Current cancer stem cell biomarkers in laryngeal cancer

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ABSTRACT

Larynx cancer (LCa), being an aggressive malignancy, is the second most commonly diagnosed malignant type of head and neck squamous cell carcinoma worldwide. Although there have been significant improvements in the detection and diagnosis of LCa in the last decades, it is still one of the considerable causes of cancer deaths and an urgent need for identification of novel biomarkers with diagnostic and prognostic significance still remains. The cancer stem cells (CSCs) resemble normal stem cells in terms of biological features and are considered to play critical roles in biological aggressiveness of tumors. Accumulating evidences have proven the existence of CSCs in various tumors including LCa and they are considered as driving force for tumor relapse, metastasis, and chemo-radioresistance. Comprehensive identification and characterization of the CSCs is of paramount importance for their further characterization to develop more effective and targeted therapeutic strategies against cancer. Here, we reviewed and summarized the most current literature to provide an insight into the functions and roles of current CSCs biomarkers in human LCa. We believe that this review will contribute to the knowledge of scientists especially working with LCa CSCs and will help understanding the significance of CSCs biomarkers implicated in LCa pathogenesis.

Introduction

Larynx cancer (LCa), being an aggressive malignancy, is the second most commonly diagnosed malignant type of head and neck squamous cell carcinoma worldwide [1] and it is estimated to constitute approximately 1% (13,360) of incident cancer cases in 2017 in the United States alone. It is also predicted to constitute an important fraction of cancer deaths with 3,660 attributed cases in 2017 [2]. LCa incidences have been reported to be increasing each year and the prognosis seems to remain poor along with high mortality rates [2, 3]. Although there have been significant improvements in the detection and diagnosis of LCa in the last decades, it is still one of the considerable causes of cancer deaths and an urgent need for development of novel therapeutic approaches against advanced LCa cases and identification of biomarkers with diagnostic and prognostic significance still remains.

The cancer stem cells (CSCs), constituting a small fraction of tumor cells, resemble normal stem cells in

terms of biological features and are considered to play critical roles in the biological aggressiveness of tumors [4]. They have been proposed to have intrinsic and/or acquired capacity to promote initiation, progression, spread, recurrence of the tumor and make it resistant against current clinical treatment strategies [5]. Accumulating evidences have proven the existence of CSCs in a variety of tumors including lung, brain, breast, prostate, colon, ovarian, and head and neck cancers [6, 7] and they are considered as the driving force for tumor relapse, metastasis, and chemo-radioresistance [8-10].

Comprehensive and accurate identification and characterization of the CSCs are of paramount importance for their further characterization to develop more effective and targeted therapeutic strategies against cancer. CSCs are mostly isolated through utilization of certain cell surface markers including CD133, CD44, ALDH1, and ABCG2 [8, 11-14]. Furthermore, CSCs enriched cell populations are known to display elevated expressions of stemness genes like SOX2, OCT4, KLF4, and BMI1 [15] (Table 1 and Figure 1).

Here, we reviewed and summarized the most current literature to provide an insight into the functions and roles of current CSCs biomarkers in human LCa. We believe that this review will contribute to the knowledge of scientists not only working with LCa, but also studying the CSCs in other cancers and diseases, and will help understanding the significance of CSCs biomarkers implicated in LCa pathogenesis.

CD133

Table 1. Laryngeal CSCs biomarkers associated with prognosis of disease.

| CSCs Biomarker	Expression Level	Association	Reference
CD133	High	Chemoresistance	[21]
		TNM stage, pathological grade, lymph node metastasis, poor overall survival and disease-free survival	[25]
CD44	High	Poor patient survival	[36]
		Lymph node metastasis	[37]
OCT4, KLF4, and SOX2	High	Poor patient survival	[50]
BMI1	High	Chemoresistance	[55]
ABCG2	High	Clinical stage, lymph node metastasis, and overall survival of patients	[61]
Beta1-integrin	High	Cervical lymph node metastasis, T stage, and histologic differentiation	[68]

CSCs=cancer stem cells
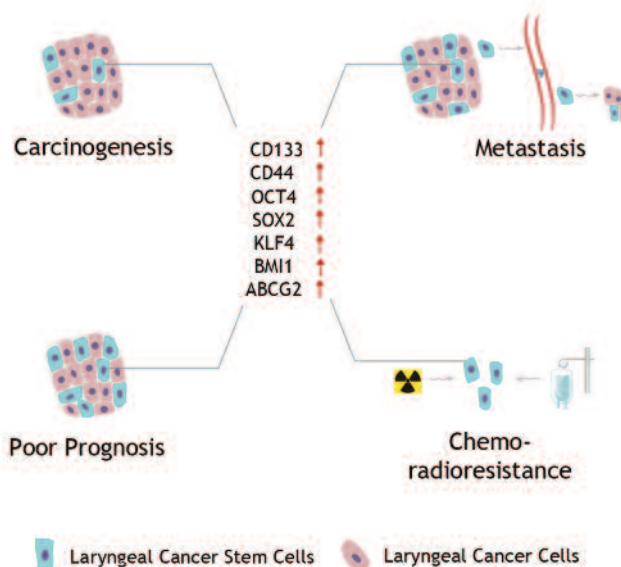
Figure 1. CSCs are characterized by significant overexpression of certain cell surface markers and stemness genes

CD133, a 120 kDa pentaspan transmembrane cell surface glycoprotein [16,17], is a commonly studied potential CSCs marker, which has been demonstrated to be expressed in distinct normal tissue stem cells [18]. It is an apical plasma membrane protein with potential to isolate stem cells from distinct tissues and tumors including LCa [19-21]. CD133 overexpressing cells were shown to possess high self-renewal capacity and multi-lineage differentiating potential both in vitro and *in vivo* [12].

CD133 positive (CD133+) cell fraction in Hep-2 cells, which is a well-studied human LCa cell line, was initially identified in 2007 by Zhou *et al.* [13], and CD133 was proposed as a candidate CSCs marker for LCa. They showed that purified CD133+ cells constituted only a small portion of total Hep-2 cell population and had profoundly increased self-renewal, proliferative, and differentiation capacity *in vitro*. Same group also evaluated the *in vivo* tumorigenic potential of CD133+ cells and found that those cells possessed significantly increased capacity for formation of new tumors *in vivo* when compared to CD133 negative (CD133-) and unsorted Hep-2 cells [14]. Furthermore, CD133+ Hep-2 cells within the side population, which was isolated through Hoechst 33342 dye exclusion, were found to exhibit enhanced cancer stem-like properties compared to corresponding CD133- side population cells both *in vitro* [22] and *in vivo* [23].

CD133, together with CD44, another important stem cell marker, were utilized to isolate cells with stem cell characteristics. When CD44 and CD133 positive cells (CD44+/CD133+) were injected to mice, they produced significantly larger tumors compared to those produced from other cell populations. CD44+/CD133+ cells, with stronger invasive potential, were also found to express several other stem cell markers [24]. In a recent study, Suer et al. [21] investigated the CSC potential of CD133+ cells isolated from freshly resected LCa specimens and found that CD133+ cells strongly express stemness genes such as SOX2, OCT4 and KLF4. Besides, overexpression of CD133 was significantly associated with TNM stage, pathological grade, lymph node metastasis, poor overall survival and disease-free survival in LCa patients [25].

CD133+ CSCs were found to exert stem cell features through upregulation of anti-apoptotic genes and activation of stem cells related signaling pathways like Hedgehog, Wnt and BMI1 [26]. Furthermore, the expression of Glut-1, which is required for transport of glucose (the essential source of energy for both stem cells and cancer cells) through cell membranes, was demonstrated to be significantly upregulated in CD133+ cells than in CD133- cells [27].

Recently, CD133+ cells were also reported to have strong resistance to irradiation and chemotherapy [28-30]. They were shown to have increased expression levels of ABCG2 and CXCR4, which were associated with resistance of tumors to regular chemotherapeutic reagents [21].

Taking these findings into account, CD133 might be considered to serve as an important target for cancer therapy against LCa. In a recent study, CD133+ cells were specifically targeted with mesoporous silica nanoparticles conjugated with chemotherapeutic drugs and siRNA against ABCG2. Interestingly, this CD133 targeted therapeutic approach enhanced the efficacy of chemotherapeutic drug-induced apoptosis through downregulation of ABCG2 in LCa CSCs and effectively inhibited tumor growth *in vivo* in a LCa mouse model, pointing the power CD133 as CSCs specific biomarker to be utilized in targeted therapies against LCa [31].

CD44

CD44 is a conserved single pass transmembrane glycoprotein, which is ubiquitously expressed throughout the body [32]. It interacts with several well-known ligands including hyaluronic acid (HA) and activates distinct signaling pathways involved in tumor progression and acquisition and maintenance of cancer stem cell characteristics [33].

The potential of CD44 in terms of its implication in stem cell characteristics of human laryngeal carcinoma cells was initially evaluated by Yu et al. [34] through isolation of CD44 positive (CD44+) cells from primary cultures of tumor samples obtained from 5 patients. They found that almost half of the cells were CD44+, which showed stronger proliferative capacity compared to CD44 negative (CD44-) cells. These findings pointed that CD44+ cells might be enriched for CSCs in laryngeal carcinoma samples [34].

As an important candidate CSCs biomarker in LCa, a significantly increased CD44 expression along with CD133 was detected in laryngeal tumor tissue samples obtained from LCa patients with lymph node metastasis, implying a potential role for CD44+ CSCs during cancer progression [35]. CD44 has been also proposed as a specific prognostic factor for LCa, where its overexpression in tumor tissues was

significantly associated with reduced 5 year overall survival of patients [36]. Interestingly, the soluble fraction of the CD44 protein in saliva samples of LCa patients served as a valuable prognostic biological marker. Patients with higher concentrations of salivary CD44 v6 tended to have larger primary tumors and metastatic lymph node involvement. Furthermore, patients with advanced tumor (stage III-IV) showed elevated levels of salivary CD44 v6 compared to those with early stage tumors [37]. These findings suggest that increased CD44 expression, which potentially points the enrichments of CSCs within the tumor tissue, would be a critical predictor for aggressiveness of the LCa.

Importantly, the number of CD44+ cells in tumors of most of the LCa patients tested profoundly increased upon fractionated irradiation at a total dose of 10 Gy, indicating their potential for resistance to radiotherapy [38]. Its expression was also demonstrated to significantly associate with response to radiotherapy in early stage LCa patients both at the mRNA and protein levels [39].

Considering the presence of CD44+ cells within the LCa tumor tissues and their potential for predicting the prognosis of patients, CD44 might be considered as an important biomarker for specific targeting of CSCs to eradicate the cancer. In an *in vivo* LCa model, targeting of CD44+ LCa cells via peri-tumoral injection of cisplatin conjugated with HA (a highly specific ligand for the CD44 surface receptors) provided a superior antitumor efficacy and a significant reduction in CD44+ positivity on ex vivo analysis [40]. In another study, a monoclonal antibody recognizing the extracellular domain of a CD44R1 was prepared as a potential immunotherapy tool. When it is injected intraperitoneally one week after the subcutaneous transplantation of HSC-3 human larynx carcinoma cells, it significantly suppressed the tumor growth in mice, which proposed CD44R1 as a possible molecular target for LCa therapy [41].

OCT4, KLF4, and SOX2

OCT4, KLF4, and SOX2 (OKS), as essential stemness factors, are crucial for early development and have been shown to participate in the tumorigenesis of various cancer types [42-44]. They are required for maintenance of self-renewal as well as pluripotency features of embryonic stem cells and CSCs [45-47]. Recent investigations have demonstrated that CSCs obtained from distinct cancer tissues and cell lines express several stem cell markers

including OKS factors. They have also been studied in LCa to investigate their involvement in the laryngeal carcinogenesis process and their potential as CSCs markers.

Their expressions have been found to be significantly upregulated in LCa tissue samples [48] and introduction of SOX2 into human laryngeal cancer cell line Hep-2 cells resulted in promoted migratory and invasive capabilities and its overexpression caused induction of epithelial mesenchymal transition [49]. Elevated SOX2 levels was also demonstrated to significantly correlate with poor prognosis of LCa patients [50].

Hypoxia induced CSCs enriched in Hep-2 cells [51] and CD133+ cells isolated from freshly resected laryngeal tumor specimens [21] displayed significantly elevated OKS levels, pointing in their importance as critical CSCs biomarkers for LCa.

However, current literature is limited and further studies are needed to understand the power of OKS factors as CSCs biomarkers for LCa and to enlighten the underlying mechanisms of laryngeal carcinogenesis through unraveling the molecular circuitries of stem cell biology in association with OKS factors.

BMI1

BMI1, with tumorigenic potential in a variety of cancers, has been shown to play critical roles in normal stem cell proliferation [52]. It has been found to induce tumor invasion, metastasis and chemoresistance of solid tumors [53, 54]. As an initial study, Yu et al. [55] demonstrated significant overexpression of oncogenic BMI1 protein,which involves in gene silencing, through chromatin modifications [55], in CD44+/CD133+ cells [34]. It was reported to be upregulated in laryngeal tumor tissue samples and CD133 positive Hep-2 cells, which provided maintenance of cell proliferation and prevented apoptosis [52, 56]. Its knockdown resulted in inhibition of *in vitro* proliferative, clonogenic, invasive capacity, and *in vivo* tumorigenic potential of CD133+ cells through up-regulation of p16(INK4A) and p14(ARF) [57]. More importantly, BMI1 expression was demonstrated to be co-localized with CD133 in LCa specimens [52, 58]. These findings suggested BMI1 as a molecular target to treat patients with LCa.

ABCG2

ABCG2 is known to participate in

chemoresistance and acquisition of stem cell features of tumor cells and its upregulation has been demonstrated in various cancer types [59]. Side population cells with CSCs features isolated from the laryngeal carcinoma cell line as well as primary laryngeal carcinoma cells [60] and CD133+ larynx CSCs isolated from freshly resected laryngeal tumor specimens [21] had significantly higher levels of ABCG2. The presence of ABCG2 in LCa tissue samples was significantly associated with clinical stage, lymph node metastasis, and overall survival of patients [61]. Besides, CD133+ cells, having elevated potential for clonogenicity and invasion, were demonstrated to be more resistant to chemotherapy, which was strongly correlated with higher expression of ABCG2 [62].

Taking these findings into consideration, elevated ABCG2 expression in CSCs population strengthens the idea that these cells are among the strong candidates for chemoresistance and recurrence of cancer.

Other Potential Cancer Stem Cell Biomarkers

Apart from well-characterized surface markers and stemness genes, there are also other factors reported to be as potential CSCs factors including Aldehyde dehydrogenase isoform 1 (ALDH1), h-TERT, and beta1-integrin.

Hep-2 cells overexpressing ALDH1, as a marker of CSCs in head and neck cancers, were reported to exhibit elevated proliferative and tumorigenic potential *in vitro* and *in vivo*, respectively [63]. However, in a very recent study, the relapse rate in patients who underwent curative-intent radiotherapy or chemo-radiotherapy was lower in those with ALDH1 positive tumors [64]. These two studies providing conflicting data about the biomarker potential of ALDH1 for laryngeal CSCs necessitates further investigations to make certain of its potential as a CSCs biomarker.

Another potential CSCs biomarker is human telomerase catalytic subunit (hTERT), which regulates telomerase activity in stem cells. Its expression was found to be increasing from non-cancerous laryngeal samples to grade III LCa specimens [65]. Knockdown of hTERT in Hep-2 cells significantly decreased telomerase activity and cell viability in Hep-2 cells [66]. Besides, its promoter was reported to have lower activity in LCa cells compared to that of radioresistant variant cells [67], pointing its potential as a CSCs biomarker.

CD44+/CD133+ cells obtained from human laryngeal primary carcinoma cells exhibited elevated levels of beta1-integrin (also known as CD29) [24]. Its expression was also significantly associated with cervical lymph node metastasis, T stage, and histologic differentiation [68].

These biomarker candidates need further research to clearly characterize their roles and functions in laryngeal carcinogenesis and their effects on the features of CSCs present in the laryngeal tumor tissue.

Future Perspectives

Experiencing a recurrence after a successful treatment is quite common in various cancer cases including LCa. In addition, current traditional therapies mostly fail to result in a positive outcome in advanced and metastasized tumors, which ultimately causes cancer related death due to disease progression and related organ failure. Recent studies noted that CSCs are crucial contributors of cancer initiation, progression, metastasis, recurrence, and chemo-radioresistance (Figure 2). Although underlying molecular mechanisms of how CSCs participate in cancer pathogenesis is not completely clarified and understood, scientists and clinicians aim to utilize them in therapeutic applications in fight against cancer. Unraveling both genetic and epigenetic circuitries of CSCs is essential to develop effective and successful therapies.

One of the major challenges of CSCs research is the true identification and characterization of CSCs, which is essential for specific targeting of CSCs. Therefore, to develop a therapeutic tool aiming to eradicate CSCs preferentially, the initial goal must be the determination of accurate surface marker(s) and stemness genes in CSCs. By this way, further development of anti-CSCs agents will help overcoming the acquired chemotherapy or radiotherapy resistance of tumors.

Furthermore, early detection and correct diagnosis are especially crucial for determination and application of accurate and effective cancer therapy alternatives in the clinical decision making process. Detailed characterization of circulating tumor cells, especially CSCs, will give opportunity to estimate the prognosis of the disease and decide the therapy strategy for each patient.

Conclusion

As a conclusion, the area of CSCs research is in

CD133

✓ CD133 overexpressing cells were demonstrated to possess high self-renewal capacity and multi-lineage differentiating potential both in vitro and in vivo

✓ CD133+ cells also reported to have strong resistance to irradiation and chemotherapy

✓ CD133 might be considered to serve as an important target for cancer therapy against LCa

CD44

✓ CD44+ cells exerts stronger proliferative, invasive capacity *in vitro* and produce larger tumors *in vivo*

✓ CD44 is implicated with metastasis and poor pateint survival

✓ CD44 might serve as an important biomarker for specific targeting of CSCs to eradicate the cancer

ABCG2

✓ ABCG2 participate in chemoresistance and acquisition of stem cell features of tumor cells

✓ CSCs enriched LCa cells have significantly higher levels of ABCG2

✓ Chemoresistant LCa cells strongly express ABCG2

Laryngeal CSCs

OCT4, KLF4, and SOX2

✓ OKS factors are required for maintenance of self-renewal and pluripotency features of CSCs

✓ Their expressions are significantly upregulated in LCa

✓ CSCs enriched LCa cells display significantly elevated OKS levels

BMI1

✓ BMI1, with tumorigenic potential in a variety of cancers, play critical roles in stem cell proliferation

✓ BMI1 expression was demonstrated to be co-localized with CD133 in LCa specimens

✓ Its knockdown results in inhibition of *in vitro* carcinogenic and *in vivo* tumorigenic potential of CD133+
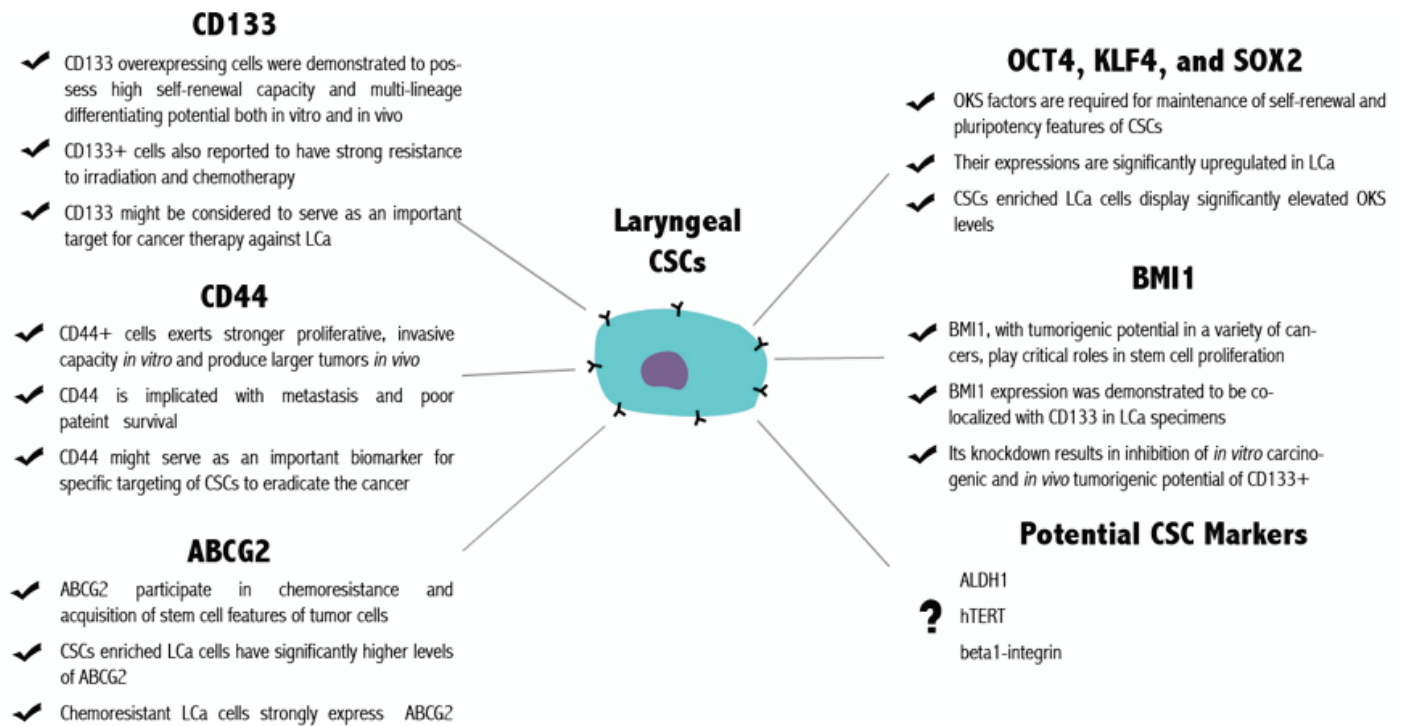
Potential CSC Markers

? ALDH1
hTERT
beta1-integrin

Figure 2. Certain biomarkers are associated with acquisition of stemness features in CSCs, which are crucial contributors of cancer initiation, progression, metastasis, recurrence, and chemo-radioresistance

its infancy and current understanding of the CSCs biomarkers in laryngeal pathogenesis is limited. Therefore, further intense investigations are needed for better understanding of the roles and functions of CSCs biomarkers in LCa biology.

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