvement in treatment, more targets specific to CSCs need to be explored.

There have been few clinical trials worldwide as well as those involving Indian institutes for newer molecules targeting EGFR pathway, immune checkpoints, PD-L1, cyclin dependent kinases *etc.* as single treatment agent or in combination with other chemotherapeutic agents/radiation in HNSCC (Table 1). However, these trials are still in

early phases and do not particularly target CSCs. Therefore, further detailed study is essential in finding newer targets specific to CSCs in HNSCC.

CONCLUSION

CSC population plays an important role in the therapy resistance, recurrence and metastasis. These factors adversely affect the patient survival. Inspite of several years of research, most of the treatment regime employed currently target the tumor bulk. CSCs possess self-renewing property, slow cycling, aberrant cell signaling, dynamic ABC transporter system, DNA repair mechanism, epigenetic modifications and metabolic regulation *etc.*, enable CSCs to escape this therapy and thereby play an important role in recurrence, therapy resistance and loco-regional/distant metastasis.
A better understanding of the aberrant signaling pathways involved in poor prognosis and maintenance of the CSC population would be more effective in improving treatment outcome. Such an understanding would also be important in the prognosis, prediction and designing treatment regime that not only reduce the bulk of the tumor, but also effectively eliminate the CSC population thereby improving patient survival.

Molecules specifically targeting pathways that regulate CSC population administered in combination with conventional therapy or as a single treatment modality need to be studied in TSCC. Uncovering the signaling pathways for CSCs, and targeting them would enable better clinical outcomes.

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