

The significance of Cancer stem cell markers' gene expression and Relevance for Survival Outcomes

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Abstract: Solid tumors display complex biology and most therapies including chemotherapy cannot prevent therapy resistance and relapse. Most therapeutics target cancer cells, but recent data suggest the presence of cancer stem cells as cells with self-renewal and tumorigenic abilities. Cancer stem cell markers have been suggested to have prognostic value and can be targeted during cancer treatment and in resistant disease. CSCs have been postulated to play significant contextual roles in tumor initiation, progression, therapy resistance and metastasis. CSCs have thus been targeted by new generation cancer drugs. The transcriptional expression of several CSC markers in different cancers was evaluated by searching publicly available The Cancer Genome Atlas (TCGA) and Gene Expression Profiling Interactive Analysis (GEPIA) databases. We report here new findings on expression and prognostic significance of CSC markers in several cancers by examining the expression of CSCs markers in tumor tissues versus the adjacent normal tissues. We found that CSC markers were mostly highly expressed various tumors such as colon, lung, pancreatic and esophageal cancers. No CSC marker is expressed in the same pattern in all cancers and individual CSC marker expression was not linked to patient survival. This analysis calls for continued research on CSCs and clinical evaluation of the CSC markers in relation to prognosis of cancers in large population samples. Novel cancer drugs ought to target CSCs, cancer cells and tumor microenvironment variations.

Keywords: tumor microenvironment, biomarkers, solid cancers, computational biology, cancer stem cells, anti-cancer stem cell therapy, chemotherapy.

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1.0 Introduction

Huge progress has been made in the treatment of cancer but its health burden continues to increase globally, raising the need for novel therapeutic strategies to contain cancer [1, 2]. Whilst current anti-cancer strategies target genetic and epigenetic changes in cancer cells, most of these have proved to be unsuccessful [3-7]. Detailed analyses of tumors have revealed the important role the tumor microenvironment (TME) play in tumor maintenance and therapy resistance [8-14]. Within the tumor microenvironment are several stromal cells and the extracellular matrix (ECM) that contributes to tumor response to therapies and metastasis [5, 6, 15].

For many cancers, clinical factors, such as tumor stage and differentiation, have been reported to be associated with prognosis of the disease [5, 6, 16-22]. Many times the utilisation of these clinical factors is not enough for risk stratification and prediction of disease outcome. This can result in wrong clinical prognosis predictions. For many cancers, including those that are not well studied such as esophageal cancer, there is an urgent need for the identification of reliable prognostic factors that can accurately predict clinical prognosis. Several studies have shown that the prognostic value of cancer stem cells depends on the type of cancer and the histological subtype [23-28]. In addition, several recent studies have revealed that a major mechanism for post-therapeutic recurrence and metastasis of cancers is the presence of therapy-resistant cancer stem cells [5, 6, 29-31]. It has been demonstrated that chemoresistance to 5-fluorouracil and cisplatin in several cancer patients may occur through the increased expression of microRNAs such as miR-200 and cancer stem cell-related proteins [32, 33]. Furthermore, increased expression of the multidrug resistance

protein 2 (MRP2) has been shown in the tissue samples of patients resistant to neo-adjuvant chemotherapy including 5-fluorouracil, doxorubicin and cisplatin [34-36].

Thus the focus of many studies has been on a subpopulation of cells with stem cell-like characteristics called cancer stem cells (CSCs) [5, 6, 37]. CSCs have been identified in many cancers such as breast, glioma, melanoma, ovarian, head and neck cancer [5, 38-45]. Besides being important in the initiation, maintenance and relapse of tumours, CSCs have been shown to modify neoplastic cell behaviour and aggressiveness as well as therapeutic response. CSCs are rare tumour cells with the ability to self-renew and can proliferate extensively. They also have the ability to resist chemotherapy and radiotherapy treatments [5, 38, 40-42, 46-49]. These cells can form tumour-spheres *in vitro* and have been shown to be enriched for tumorigenic cells by their ability to form xenograft tumours in severe combined immunodeficient (SCID) mice [50-54]. CSCs can be isolated through various methods including the use of antibodies against various surface markers such as cluster of differentiation 44 (CD44), CD24, CD133, and CD166 [46, 53-57]. Many studies have shown that CD44⁺/CD24⁻ phenotype is associated with a worse prognosis in many cancers including breast cancer [58-61]. Other markers such as CD90, aldehyde dehydrogenase 1 (ALDH1), EpCAM and p63 have also shown to be useful in this regard [62, 63]. Besides the use of antibodies, CSCs can be isolated and identified via the use of side population cells. Side population cells in tumours are a small subpopulation of cancer cells with stem cell-like properties and can be isolated and identified by dual wavelength FACS analysis [56, 57, 64-66]. In several cancers, it has been shown that the side population tends to enrich CSCs and can be isolated using Hoechst 33342 dye [64-66].

Overall, CSCs are an emerging target for cancer therapy and any therapy targeting CSCs hold great potential for improving cancer treatment and outcome. In this study we performed a bioinformatic analysis to determine the prognostic value of CSC markers in several cancers. We utilized the publicly available databases The Cancer Genomic Atlas (TCGA) (<http://cancergenome.nih.gov>) and Gene Expression Profiling Interactive Analysis (GEPIA) (<http://gepia.cancer-pku.cn>) to determine CSC markers' (*ABCB1*, *ABCG2*, *ALDH1*, *CD24*, *CD44*, *CD90*, *CD133*, *CXCR4*, *EpCAM*, *ICAM1* and *NESTIN*) expression in tumor tissues versus the adjacent normal samples and in relation to patients' overall survival. Our analysis reveal that whilst CSC markers' expression is upregulated in tumor tissues versus normal samples, individual CSC markers expression may not be associated with overall patients' survival.

2.0 Materials and Methods

2.1 Bioinformatic Analysis of CSC markers' RNA-seq analysis

This study utilised the publicly available TCGA and GEPIA databases and did not involve human subject recruitment nor animal studies. Whole genome messenger RNA expression levels of *ABCB1*, *ABCG2*, *ALDH1*, *CD24*, *CD44*, *CD90*, *CD133*, *CXCR4*, *EpCAM*, *ICAM1* and *NESTIN* were examined in tumor and normal adjacent tissues (Match TCGA normal and GTEx data) in relation to patients' survival outcomes. TCGA and GEPIA data was accessed for different cancers in March 2020 via the respective portals. The GEPIA website contains web-based tools that allow in-depth analysis of the CSC markers expression within the TCGA and GEPIA databases.

2.2 Statistical Analyses

Statistical analyses were performed using GraphPad Prism software (version 6; San Diego, USA). In addition, significance of differences was tested by Student's t-test and one way analysis of variance (ANOVA) test. Statistical significance between groups regarding overall survival was performed by the Kaplan-Meier analysis with the log-rank test (95 % confidence interval). GEPIA use statistical analysis that divided patients into high and low expression of CSC markers. The same number of patients for each group was used to analyze survival chance. Statistical significant was set as $P < 0.05$.

3.0 Results

3.1 Significance of Cancer stem cells in tumorigenesis

3.1.1 Colon Cancer

One of the most deadly cancers, colorectal cancer, has been reported to contribute above 10% of diagnosed cancers worldwide and causes approximately 9 % of all cancer deaths each year [67, 68]. The incidence of colorectal cancer is increasing globally and patients' survival varying greatly among countries. Intense research continues to unravel the biology and mechanism of progression of colorectal cancer [69].

Bioinformatic analysis showed that CSC markers, *CD24*, *CD44*, *CD90* and *CD133* expression was significantly enhanced in colon adenocarcinoma (COAD) compared to adjacent normal tissues (**Figure 1**). Surprisingly, another CSC marker, ALDH1 expression was significantly reduced in tumor samples versus normal samples (**Figure 1**). Consistent with the above results, 10 out of 12 colorectal cancer specimens showed medium to high

CD44 protein expression based on immunohistochemistry-based data available at Human Protein Atlas database (www.proteinatlas.org). In the same vein, 12 out of 12 colorectal cancer specimens showed medium to high CD133 protein expression based on immunohistochemistry-based data available at Human Protein Atlas database. However, association analyses of CSC markers' expression with prognosis of COAD revealed that there were no significant differences in survival between patients expressing high CSC markers compared to patients expressing low levels of the same CSC markers (**Figure 1**).

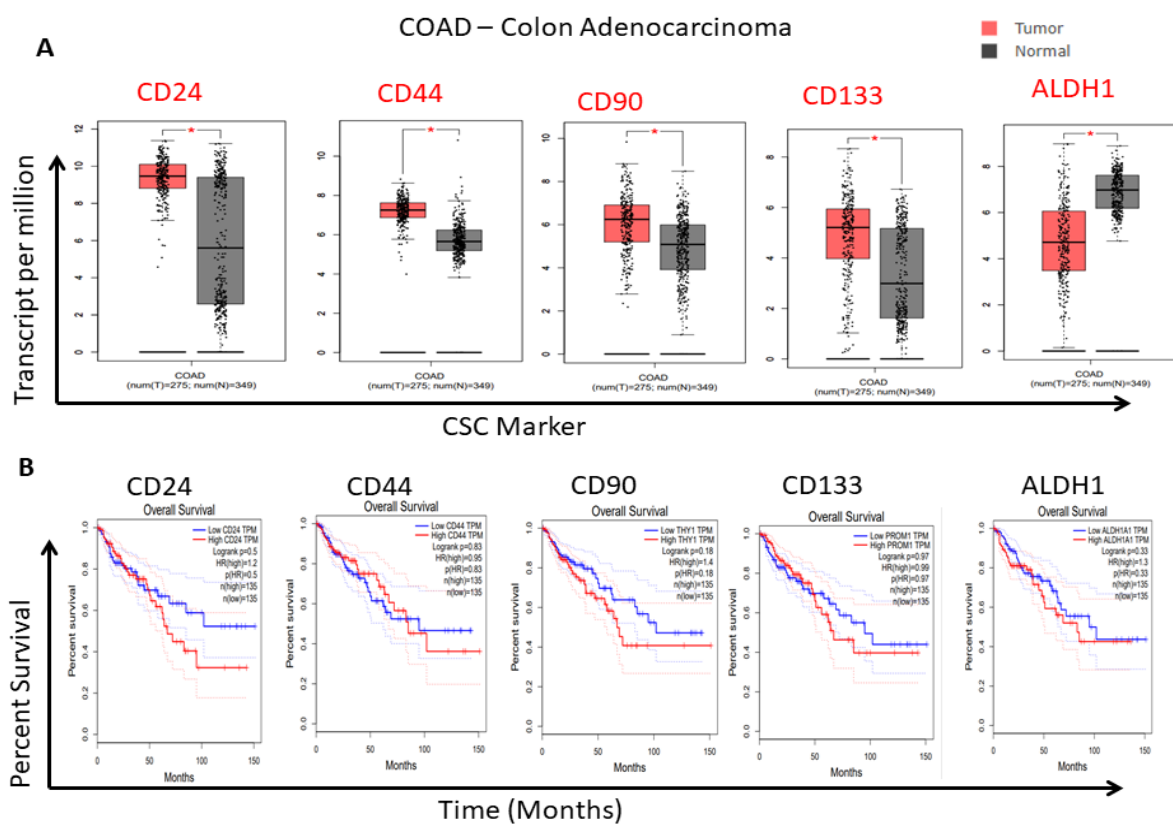


Figure 1. (A) Cancer stem cell markers' gene expression profiles in colon adenocarcinoma (COAD). The expression of CSC markers CD24, CD44, CD90, CD133 and ALDH1 in colon adenocarcinoma tissues and adjacent normal tissues (Box plot) based on TCGA/GEPIA database. Data is based on TCGA COAD samples $n = 275$; normal = 349. CSC markers showing significant differences between tumor and normal samples are shown in red with * indicated for $p < 0.05$ (B) Kaplan-Meier overall survival analysis of COAD patients by CSC markers expression in TCGA data set (high expression ($n=135$), low expression ($n=135$)).

3.1.2 Lung cancer

The most commonly diagnosed cancer globally is lung cancer and its incidence reported to be around 11.6 % of the total cases of cancer [1]. Lung cancer results in more cancer deaths than any other cancer, accounting for around 18.4 % of the total cancer deaths [1]. Several risk factors have been identified and include exposure to chemicals, tobacco, smoke and asbestos [4, 67, 70, 71].

With the exception of *CD133* and *ABCG2* expression, which showed similar expression in lung adenocarcinoma (LUAD) tissues versus controls, the expression of CSC markers, *CD24*, *CD90* and *EpCAM* was significantly upregulated in TCGA LUAD samples compared to adjacent normal samples (Figure 2). Consistent with the above results, 10 out of 12 lung cancer specimens showed medium to high EpCAM protein expression based on immunohistochemistry-based data available at Human Protein Atlas database (www.proteinatlas.org). Analysis of association of CCS markers expression with prognosis of LUAD cancer patients revealed no significant difference in overall survival between LUAD cancer patients expressing low and high CSC markers (Figure 2).

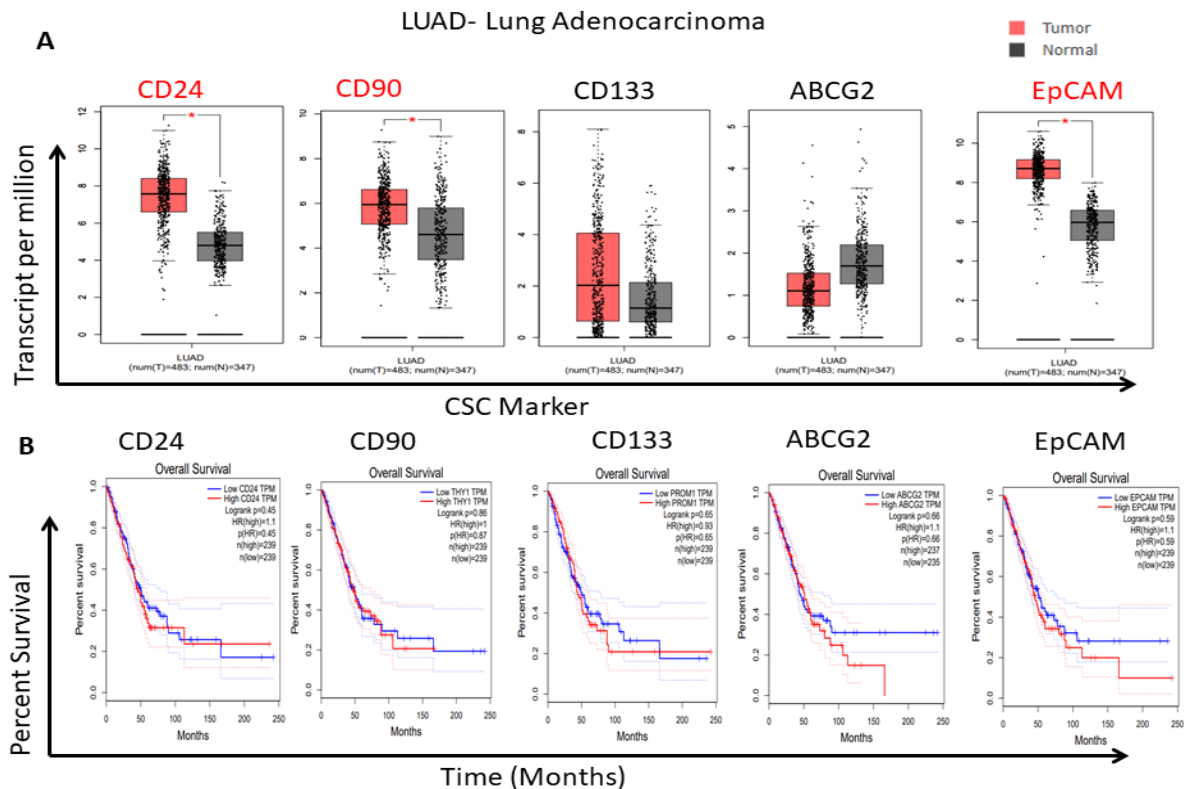


Figure 2. Cancer stem cell markers' gene expression profiles in lung adenocarcinoma (LUAD). The expression of CSC markers CD24, CD90, CD133, ABCG2 and EpCAM in lung adenocarcinoma tissues and adjacent normal tissues (Box plot) based on TCGA/GEPIA database. Data is based on TCGA LUAD samples $n = 483$; normal = 347. CSC markers showing significant differences between tumor and normal samples are shown in red with * indicated for $p < 0.05$ (B) Kaplan-Meier overall survival analysis of LUAD patients by CSC markers expression in TCGA data set, high expression ($n=239$), low expression ($n=239$)).

3.1.3 Pancreatic Cancer

One of the most lethal cancers is pancreatic cancer with estimates showing that above 338,000 people were diagnosed with the disease worldwide in 2012 [72, 73]. One of the major histological subtypes is pancreatic ductal adenocarcinoma and show aggressive growth resulting in high mortality rate [74, 75]. Pancreatic adenocarcinoma (annotated as PAAD within TCGA and GEPIA databases) is the most common pancreatic cancer, with estimates showing that 9 out of 10 people with pancreatic cancer have this type of cancer [76-78].

Using the TCGA database samples, our bioinformatic analysis showed that, *ABCB1*, *CD133*, *CXCR4*, *EpCAM* and *NESTIN* expression was significantly upregulated in pancreatic adenocarcinoma (PAAD) tumor samples versus adjacent normal samples (Figure 3). In agreement with the above results, 9 out of 12 pancreatic cancer specimens showed medium to high CD133 protein expression based on immunohistochemistry-based data available at Human Protein Atlas database (www.proteinatlas.org). Assessment of the association of CSC markers expression with prognosis of PAAD cancer patients revealed no significant difference in overall survival between PAAD cancer patients expressing low and high *CD133*, *CXCR4*, *EpCAM* and *NESTIN* CSC markers (Figure 3). Low expression of *ABCB1* was associated with low survival in PAAD patients (log rank $p = 0.0059$) (Figure 3).

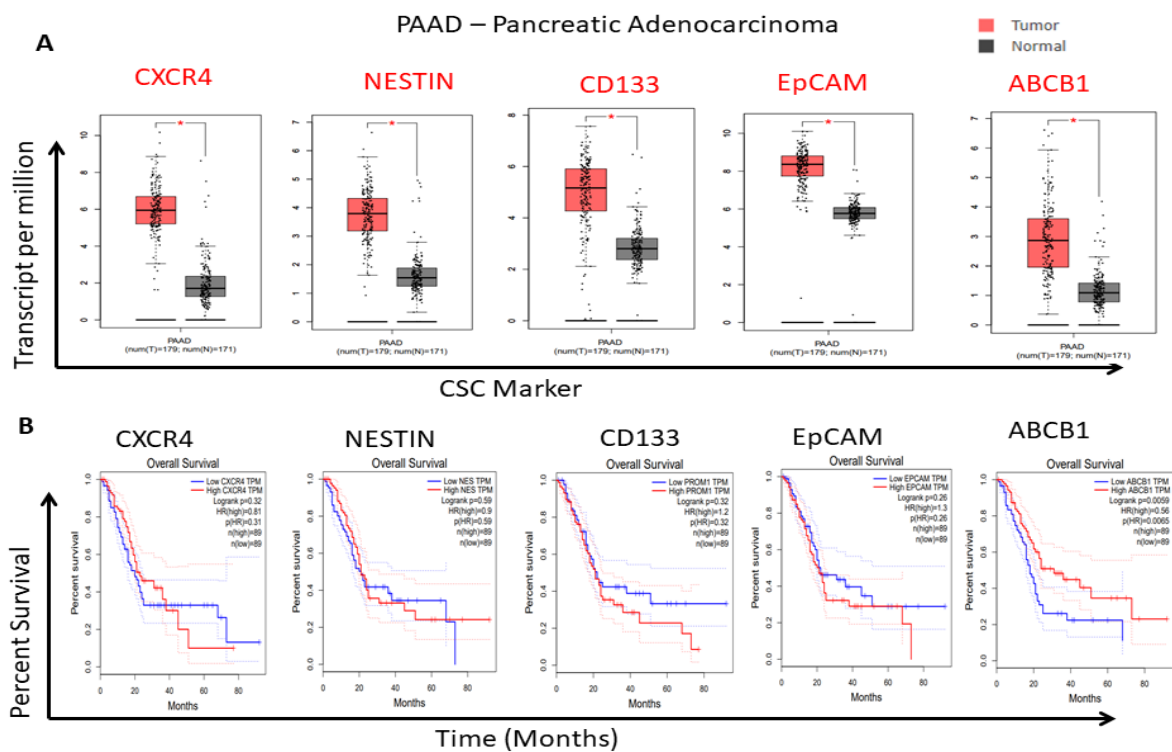


Figure 3. Cancer stem cell markers' gene expression profiles in pancreatic adenocarcinoma (PAAD). The expression of CSC markers CXCR4, Nestin, CD133, EpCAM and ABCB1 in pancreatic adenocarcinoma tissues and adjacent normal tissues (Box plot) based on TCGA/GEPIA database. Data is based on TCGA PAAD samples $n = 179$; normal = 171. CSC markers showing significant differences between tumor and normal samples are shown in red with * indicated for $p < 0.05$ (B) Kaplan-Meier overall survival analysis of PAAD

patients by CSC markers expression in TCGA data set, high expression (n=89), low expression (n=89).

3.1.4 Esophageal cancer

Esophageal cancer is one of the most understudied cancers and has been associated with a poor prognosis [4, 79]. Several risk factors have been identified but new molecular targets are needed for effective therapy and improvement in patients' outcomes.

Our analysis revealed no difference in expression of commonly used CSC marker, CD44, in tumor tissues compared to normal tissue (Fig 4). Other CSC markers, including ALDH1A1, CD90, ICAM1 and EpCAM showed upregulated expression in ESCA tumor samples versus adjacent normal tissues (Fig 4). Our analysis revealed no association between CSC markers' expression and overall survival between patients with low and high expression of CSC markers (Fig 4).

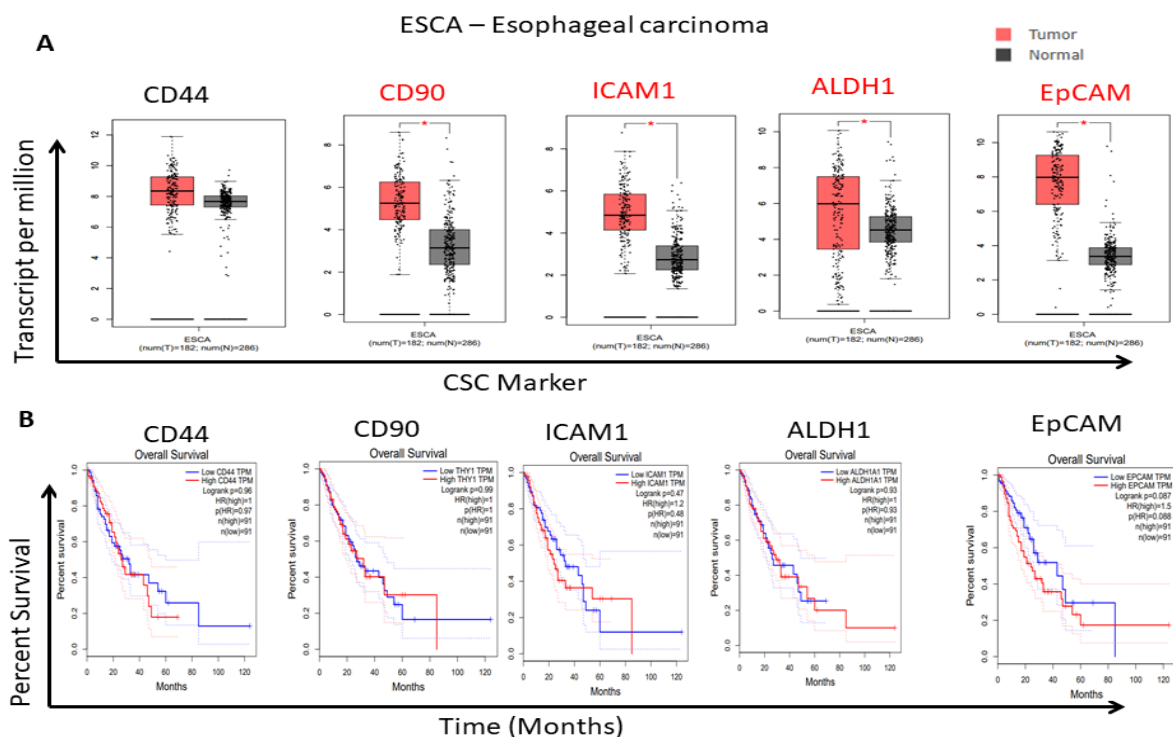


Figure 4. Cancer stem cell markers' gene expression profiles in esophageal carcinoma (ESCA). The expression of CSC markers CD44, CD90, ICAM1, ALDH1 and EpCAM in esophageal carcinoma tissues and adjacent normal tissues (Box plot) based on TCGA/GEPIA database. Data is based on TCGA ESCA samples n =182; normal = 286. CSC markers showing significant differences between tumor and normal samples are shown in red with * indicated for $p < 0.05$ (B) Kaplan-Meier overall survival analysis of ESCA patients by CSC markers expression in TCGA data set (high expression (n=91), low expression (n=91)).

4.0 Discussion

Cancer continues to cause morbidity and mortality globally, with its incidence on the rise in many developing countries. Current cancer therapies eliminate most cells within a tumor but well advanced cancer can progress to drug-resistant disease and metastasis [80]. In addition, tumor heterogeneity contributes to therapy failure and fatal disease outcomes. Several lines of evidence point to therapy itself, especially chemotherapy, causing tumor heterogeneity and thus poor outcomes in patients. Furthermore, according to the CSC theory, most therapies fail to prevent relapse partly due to the presence of a small subpopulation of tumor cells called cancer stem cells [3, 5, 6, 37, 81]. CSCs reside in the tumor microenvironment and have been proposed to contribute towards the development of therapy resistance and relapse as they can become quiescent. Developing novel strategies against treatment-resistant cancer cells, including CSCs, remains a significant challenge. Despite the considerable progress made in the treatment of cancer in recent years, challenges still remain. Among the many challenges faced in drug development include high cost, low success rates and the poor understanding of the cellular mechanisms driving the disease [3, 81]. Thus, there is need for new targets and novel drugs or therapies. A new era of CSC-targeted therapies, in combination to conventional therapy, require a deeper understanding of CSCs properties and mechanisms of resistance to therapy.

Whilst several studies have demonstrated the presence of tumor cells with self-renewal and tumor initiating properties, the identification of markers for such cells is still an ongoing process [5, 17, 38, 39, 82-84]. Elaborate experiments by Bonnet and Dick revealed that leukemia cancer initiating cells (CSCs) express specific surface markers [85]. In addition, CSCs have been implicated in metastasis. The origin(s) of CSCs is an area under intense scrutiny at the moment with new evidence pointing to normal stem cells [86]. To complicate the matter further, markers for tumor initiating cells are not universal, with CSCs from different tissues expressing different markers. Our bioinformatic analysis showed that CSC markers are mostly highly expressed in patient tumor samples compared to adjacent normal tissues. For example, the expression of CD44, also referred to as Homing Cell Adhesion Molecule (HCAM), was expressed highly in colon adenocarcinoma samples. This suggests that CD44 expression may be used during diagnosis as well as linked to development of therapy resistance. Overall, CD44 may predict COAD prognosis. In many cancers including breast cancer, CD44 together with CD24 are used to isolate and characterise CSCs [5, 46]. The utility of individual CSC markers is not proven hence markers are usually used in combination.

The presence of CSCs in tumors may explain the high occurrence of development of drug-resistant disease and relapse [87, 88]. The results obtained in this analysis clearly suggest novel and better chemotherapeutic drugs must be developed that can provide better efficacy and clinical outcome for cancer patients by targeting not only cancer cells but CSCs as well. In addition, our analysis of the association between CSC marker expression and patients' survival suggest that targeting CSCs using a single marker such as CD44 might not be enough to eradicate cancers. Instead the use of anti-CSC therapy in combination with chemotherapeutic agents could be better at eradicating cancers. For example, monoclonal

antibodies are one of the new strategies to treat chemo-resistant cancers [89-91]. Due to their specificity, monoclonal antibodies represent a promising method for interfering with a single target molecule with high selectivity [92].

The mechanisms through which CSCs develop therapy resistance including chemoresistance have been under intense investigations [3, 5, 41, 50, 53, 65, 66, 77]. These mechanisms include epithelial mesenchymal transition, quiescence or dormancy, contribution of the tumor microenvironment factors, high expression of drug transporter proteins and enhanced DNA damage repair (Figure 5) [5-7, 53, 77, 93-95]. Markers of EMT and CSCs have been found to be co-expressed, thus linking EMT and CSCs [96]. High expression of drug transporter proteins including ABCG2 and ABCB1 allow CSCs to expel chemotherapeutic drugs and thus attain better survival than cancer cells and stroma in a tumor [97].

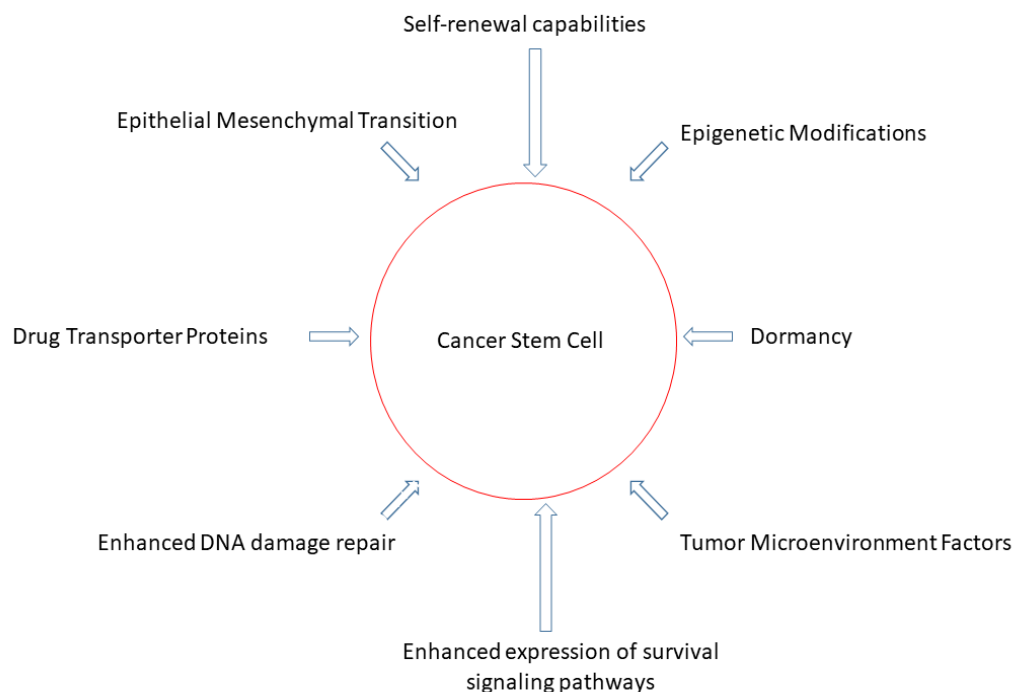


Figure 5. Cancer stem cell properties contributing to development of therapy especially chemoresistance.

CSCs have been shown to undergo dormancy and during treatment some CSC clones can be induced to grow [98]. In addition CSCs display enhanced reactive oxygen scavenging limiting DNA damage in the process [99]. Tumor microenvironment factors and cells have been shown to aid cancer cells to survive chemotherapy via induction of several survival pathways including the MEK-ERK and TGF- β pathway [8, 9, 100]. In addition, hypoxia, cancer-associated fibroblasts and cancer associated macrophages are known to induce stem cell-associated genes, sustaining CSCs within the tumor microenvironment [101-103]. Some of the therapeutic strategies against CSCs include targeting the tumor microenvironment, CSC markers, survival pathways and drug transporters proteins (Figure 6).

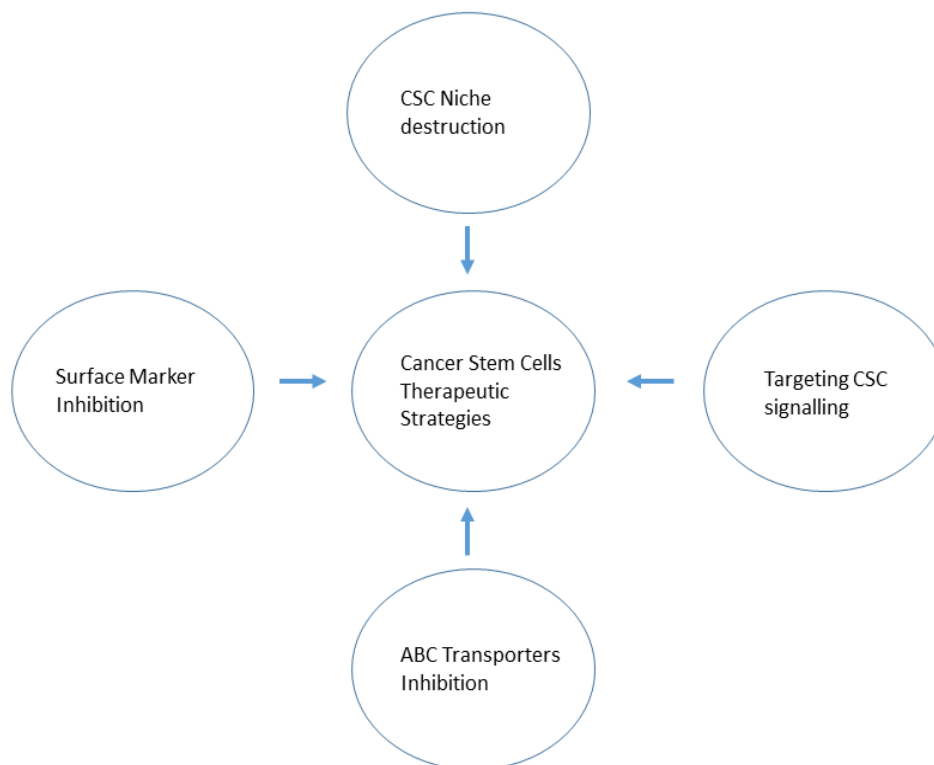


Figure 6. Cancer stem cell-targeted therapies can take the form of surface marker inhibition, drug transporter proteins inhibition, targeting survival signaling pathways and the CSC niche or tumor microenvironment. Figure adapted from Dzobo et al, 2016 [5].

5.0 Study Limitations

Further study to validate the involvement of these CSC markers in tumor initiation and progression is in progress and much needed. Future studies should carefully validate the results obtained in this study and others to evaluate CSC markers as biomarkers with putative prognostic roles.

6.0 Conclusions

This study show that CSC markers are expressed in many cancers and the targeting of these cells would be beneficial to cancer patients. CSC properties are relevant to our understanding of therapy resistance especially chemoresistance. Research into the role of CSCs in cancer initiation and progression holds great potential in the development of novel therapeutic strategies effective in eradicating ESCC. Importantly, this study provides evidence that individual CSC markers may not be useful as valuable predictors of poor prognosis in cancer. Such markers may be useful when used in combinations.

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Conflict of interest

The authors declare no conflict of interest

LIST OF ABBREVIATIONS

ALDH	aldehyde dehydrogenase
ANOVA	analysis of variance
CD	cluster of differentiation
COAD	Colon adenocarcinoma
CSCs	Cancer stem cells
ECM	Extracellular Matrix
EMT	Epithelial to mesenchymal transition
ESCA	Esophageal carcinoma
GEPIA	Gene expression profiling interactive analysis
HCAM	Homing Cell Adhesion Molecule
LUAD	Lung adenocarcinoma
MMPs	Matrix metalloproteases
MRP2	multidrug resistance protein 2
MSCs	Mesenchymal stem cells
PAAD	Pancreatic adenocarcinoma
PAAD	Pancreatic adenocarcinoma
SCID	severe combined immunodeficient

TCGA	The cancer genome atlas
TME	Tumor Microenvironment

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