Esophageal cancer stem cells and implications for future therapeutics

Abstract: Esophageal carcinoma (EC) is a lethal disease with high morbidity and mortality worldwide, and the incidence has been increasing in recent years. Although the diagnosis and treatment of EC have improved considerably, EC has rapidly progressed in the clinical setting and has a poor prognosis for its metastasis and recurrence. The general idea of cancer stem cells (CSCs) is primarily based on clinical and experimental observations, indicating the existence of a subpopulation of cells that can self-renew and differentiate. The EC stem cells, which can be isolated from normal pluripotent stem cells by applying similar biomarkers, may participate in promoting esophageal tumorigenesis through renewal and repair. In this review, major emphasis is given to CSC markers, altered CSC-specific pathways, and molecular targeting agents currently available to target CSCs of esophageal cancer. The roles of numerous markers (CD44, aldehyde dehydrogenase, CD133, and ATP-binding cassette subfamily G member 2) and developmental signaling pathways (Wnt/β-catenin, Notch, hedgehog, and Hippo) in isolating esophageal CSCs are discussed in detail. Targeting CSCs can be a logical strategy to treat EC, as these cells are responsible for carcinoma recurrence and chemoradiation resistance.

Keywords: esophageal cancer, cancer stem cells, CD44, ALDH, CD133, ABCG2

Introduction

Esophageal carcinoma (EC) is one of the most common malignancies worldwide. EC can be classified into two major pathological subtypes: esophageal adenocarcinomas (EACs) and esophageal squamous cell carcinomas (ESCCs). The incidence of EC has obvious regional characteristics. The prevalence of EC in Asian countries is mainly due to the increasing trend of established risk factors such as diet, alcohol, tobacco chewing, smoking, and physical inactivity. Smoking is a well-known risk factor for ESCC. The People’s Republic of China has a high incidence of EC, where ~90% of all cases are ESCCs, and the annual incidence and deaths in the People’s Republic of China account for 52.5% and 41.8% of those throughout the world, respectively. No efficacious treatment is currently available to cure this disease. The overall 5-year survival rate still varies from 15% to 25%. Several studies have shown that 70% of patients already suffer from cancer metastases, with a cervical lymph node metastatic rate ranging from 73.0% to 74.5% when dysphagia was first presented, which is the main clinical symptom of EC. The most important reasons for poor EC prognosis are delayed diagnosis, metastasis, high relapse rate, and a poor knowledge of the cellular and molecular mechanisms underlying the initiation, promotion, and progression of EC. Recent studies have shown that many solid tumors contain a cell subpopulation known as cancer stem cells (CSCs), which are present in many cancers, are thought to lead to aggressive tumor behavior and have exceedingly high...
The presence of cell-surface markers such as cluster of differentiation (CD)271 and CD90 on the CSCs has been routinely exploited to isolate cell subpopulations from the pool of cancer cells with stemness properties such as enhanced tumor-initiating capacity, increased recurrence rate, and increased resistance to therapy. Therefore, a thorough analysis of various esophageal CSC-associated markers is crucial to appreciate their potential fully before putting them into application.

Markers used in the identification of ECSCs

As a large number of CSC markers have recently become available in the literature, only very few are eventually validated. However, the notable variation makes it possible to identify the different CSCs from other cancer cells from the elaborate studies performed within this area. Different surface markers are simultaneously expressed in CSCs, and the use of a single marker may not be sufficient to isolate a pure subpopulation of stem-like cells from the pool of cancer cells. Consequently, studying the available CSC markers is urgently needed in relation to EC, and such markers may ameliorate the prognosis and overall therapeutic intervention for this type of cancer. In the following paragraphs, we discuss in detail the present knowledge about the ECSC markers CD44 and CD24, ABC subfamily G member 2 (ABCG2), CD133-C-X-C chemokine receptor type 4 (CXCR4), and aldehyde dehydrogenase (ALDH), since they are the most popular molecules in EC research (Table 1).
**Table 1** Cell-surface markers in cell lines of EC

<table>
<thead>
<tr>
<th>Cell markers</th>
<th>EC cell lines</th>
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<tbody>
<tr>
<td>CD271</td>
<td>KYSE620, KYSE210, KYSE30, KYSE40, KYSE180, KYSE410, KYSE510, and KYSE520</td>
</tr>
<tr>
<td>CD90</td>
<td>ECA-109, HKE15C1, KYSE30, KYSE40, KYSE180, and KYSE140</td>
</tr>
<tr>
<td>α6CD71</td>
<td>The mouse esophagus cells</td>
</tr>
<tr>
<td>TACC3</td>
<td>Eca-109, ECA-18, HKE-S1, KYSE30, KYSE140, KYSE150, KYSE410, and KYSE510</td>
</tr>
<tr>
<td>Lgr5</td>
<td>OE-33</td>
</tr>
<tr>
<td>CD44</td>
<td>YES-2</td>
</tr>
<tr>
<td>CD44+/CD24−</td>
<td>OE-33 and OE-21</td>
</tr>
<tr>
<td>ICAM1 and CD44</td>
<td>CE81/VT/VG, CE146/VGH</td>
</tr>
<tr>
<td>ABCG2</td>
<td>NCI-SB-ESC1 and NCI-SB-ESC2</td>
</tr>
<tr>
<td>CXCR4+</td>
<td>TE-1</td>
</tr>
<tr>
<td>ALDH1</td>
<td>EC109,64–49</td>
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**Abbreviations:** EC, esophageal carcinomas; CD, cluster of differentiation; TACC3, transforming acidic coiled-coil protein 3; Lgr5, leucine-rich G-protein coupled receptor 5; ICAM1, intercellular adhesion molecule 1; ABCG2, ATP-binding cassette subfamily G member 2; ALDH1, aldehyde dehydrogenase 1; CXCR4, C-X-C chemokine receptor type 4.

**CD44 and CD24**

CD44 is an extensively distributed cell-surface transmembrane protein that belongs to the adhesion molecule superfamily. Based on the special link between cells and the matrix, CD44 plays a supporting role in tissue development, inflammation, wound healing, and many other pathological processes. CD44 has a close relationship with cell motility, tumorigenesis, invasion, and metastasis, and it has received universal attention in the study of gastrointestinal cancer. CD44, which greatly influences tumor initiation and progression, is still considered an important biomarker for CSCs in ESCCs despite being expressed by the majority of ESCCs. Another stem cell-surface marker called CD24 is a heat-stable antigen that is expressed in many cancers. CD24 is a central marker for the diagnosis and prognosis of tumorigenesis because of its positive influence on cell–matrix and cell–cell interactions; thus, CD24 is involved in cell adhesion and migration. Almanaa et al isolated CSCs from a human EC cell YES-2 parental line using standard Aldefluor flow cytometry and then created a cell line enriched in CSCs (YES-2CSC). It showed that in YES-2CSC cells, CD44 levels are higher than in YES-2 cells, suggesting that CD44 may be one of the CSC markers in EC. According to current knowledge, the CD44+/CD24− subpopulation of EC cells exerts a higher proliferation rate and sphere-forming potential and is more tolerant to radiation in vitro than unselected or CD44+/CD24+ cells. Furthermore, CD44+/CD24− cells rapidly form xenograft tumors and are often deposited in hypoxic tumor areas. Based on current data and evidence from other groups, CD44+/CD24− may be an esophageal tumor stem-like cell marker. In addition, a study has reported that the intercellular adhesion molecule 1 and CD44 could have a compensatory effect on maintaining the stemness characteristics of ESCCs, suggesting that the combination of multitargeting therapies should be seriously considered to acquire a more potent therapeutic effect on CSCs of ESCCs. Furthermore, Honing et al found that CD44, sonic Hh, and sex-determining region Y-box 2 (SOX2) are associated with systemic recurrence and a poor survival outcome in esophageal cancer patients treated with neoadjuvant chemoradiotherapy.

**ATP-binding cassette subfamily G member 2**

Another important marker used in identifying ECSC is the ABC reporter. ABCG2, the second member of group G in the ABC family, is abundantly expressed in all kinds of cells, including placental trophoblast cells, intestinal epithelial cells, and hepatic tubule membrane and brain microvascular endothelial cells. Moreover, ABCG2 expression plays a role in the proliferation, initiation, and promotion of tumor cell development. V-ATPase, a kind of ATPase produced on the microsomes and presented on the cell membrane, is related to therapy resistance because of changes in intracellular and extracellular pH and environment. Huang et al sought to determine the role of ABCG2/V-ATPase in ESCC cells and observed that both ABCG2 and V-ATPase are highly expressed. ABCG2/V-ATPase expression and staining intensity are positively correlated with the pathological grade. In addition, ABCG2/V-ATPase expression may result in cell invasion, metastasis, and drug tolerance of EC. Exposure of EC cells to cigarette smoke condensate leads to upregulation of the xenobiotic pump ABCG2 and confers treatment resistance in EC. These results show that ABCG2 is indeed related to the ECSC phenotype.

**CD133 and CXCR4**

CD133 and CXCR4 are frequently adopted markers for detecting and isolating CSCs. CD133 is a kind of 5-transmembrane glycoprotein and a CSC marker in the esophagus, brain, colon, pancreas, prostate, liver, lung, and kidney. CXCR4, a membrane-bound receptor, selectively binds to the CXC chemokine ligand 12, which is also named “stromal cell-derived factor 1”. The CXC chemokine ligand 12/CXCR4 signaling pathway is closely related to carcinogenesis, progression, and metastasis, resulting in low overall survival and poor prognosis. In a study...
involved 154 tissues of ESCC patients and ESCC cell line TE-1, immunohistochemical staining and flow cytometry were performed to examine CD133 and CXCR4 expression in tissues and TE-1. The concomitant expression of CD133 and CXCR4 was up to 20.78% among 154 patient tissues. In cultured cells, CXCR4+ cells (CD133+CXCR4+ and CD133–CXCR4+) showed high invasive capacity. Furthermore, CD133+CXCR4+ cells displayed high proliferative and metastatic potential. Patients who synergistically expressed high CD133–CXCR4 had remarkably decreased disease-free survival and overall survival. These findings proved that concomitant high CD133–CXCR4 expression may be a novel marker for prognosis assessment and may be useful as a potential therapeutic target for ESCC patients. These results were consistent with the findings of other studies.

ALDH
ALDH1A1, a predominant isoform of ALDH family in the cytoplasm of mammals, is usually regarded as a marker for normal hematopoietic progenitor cells. It also appears in several malignant tumors with stem cell features, including leukemia, breast cancer, and lung cancer. Wang et al reported that ALDH1A1 is mainly expressed in the nuclei of ESCCs and associated with EC tumorigenesis and invasion. The expression of ALDH1A1 was recently found to be significantly increased in tumor spheres and 3D cultured cancer stem-like cells of ESCCs. This finding suggested that ALDH1A1 may be a good biomarker for the CSCs of ESCCs. Yang et al found that isolated cancer stem-like cells from an ESCC cell line EC109 depended on ALDH1A1 and that those cells that express ALDH1A1 high possessed stronger ability of self-renewal, repair, and tumorigenesis, indicating stem-cell properties. The degree of malignancy in cells was compared between ALDH1A1 high and ALDH1A1 low cells to further verify their stemness. ALDH1A1 high ESCCs express increased vimentin and matrix metalloproteinases 2, 7, and 9 at mRNA levels but decreased E-cadherin at mRNA level, indicating that the high invasive and metastatic capabilities of ALDH1A1 high cells may be achieved by the epithelial–mesenchymal transition (EMT) and secretory matrix metalloproteinases. Therefore, ALDH can be successfully utilized in the selection of EC subpopulation with stem-like properties that capture greater potentiality of being tumorigenic.

**Pathways involved in EC stem cells**
The signaling pathways involved in embryogenesis also play a pivotal role in oncogenesis. Some of the important pathways highly involved in the sustentation of ECSCs include Wnt/β-catenin, Notch, Hh, and Hippo. These pathways are associated with the balance of tissue homeostasis and normal stem cell renewal. Any dysregulation of such signaling drives CSC activity in diverse cancer types.

**Wnt/β-catenin pathway**
The Wnt/β-catenin signaling pathway is famous for regulating the generation and oncogenesis in many systems. In the normal physiological state, the Wnt/β-catenin signaling pathway regulates downstream genes involved in basic cellular biological functions, such as cell proliferation, differentiation, apoptosis, and cell death. Thus, Wnt/β-catenin signaling should be kept at the normal level to exert normal physiological function. However, a number of findings have shown that the Wnt/β-catenin pathway is constitutively activated in many cancers. The aberrant β-catenin expression could be an adverse underlying factor of ESCC in cancer progression, metastasis, and invasion. Ge et al revealed a new mechanism by which miR-942 triggers ESCC stem cell-like traits by activating the Wnt/β-catenin pathway. They found that miR-942 induces ESCC stem-like traits by directly targeting secreted frizzled-related protein 4, glycosynthase kinase 3 beta, and transducin-like enhancer protein 1, which are all vital negative regulators of the Wnt/β-catenin pathway. Furthermore, miR-942 was powerfully overexpressed in ESCCs and negatively correlated with EC prognosis, as seen from microRNA screening and real-time polymerase chain reaction assays. In a recent study, several critical stem-cell-related genes, such as Octamer-4 (Oct-4), Frizzled 10 (FZD10), prostaglandin-endoperoxide synthase 2 (PTGS2), and Krüppel-like factor 5 (KLF5), were upregulated in diverse histological subtypes in EC cell lines, as seen from quantitative real-time reverse transcription polymerase chain reaction, which was parallel to the results of Western blot. Moreover, the expressions of stem cell markers β-catenin, Oct3/4, and β1-integrin were remarkably higher than that of parental cells in EC cells following fractionated irradiation. In a study involving 136 patients with ESCC, the correlations between Wnt6 expression and survival parameters were analyzed. The results found that Wnt6, considered as a predictor for recurrence, may play a central role in tumor progression and inversely correlate with the poor long-term survival in ESCC patients.
differentiation, and stem cell maintenance. The Notch family may play an important role in cell differentiation and EMT in tumorigenesis because of its principal functions in CSCs.\textsuperscript{59} HEY1 and HEY2, which belong to a family of basic helix–loop–helix transcription factors, are two key target genes of the Notch signaling pathway. Forghanifard et al\textsuperscript{60} sought to explore the activation of the Notch signaling pathway in ESCC through expression analysis of the signaling target genes. Their results elucidated that HEY1 and HEY2 were significantly overexpressed in association with the clinicopathological features of ESCC patients, confirming that deregulated activation of the Notch signaling pathway plays a role in the development and progression of the malignancy and may be considered in therapeutic modalities to restrict ESCC progression. In another study, the expression of the Notch family in the ESCC lines KYSE70, KYSE140, and KYSE450 and virus-transformed squamous esophageal epithelial cell line Het-1A was evaluated at the mRNA and protein levels in vitro. The overexpression of Notch1 and Notch3 was compared in the above cell lines. In clinical samples, Notch1 protein expression was tested in the basal cells of human esophagus epithelium and squamous cell carcinomas. The authors reported that overexpression of Notch1 protein in ESCC tissues was significantly associated with advanced histological grade and poor patient survival. In addition, 5-fluorouracil (5-FU) resistance is correlated with Notch1 expression, as verified by Notch1 small interfering RNA experiments.\textsuperscript{59} Nonetheless, Notch signaling appears to be an attractive target for anticancer therapeutic interventions because of its role in stem cell maintenance and tumorigenesis.

**Hh signaling**

Although the presence of Hh signaling is almost negligible in adult tissues, it is an important component of embryogenesis and regulates the proliferation, differentiation, and migration of embryonic stem cells. Activation of Hh signaling by binding secreted Hh ligands to the membrane receptor results in nuclear translocation and activation of transcription factors of the glioma-associated oncogene (Gli) family, whose targets are composed of genes controlling the cell cycle, cell adhesion, and signal transduction.\textsuperscript{61} Hh–Gli signaling has been involved in the maintenance of different cancers, including breast, melanoma, and lung cancers.\textsuperscript{62–64} Sims-Mourtada et al\textsuperscript{65} showed that Hh pathway activity affects the proliferation rates of EC cell lines through upregulation of the G1–cyclin–Rb axis. In addition, they found that blocking Hh signaling enhances radiation sensitivity of EC cells. These results suggest that activating the Hh pathway may contribute to chemoradiation resistance in EC.

**Hippo pathway**

The Hippo signaling pathway is regarded as an important player not only in organ size control but also in tumorigenesis, because the disruption of any important components (mammalian ste20-like kinase [Mst] 1/2, salvador homolog 1 [Sav1] and large tumor suppressor [Lats] 1/2, and YAP-1) in this pathway can lead to tumorigenesis.\textsuperscript{66} YAP-1, the downstream effector of the Hippo signaling pathway, is constantly overexpressed in many cancer types and mediates the constitutive and acquired treatment resistance in EAC cells.\textsuperscript{21,67} Emerging evidence indicates the importance of YAP-1 and deregulation of the Hippo pathway during cancer development and progression. In a clinical research, EAC and ESCC were characterized by a significant increase in YAP-1 in cytoplasmic and nuclear cells.\textsuperscript{68} Recent studies have identified YAP-1 as a major inducer of CSC properties in non-tumorigenic cells and EC cells through direct upregulation of SOX9. Thus, the YAP-1–SOX9 axis can be an important therapeutic target in EC.\textsuperscript{19}

**Targeting ECSCs for therapy**

ECSCs, as well as local tumor microenvironment, play a significant role in tumor initiation and progression. The signal a cell receives from its microenvironment determines the cell’s fate. EC therapy targeted at the CSCs or the tumor microenvironment has been developed accordingly. In recent years, it has been shown that the enrichment of CSCs through transforming growth factor beta 1 (TGF-β1)-mediated EMT in ESCCs can be overcome by using erlotinib and cetuximab, which target CD44, Notch1, and Notch3 by suppressing expression of zinc-finger E-box binding homeobox 1 (ZEB1) and ZEB2, which are known regulators of EMT. Epidermal growth factor receptor inhibitors could be one effective therapeutic strategy targeting against the generation of CSCs in the ESCC microenvironment.\textsuperscript{69} All-trans retinoic acid can also induce ESCC differentiation and dramatically downregulate the expression of CD44 mRNA and protein levels, which is a platform that integrates cellular microenvironmental cues with growth factor and cytokine signals, suggesting a potential application of all-trans retinoic acid differentiation therapy for CD44.\textsuperscript{27} CXCR4 silenced by small interfering RNA can suppress the proliferation, invasion, and metastasis of EC cell lines KYSE-150 and TE-13 in vitro and in vivo. These results provide a theoretical and experimental basis for the gene therapy of ESCC using RNA interference technology.
based on the CXCR4 target site. Methramycin could repress basal- and CSC-mediated induction of ABCG2 and dramatically decrease the prolifand tumorigenicity of EC cells, thereby showing the potential activity for targeting ABCG2 and other stem-cell-related genes in thoracic malignancies. Metformin, usually used as an oral anti-hyperglycemic agent, may reduce cancer risk and have antitumor effects in many cancer types. The synergy between metformin and 5-FU can potentially treat CSCs and simultaneously increase the sensitivity of chemoradiation in EAC patients through ribosomal S6 kinase (S6K) phosphorylation, and the crosstalk between mTOR and Hh signaling confers tumor cell growth advantage and resistance to therapy. Others have found that YAP-1 knockdown by lentivirus short hairpin RNA in EC cells decreases tumor cell growth and increases 5-FU sensitivity in EC. These results were consistent with the recent finding of Huang et al, who reported that YAP-1 knockdown sensitized ovarian cancer cells to cisplatin and survivin inhibitors. Moreover, Verteporfin, a novel YAP-1 inhibitor, effectively inhibits YAP-1 and epidermal growth factor receptor expression and sensitizes the cytotoxic effects of 5-FU or docetaxel on EC cells. These data demonstrate that YAP-1-targeted modalities are a promising strategy when combined with cytotoxics to achieve maximal therapeutic effects. ABT-263, a potent Bcl-2 family inhibitor, is active against many tumor types. ABT-263 was reported to sharply suppress the CSC properties by Wnt/β-catenin and YAP/SOX9 axes in EC cells. The combination of ABT-263 and 5-FU significantly reduces tumor growth in vivo and suppresses the expression of stemness genes. Based on these findings, a novel mechanism of ABT-263 antimutumor effect is demonstrated, indicating that combining ABT-263 with cytotoxic drugs is worthy of pursuit in EC patients. The majority of these studies reported the effectiveness of targeting ECSCs in controlling different EC subtypes.

**Conclusion**

EC occurs with high prevalence in different regions of the world, and the high-incidence areas include the People’s Republic of China, Iran, South Africa, and France. EC is the fourth highest cause of cancer-related mortality in the People’s Republic of China and seventh in the world. Surgical resection and neoadjuvant chemotherapy or radiotherapy are the common treatment modalities used nowadays. However, EC is difficult to treat because of its resistance to current standard therapies. CSCs are thought to be the basic cause of tumor formation, metastasis, recurrence, and therapy failure. Therefore, an accurate understanding of ECSC is critical for the comprehension of the biology of esophageal epithelium in health and disease. Different markers and pathways, such as CD44/CD24, CD133, Notch, and Wnt/β-catenin, are deregulated in specific cancer cases, even in EC. Thus, their roles need to be elucidated so that these markers and pathways can be targeted to design definitive therapeutic modalities against drug-resistant EC and solve the problem of tumor relapse.

**Disclosure**

The authors report no conflicts of interest in this work.

**References**


