

Review Article

Cancer Stem Cell Markers in Esophageal Cancer

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Abstract

Esophageal carcinoma is one of the most malignant of all tumors, and affected patients have low survival rates. The lack of good prognostic and therapeutic targets indicate the mysterious biology of this cancer. Recently, studies with cancer stem cells (CSCs) revealed some clues for better understanding of cancer biology and development. CSCs are derived from normal stem cells and have essential roles in tumor initiation and development of malignancies, such as esophageal carcinoma. Self-renewal studies in CSCs have improved our understanding of the factors that regulate CSCs behaviour and may result in improved prognostic markers and new therapeutic targets. Abnormal activity of major cell signaling pathways such as Shh, Notch, and Wnt play important roles in converting stem cells from normal to cancerous. This manuscript reviews the importance of several processes in the maintenance of esophageal CSCs and introduces probable useful markers for CSC based ESCC therapy.

Keywords: Cancer Stem Cells; Esophageal Cancer; Signaling Pathways; Prognostic Markers

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Background

Stem cell biology is one of the most important topics of the century in biology and medicine. Understanding the mechanisms by which stem cells self-regenerate in an undifferentiated state will improve our understanding of human development and diseases.

Stem cells are known to have important characteristics such as self-renewal, in which they undergo unlimited asymmetric division [1, 2]. Somatic stem cells are able to maintain and regenerate normal tissues by means of asymmetric and self-renewing divisions; therefore, defects in their proliferation capacity may result in cell aging or tumorigenesis. In contrast to somatic stem

cells, other cells within tissues are transient, have limited proliferation, and eventually differentiate.

It has been shown that most cancers originate from a subpopulation of proliferating cells in tissues known as cancer stem cells (CSCs). CSCs were first observed in acute myelogenous leukemia [3-5], and subsequently have been observed in breast, colon, and prostate cancers [6-9]. The presence of CSCs in different tumor types indicates the tumorigenic role of a small subpopulation of cancer stem cells that support the maintenance of tumor cell growth [5, 10].

Three cancer propagation models have been proposed. The CSCs model describes two different kinds of cells; a small population of tumor cells with tumorigenic ability (tumorigenic cells), and their derived cells (non-tumorigenic cells), which have relatively less proliferative ability than tumorigenic cells and no potential for tumor formation. The stochastic model refers to a high proportion of tumorigenic cells in a tumor with genome instability, resulting in mutations and the generation of different genetically malignant clones in a tumor [11]. Finally, the interconversion model refers to tumorigenic cells switching between hyper- and hypo-proliferation statuses, which represent tumorigenic and non-tumorigenic cells, respectively [12].

It is believed that the derivation of non-tumorigenic cells from tumorigenic cells in some tumors is similar to the generation of differentiated cells from stem cells by asymmetric division in normal tissues. These tumorigenic cells are a result of initiating and required carcinogenic mutations in most malignancies, such as gastrointestinal tumors, because mature cells have not had sufficient time to accumulate such mutations [5].

Strict genetic programs regulate the relatively stable population of stem cells in normal tissues and organs [13, 14], whereas lack of such homeostatic regulation in cancer cells results in an elevated number of self-renewing cells, which cause an increase in tumor size. Elucidation of these regeneration mechanisms is critical to our understanding of cancer cell biology [15].

Esophageal carcinoma is one of the most malignant of all tumors. It is the sixth leading cause of cancer-related deaths in the world, and the 5-year survival rate in affected patients is approximately 42%

[16]. Incidence of esophageal carcinoma has increased significantly in western countries in the past 30 years, and 95% of the histological subtype is esophageal squamous cell carcinoma (ESCC) or adenocarcinoma [17-20]. Northeast Iran is one of the high-risk areas in the world; this area is located in the "Central Asian Esophageal Cancer Belt," which is along the Silk Road and extends from north Iran eastward to China [21, 22].

Understanding the molecular mechanisms involved in esophageal tumorigenesis is necessary for the improvement of prognosis and treatment. Of the many genetic alterations involved in the tumorigenesis of esophageal cancer [23], only a small part are associated with the clinicopathological features of tumor cells; hence, identification of genes associated with the clinicopathological features is necessary.

It has been shown that signaling pathways such as Shh, Notch, and Wnt, regulating the self-renewal of stem cells, play important roles in tumorigenesis [24, 25]. Here we review correlations between these pathways and self-renewal properties of esophageal cell carcinoma.

Wnt signaling and its role in regulation of CSCs

The Wnt/ β -catenin signaling pathway responsible for the regulation of self-renewal in normal stem cells and also plays an important role in tumorigenesis [26, 27].

The Wnt proteins prime the Wnt signaling pathway, bind to Frizzled receptors (Fz), and activate an inhibitor called Dishevelled (Dsh). Dsh acts as an inhibitor for a destruction complex (APC, Axin, and Gsk3) responsible for the ubiquitination and degradation of β -catenin. Undegraded β -catenin enters the nucleus and activates transcription factors involved in oncogenesis, such as T-cell factor (TCF), whereas in the absence of Wnt signals, the destruction complex ubiquitinates β -catenin for degradation by proteasomes (Figure 1) [28, 29]. Wnt signaling has been reported to be involved in the maintenance of intestinal epithelial [30] and skin stem cells [31].

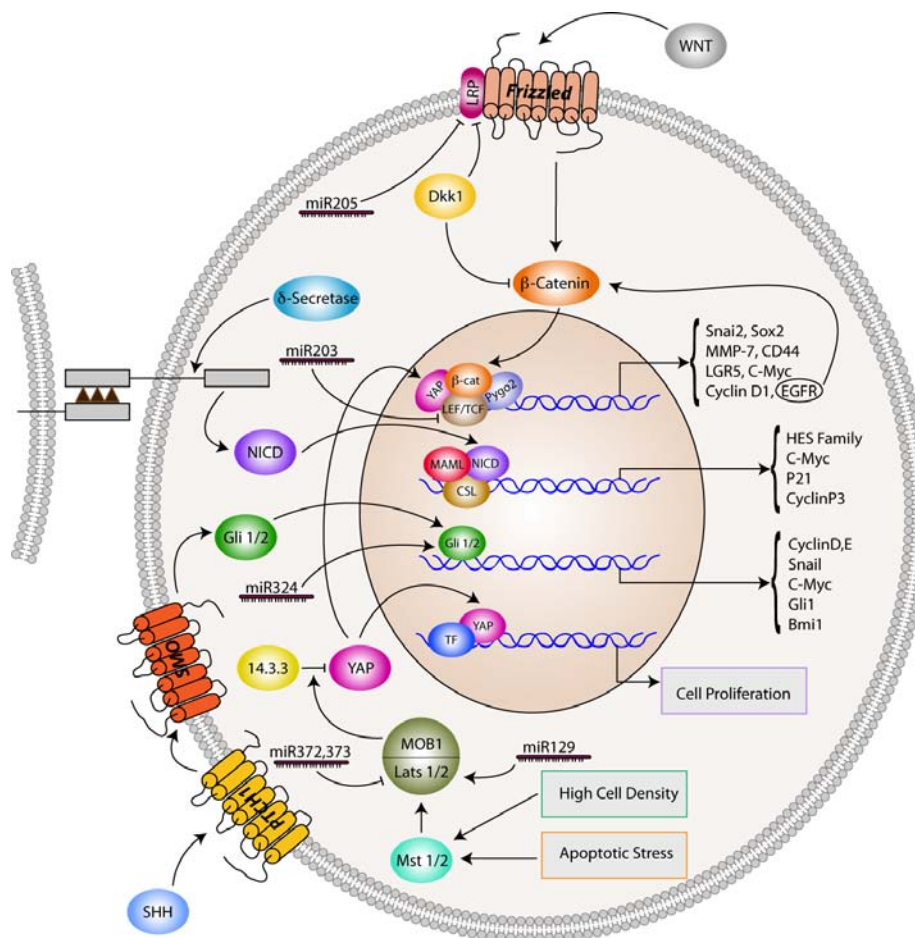


Figure 1 Actions and interactions of major cell signaling pathways in Esophageal CSCs

There are several important signaling pathways which have complex interactions together in Esophageal CSCs maintenance and miRNAs have a noticeable role in regulation of such pathways (see the text for details).

Axin, a suppressor in the Wnt signaling pathway, induces the ubiquitination and degradation of β -catenin and inhibits β -catenin from entering the nucleus. Axin underexpression has been observed in ESCC [32]. The Axin-ectopic expression targets β -catenin for ubiquitination and degradation, and inhibits proliferation of stem cells both in vivo and in vitro [33]. Although the main role of Axin is regulation of the Wnt/ β -catenin pathway, it is also involved in other signaling pathways, such as that of TGF- β , an inducer for smad3 activation and translocation to the nucleus, where smad3 acts as a transcription factor for several target genes that include p21, p15, p16, and RUNX3 [34]. Dickkopf-1 (DKK1), another Wnt signaling inhibitor, binds to the low-density lipoprotein receptor-related protein-5/6 (LRP5/6) and blocks the interaction between Fz and Wnt-1. This results in

β -catenin degradation and slowing of proliferation [35]. A relationship between DKK1 overexpression and esophageal tumorigenesis has been previously reported [36]. Recently we have analyzed the Msi1 expression in ESCC patients and showed its significant overexpression in 41.5% of tumors (unpublished data). Msi1 is one of the DKK3 inhibitors and can induce Wnt pathway via the DKK1 degradation. This result may also confirm the involvement of Wnt signaling in the ESCC tumorigenesis. Mutations in some subunits of destruction complexes such as APC can inactivate the complex in colon cancer and other malignancies [37]. Furthermore, overexpression of some components of this pathway, such as Wnt gene products, has been observed in epithelial cancers [38]. Wnt signaling, one of the most common signaling pathways in CSCs, targets several gene products. LGR5, one of the Wnt

signaling targets, is a G protein-coupled receptor that has been shown to be expressed in cryptal precursor cells and involved in esophageal [39], colorectal [40], hepatocellular [41], and endometrial cancers [42]. Abnormal nuclear E-cadherin expression is associated with esophageal carcinogenesis by regulation of β -catenin availability, resulting in altered expression of Wnt target genes [43, 44]. It is believed that E-cadherin overexpression and binding to β -catenin competitively inhibits β -catenin binding to Axin and the APC destruction complex proteins, resulting in an increase in cytoplasmic β -catenin levels, which then enter the nucleus and activate Wnt target genes [44]. Additionally, β -catenin is involved in cell-cell adhesion via an E-cadherin-mediated cell adhesion system, in which it binds to the cytoplasmic domain of cadherins to link the cadherin to the actin cytoskeleton; therefore, a decrease in β -catenin levels leads to tissue disorganization [45]. Underexpression of β -catenin in ESCC patients is associated with tumour undifferentiation and poor prognosis, and is also involved in the early stages of esophageal tumorigenesis [46].

CD44, a type I transmembrane glycoprotein, is one of the major CSC surface markers. It monitors the extracellular environment and plays essential roles in cell proliferation, migration and intercellular interactions [47-49]. Moreover, it facilitates the tumor cell distribution via the vessels during metastasis through its ligand-analogy for p-selectin receptor on endothelial cells [50]. CD44 is a Wnt target gene in ESCC [51].

Recently, we have revealed a significant correlation not only between PYGO2 and EGFR mRNA expression in ESCC, but also between PYGO2/EGFR expression and clinicopathological features. There is a mutual positive correlation between PYGO2 and EGFR in ESCC progression. EGFR may stabilize the cytoplasmic β -catenin and consequently increases its accumulation in the nucleus where it can prime gene expression of Wnt target genes in presence of other factors such as BCL9 and PYGO2 (Figure 1) [52]. Therefore, Wnt signaling pathway plays important roles in the CSCs of ESCC.

Notch signaling and its role in regulation of CSCs

Notch signaling is involved in cell proliferation and apoptosis. The Notch gene family encodes the membrane receptors Notch1 and Notch4, which are activated by ligands such as Delta and Jagged. Notch activation occurs through cleavage via a specific metalloprotease and γ -secretase, in which an intracellular domain is released. The intracellular domain of Notch (ICN) translocates to the nucleus where it binds to the CSL complex (CBF1, suppressor of Hairless/ Lag1) and activates several Notch signaling target genes, including cyclin D1, Hes1, and Nuclear Factor- κ B (NF- κ B) (Figure 1). In the absence of ICN, the CSL complex in the nucleus inhibits Notch target gene expression [53]. Deregulation in Notch signaling results in unlimited cell proliferation and tumorigenesis in colon, lung, head and neck, cervical, renal, and pancreatic cancers [53, 54]. In addition, it has been reported that deregulation of Notch signaling leads to a tumor suppressor effect seen in skin and hepatocellular cancers [53]. The exact role of Notch signaling depends on the cell context, in which it may have either oncogenic or tumor suppressor roles in tumorigenesis. Notch1 and ICN overexpression in SHG-44 glioma cells have significant roles in colony formation and the development of neurosphere-like colonies [55]. Notch1 overexpression in erythroleukemia cells influences the cell cycle and apoptosis by regulation of Bcl-xL, p21cip1, p27kip1, NF- κ B and Rb [56].

Deregulation of TGF- β and Notch signaling may result in Barrett's-related adenocarcinoma. Moreover, expression of Hes-1 and Sox-9, two important Notch signaling targets, is upregulated in Barrett's-associated adenocarcinoma tissues and cell lines [57]. Epithelial-mesenchymal transition (EMT), an important process during embryogenesis and tumor metastasis, begins with a decrease in cell adherence [58]. Twist1 and Mastermind-like 1 (MAML1) transcription factors, which are involved in Wnt and Notch signaling, respectively, are the main factors in the EMT process [59, 60]. Twist1 transcription factor is an inhibitor of E-cadherin expression and one of the

most important proteins in cell adherence [61].

We previously showed the probable correlation between Wnt and Notch signaling pathways in ESCC tumorigenesis by means of MAML1 and TWIST1 causing aggressive behavior in ESCC tumor cells through the EMT process. We demonstrated a significant correlation between MAML-1 and Twist1 overexpression and lymph node metastasis and surgical staging of tumors. Twist1 overexpression was associated with tumor depth. Moreover, Twist1 and MAML1 mRNA expression was significantly higher in advanced tumor stages (stages 3 and 4) than in less advanced stages in ESCC patients [62].

The hairy and enhancer of split gene families (HES/HEY), basic helix-loop-helix (bHLH) transcription factors, are the major downstream target genes of Notch signaling pathway. They are included in both self renewal and tumorigenesis [63]. Expressional analyses showed that the members of these families are either misregulated or significantly correlated with indices of poor prognosis in ESCC (data not shown). All together, these data prove the involvement of Notch signaling pathway in ESCC progression.

Hedgehog signaling and its role in regulation of CSCs

The Hedgehog (Hh) signaling pathway has multiple roles in embryogenesis, tumorigenesis, tissue regeneration, and homeostasis [64, 65]. It begins with the binding of secreted Hh factors to Ptch1 receptors, resulting in Ptch1 deactivation. Ptch1 is an inhibitor of Smo. Subsequently, Smo activation triggers a cytoplasmic cascade that translocates the Gli1 zinc finger transcription factor into the nucleus as an activator of hedgehog target genes BMP4, FOXA2, ISL1 and FOXM1 (Figure 1) [66-68]. The Hh pathway is associated with carcinogenesis in skin, prostate, and breast [69]. Esophageal cancer is one of the most malignant of all tumors and affected patients have low survival rates. The best treatment is tumor resection and chemoradiotherapy (CRT); however, only 25% of patients respond to CRT, whereas in 75% of patients we see no response due to the emergence of tumorigenic cells, which self-renew and are

CRT-resistant [70, 71]. CRT-resistant patients with relapsed tumors express Gli-1, one of the Hh signaling components [72]; therefore, Gli1 is a suitable marker for diagnosis of CRT-resistant patients. Moreover, Gli1 is expressed in half of ESCC tumors [73]. In general, Hh signaling activation in normal tissues is under strict control and every aberrant activation results in tumorigenesis via cancer stem cells, in which only a small fraction of tumor cells can repopulate.

Hippo signaling and its role in regulation of CSCs

The Hippo signaling pathway has an important role in the balance between cell proliferation and apoptosis in response to cell density changes [74]. Additionally, this pathway is involved in stem cell expansion and tissue regeneration [75, 76]. Mst1/2 kinases are the main components of Hippo signaling, and are activated by apoptotic stress. Mst1/2 and other mediators, such as Lats1/2 and Mob1, inhibit the YAP/TAZ transcription co-activators from entering the nucleus by binding to the cytoplasmic anchor 14-3-3 protein (Figure 1) [77]. Mouse studies revealed that YAP is involved in the Wnt signaling pathway through the interaction with β -catenin, leading to overexpression of the Wnt target genes Sox2 and Snai2 [78]. Moreover, YAP is involved in the regulation of several transcription factors, such as RUNX2, SMAD7, ERBB4, and P73 [79-82]. YAP overexpression is involved in the tumorigenesis of multiple human malignancies that include colon, ovarian, and prostate cancers [83] by inducing EMT, apoptosis inhibition, and other processes [84]. Overexpression of YAP is associated with poor prognosis and low survival rates in ESCC patients [85].

Cancer testis antigens in CSCs

Another group of proteins linked to cancer stem cells are the cancer testis antigens (CTAs) [86]. Although CTAs are expressed in a limited spectrum of normal tissues such as testis, placenta, and the other immature cells, they are expressed widely in several tumors [87-89]. SSX is one of the most important CTAs that

binds to domains associated with Bmi1 [90]. SSX is involved in embryogenesis by means of its role in EMT, an essential process during embryogenesis [86]. MAGE-A4 is another member of the CTA family involved in regulation of gene expression by association with the PIAS2 transcription factor. The C-terminal domain (CTD) of MAGE-A4 inhibits Miz1, a transcription factor involved in p21 expression; therefore, MAGE-A4 indirectly inhibits cell cycle arrest by p21 [91]. The CTD of MAGE-A4 also induces apoptosis through p53 [92]. MAGE-A4 overexpression has been reported in ESCC and bladder cancer [93, 94]. We observed overexpression of the CTAs MAGE-A4, LAGE1, and NY-ES O1 in 90.2, 39.0, and 41.4% of ESCC patients respectively, and 97.5% of our samples overexpressed at least one CTA. Moreover, we observed a direct correlation between MAGE-A4 overexpression and lymph node metastasis and tumor stage ($p < 0.05$), which implicates MAGE-A4 as a good prognostic marker for ESCC [94].

Chromatin structure in CSCs

Although signaling pathways and CTAs have important roles in the regulation of stem cell function, it is believed that epigenetic modifications in chromatin, such as histone modifications, DNA methylation, and nucleosomal remodelling also influence self-renewal in stem cells [95], and epigenetic aberrations are involved in carcinogenesis and tumor progression [96].

Many pluripotent genes, such as OCT4, SOX2, and NANOG, which are transcriptionally silenced by the polycomb group (PcG) proteins, are located in chromatin areas with trimethylated H3K27 histones [97]. Addition of the demethylator 5-azacytidine resulted in overexpression of these self-renewal markers [98]; however, methylation can result in diverse outcomes; for example, trimethylation of lys9 and 27 of histone 3 (H3K9, H3K27) resulted in chromatin inactivation, whereas H3K4 trimethylation or acetylation is associated with transcription [99]. To conserve stem cells, genes involved in stem cell differentiation must be down-regulated. PcGs, such as polycomb repressive complexes PRC1 and PRC2

are responsible for this important task. EZH2 and BMI1 are PRC2 and PRC1 members, respectively [100]. EZH2 is a histone methyltransferase that is responsible for the trimethylation of histone H3 at lysine 27 (H3K27me3) via its SET domain and acts as a suppressor of expression of tumor suppressor genes, including DAB2IP [101], E-cadherin [102], and RUNX3 [103]. EZH2 is involved in tumorigenesis of several cancers, and EZH2 gene silencing reduces tumor cell invasiveness [104]. Abnormal expression of EZH2 and BMI1 was observed in 14% and 16.9% of ESCC tumors, respectively, and EZH2 expression was associated with clinicopathological features such as size, metastasis, and survival rates in ESCC patients [105]. A significant association between clinicopathological features and BMI1 overexpression in ESCC patients has been observed, indicating that the BMI1 plays an important role in ESCC. BMI1 overexpression was higher in stage I/II tumors compared to stage III/IV tumors; whereas, BMI1 expression was significantly lower in patients without, than in those with, metastatic lymph nodes [106].

MicroRNAs as the CSC markers in ESCC

MicroRNAs (miRNAs), members of non-coding RNAs, are important molecules in regulation of a variety of biological processes such as cell proliferation and apoptosis, and their abnormal functions will result in tumorigenesis [107,108]. miRNAs are responsible for negative post-transcriptional gene regulation. They bind to the 3' untranslated region (3'-UTR) of target-mRNAs and lead mRNA degradation or cease-translation [109].

Functionally, mature miRNAs are produced via a sequential process from pri-miRNAs precursors. This process is mediated by two RNase III enzymatic complexes; Drosha/DGCR8 (Pasha) and Dicer (RLC complex) which are found in the nucleus and cytoplasm respectively. [110, 111]. In cytoplasm, mature miRNAs along with RNA-induced silencing complex (RISC) can bind to the 3'-UTR of target mRNAs and operate either cleavage of target mRNAs or miRNA-dependent translational inhibition [112].

Deregulated miRNAs which are related to the

tumorigenesis are often known as oncogenic microRNAs (oncomiRs). The first mammalian oncomiR, miR17-92 polycistron, was observed in mouse B-cell lymphoma model [113]. It is transcriptionally activated by the c-Myc and plays oncogenic role in various malignancies through targeting several tumor suppressor genes such as PTEN and E2F1 [114, 115]. miR-21 is another well known oncomiRs that targets different tumor suppressors such as MASPIN [116]. miR-21 overexpression is reported in several malignancies such as esophageal, pancreatic and colon cancers [117-119]. Some miRNAs such as miR-34a and miR-128 have suppressing roles in tumorigenesis and function as tumor suppressor via targeting Notch-1/2 and BMI-1, respectively [120, 121]. Notch 1/2 and BMI1 are the major component of Notch signaling and chromatin remodeling, respectively. In addition, underexpression of some miRNAs is involved in EMT process of tumor cells. Several studies have pointed that underexpressed miR-200 and miR-205 is significantly correlated with tumor metastasis [122, 123]. miRNAs expression has undeniable roles in ESCC. While underexpression of some miRNAs such as miR-518b, miR-143 and miR-145 is significantly correlated with lymph node metastasis and tumor invasion [124, 125] miR-21 has a remarkable higher level of expression in the sera of metastatic ESCC samples in comparison with the non-metastatic tumors [126, 127]. It has been shown that OCT4, SOX2 and KLF4 as the master regulators of CSCs are the targets of miR-145 [128, 129]. Since the most important characteristic of a biomarker is its accessibility, circulating biomarkers (such as miRNAs) in human serum are the best means of malignancies' diagnosis and can be used as a prognostic markers in a variety of cancers including ESCC [130-134]. It is worthy to note that such markers have a noticeable stability in severe conditions which is really idealistic in the case of sample preparation [127].

To sum up, deregulation of miRNAs in various cancers highlighted their critical role in cancer development acting as either oncogene or tumor suppressor [135]. Having considered the Figure1, there are strict connections among different CSC signaling pathways in ESCC, which can be governed by specific master mediators in the cell such as miRNAs.

Conclusion

CSC pathways analysis is one the main helping ways to understand CSCs involvement in tumor growth and development. Considering the higher efficiency of therapeutic modalities in the primary levels of tumor development, it is valuable to compare malignant cells biology and CSCs behavior due to prominent roles of CSCs in initiation of tumorigenesis.

Regarding to the CSCs features such as self-renewal and proliferative capacity and drug resistance, it seems that the tumor metastasis and relapse may be associated with CSCs. Although chemotherapeutic modalities discard most of the tumor cells, CSCs resist and stay dormant for a long time and they will be able to reactivate and repropagate. Besides working on mouse modeling which provides tumors resemble to the human tumors would prepare strong tools to assess the CSCs drug sensitivity.

CSCs distribution and maintenance is depended to various factors in tumor microenvironment such as oxygen [136]. Hypoxic targeted therapy would be an efficient way to ruin the CSC niches. Regarding to the presence of similar markers in normal and CSCs, most of therapeutic strategies will results in normal stem cell damage, emphasizing the introducing more specific CSC markers. Indeed, finding the efficient markers from the mentioned pathways which have discussed in this review will prepare more specific markers in CSC based ESCC therapy. Moreover, as mentioned above the miRNA networks in CSCs also play an important role in EMT, which is orchestrated by the tumor microenvironment signals [137], presenting such factors as useful targets in CSC therapy.

In this review we focused on the role of different self-renewal signaling pathways in esophageal cancer to introduce the probable more specific ESCC cancer stem cell markers. Signaling pathways such as Notch, Wnt, Sonic hedgehog (Shh), and others, form a complex net and communicate with each other, and these relationships play important roles in the preservation and function of cancer stem cells [138, 139]. It also has been demonstrated that CSC gene expression profiles in some cancers are associated with diagnosis and CRT-resistance. These signaling

pathways have helped researchers to identify new specific targets for esophageal cancer therapy. However, one challenge is the presence of these signaling pathways in somatic stem cells; therefore, targeting these components will also affect normal stem cells, and indeed, finding new methods to isolate CSCs within tumors will offer improved CT in esophageal cancer patients. Unfortunately, the majority of cancer therapeutic strategies won't be able to target only the CSCs and they target the other cancer cells which results in tumor relapses. Although, there are some specific anti CSC drugs for some cancers [140, 141], their clinical success depends on rational clinical consequences. Indeed, to reach such idealistic aims for CSC targeted therapy in solid tumors, further research should be performed to introduce the more efficient CSC markers.

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