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The role of cancer stem cells in tumor heterogeneity and resistance to therapy

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1 **The role of cancer stem cells in tumor heterogeneity and resistance to therapy**

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19 **Abstract**

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21 Cancer is a heterogenous disease displaying marked inter- and intra- tumoral diversity. The
22 existence of Cancer Stem Cells (CSCs) has been experimentally demonstrated in a number of
23 cancer types as a subpopulation of tumor cells which drives the tumorigenic and metastatic
24 properties of the entire cancer. Thus, eradication of the CSC population is critical for the
25 complete ablation of a tumor. This is however confounded by the inherent resistance of CSCs
26 to standard anti-cancer therapies, eventually leading to the outgrowth of resistant tumor cells
27 and relapse in patients. The cellular mechanisms of therapy resistance in CSCs are ascribed to
28 several factors including a state of quiescence, an enhanced DNA damage response and
29 active repair mechanisms, up regulated expression of drug efflux transporters as well as the
30 activation of pro-survival signaling pathways and inactivation of apoptotic signaling.
31 Understanding the mechanisms underlying the acquisition of resistance to therapy may hold
32 the key to targeting the CSC population.

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35 **Keywords**

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37 Cancer Stem Cells (CSCs), Heterogeneity, Therapy resistance, Metastasis, Tumorsphere
38 assay.

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Introduction

Cancer poses a major global health problem as it is one of the leading causes of morbidity worldwide and kills more than eight million men and women each year. An increasing insight into the molecular and cellular mechanisms underlying tumorigenesis has emerged leading to the development of targeted treatments. Although this has resulted in a significant decrease in mortality rates for several cancer types, many cancers still lack effective targeted treatments and thus rely on non-specific chemo- or radiotherapy that are associated with relapse and therapeutic resistance (Longo et al. 2007). An in depth understanding of the biology and molecular mechanisms driving tumor progression, metastasis, relapse and resistance to therapy in cancer is therefore urgently required in order to enable effective therapies for cancer patients.

Inter- and intra- tumoral heterogeneity

Cancer is known to be a heterogeneous disease displaying marked genetic and phenotypic variation not only between patients but also within the individual tumor (Bedard et al. 2013; Burrell et al. 2013; Polyak 2011). Inter- tumoral heterogeneity in tumors originating from different tissues could result from variations in gene expression profiles and signaling pathways of different cell types. In addition, diverse genetic events of oncogenic transformation and the interplay of each tumor with the surrounding microenvironment largely influence tumor progression, aggressiveness and the sensitivity of a tumor to therapy (Junttila and de Sauvage 2013). This tumor heterogeneity poses a significant challenge to effective cancer treatment as it may vary throughout disease progression, metastasis or drug treatment due to changes in selection pressures and the microenvironmental niche.

64 Two models have been proposed to explain tumor progression and heterogeneity, namely
65 “the clonal evolution model” in which tumor heterogeneity originates from the outgrowth of
66 sub-clones with an acquired selective advantage (Nowell 1976), and “the cancer stem cell
67 model” where tumor cells are hierarchically organized according to their tumorigenic
68 potential (Reya et al. 2001; Shackleton et al. 2009).

69 The clonal evolution model proposes that all cells within a tumor are biologically equivalent
70 and inherently possess the same tumorigenic potential (Nowell 1976). However, epigenetic
71 modifications, acquired mutations and genetic instability associated with tumor progression
72 leads to the formation of sub-clones, most of which decline due to a selective disadvantage.
73 Occasionally a mutation confers an additional growth advantage with respect to the original
74 tumor cells, leading to their selection and outgrowth as a dominant sub-population within the
75 tumor. For example, an acquired mutation conferring drug resistance could explain relapse in
76 patients due to the persistence and outgrowth of a resistant sub-clone.

77 On the other hand, the cancer stem cell model puts forward a hierarchical system in which
78 tumor growth and progression are sustained by a distinct subpopulation of tumor cells with a
79 stem-like phenotype, termed cancer stem cells (CSCs) (Reya et al. 2001). Key properties of
80 CSCs are their ability to self-renew and multi-potent differentiation. This refers to the
81 capacity of CSCs to divide symmetrically or asymmetrically (Morrison and Kimble 2006) to
82 maintain a tumor-propagating subpopulation of tumor cells, while also giving rise to a
83 heterogeneous population of more differentiated, fast dividing non-tumorigenic progenitor
84 cells that make up the remaining tumor bulk mass.

85 Variation within a tumor could also be influenced by the interplay of the tumor cells with the
86 extracellular environment which would include the immune system and other cells that make
87 up the surrounding matrix. While the clonal evolution and CSC models represent two
88 extreme hypotheses for the generation of heterogeneity in cancer, they are not mutually

89 exclusive models. It is not hard to imagine that a population of cells within the tumor (CSCs)
 90 could be more susceptible to random genetic changes due to changes in DNA repair
 91 mechanisms, which could result in phenotypes advantageous to a cancer cell. Analysis of the
 92 mutational spectrum in solid tumors like breast cancer has clearly demonstrated the existence
 93 of sub-clones which could also interact with each other (Fillmore et al. 2010; Kandoth et al.
 94 2013; Rajaram et al. 2015; X. Zhang et al. 2015). These findings have also been extended to
 95 the isolation of subclones with similar and different mutations in primary and metastatic
 96 tumors supporting a stochastic clonal evolution model (Ding et al. 2010; Vignot et al. 2013).
 97 A tumor could also follow a combination of the two models . For example, while colorectal
 98 cancers follow a CSC model, sequencing of tumors when serially passaged in mice also
 99 reveal new genetic variants that were not originally present in the patient (O'Brien et al. 2007;
 100 Ricci-Vitiani et al. 2007). While solid cancers like breast cancer demonstrate a clear
 101 hierarchical organization, the jury is still out in the case of leukemias and melanomas (Boiko
 102 et al. 2010; Quintana et al. 2010; Schatton et al. 2008). Researchers have further used single
 103 cell technology to demonstrate that metastasis is established by tumor cells with increased
 104 stem-like, mesenchymal and quiescence associated genes, which differentiate to form
 105 proliferative cells in advanced metastatic disease (Lawson et al. 2015).
 106 Experimental testing of heterogeneity can be confounded by plasticity associated with CSCs,
 107 which refers to the ability of cells to transition through different phenotypic states. One
 108 definition of plasticity could be the ability of a cell to give rise to tumorigenic and non-
 109 tumorigenic progeny. The distribution of the CSC population in tumors having a larger
 110 number of CSCs with the ability to differentiate into tumorigenic and non-tumorigenic
 111 progeny would generate more aggressive tumors. This has important therapeutic
 112 consequences where a drug targeting a particular cancer cell state would be rendered
 113 ineffective if it has reverted to a drug resistant phenotype. One of the well studied

phenomenon of plasticity deals with the transition between epithelial and mesenchymal states in breast cancer (Gupta et al. 2011). Studies have demonstrated in breast cancer that CSCs are capable of transitioning between epithelial and mesenchymal states via the process of EMT (Epithelial-Mesenchymal-Transition) and MET (Mesenchymal-Epithelial-Transition). This could potentially lead to the cancer being a constantly moving target. This is also true in melanomas which show different tumor initiating ability based on reversible activation of the histone demethylase JARID1B (Roesch et al. 2010). Plasticity is an important consideration in the clinic as it has been demonstrated that changes in the cellular state could be responsible for the acquisition of drug resistance (Sharma et al. 2010) which could be dependent on the epigenetic status of a cell. It would be important to understand if the degree and the proportion of cells exhibiting plasticity within a tumor are directly correlated to the acquisition of drug resistance.

Discovery of Cancer Stem Cells

The existence of CSCs was initially alluded to in the 19th century when histological similarities were noted between tumors and embryonic tissue (Sell 2004) and resulted in the “embryonal rest theory”. This theory proposed that a small population of dormant embryonic stem cells remained within adult tissues as remnants from embryogenesis and could be activated by an unbalance in the tissue environment to cause cancer. Due to lack of experimental evidence to support this idea, the “embryonal rest theory” was abandoned for half a century. The pioneering observation in the mid-20th century that teratocarcinomas, recognized as malignant structures arising from germ cells, were made up of not only an abnormal mixture of diverse types of mature, differentiated tissue but also of morphologically undifferentiated cells, suggesting their origin from tumorigenic stem cells. However, the first work to support the CSC model and evidence for the existence of CSCs was provided by

Dick and colleagues in the hematopoietic research field (Lapidot et al. 1994). Utilizing the observed similarity between tumors and embryonic tissue, they demonstrated the ability of a rare subset of leukemic cells expressing the normal stem cell surface markers (CD34⁺/CD38⁻) to initiate tumors when transplanted into severe combined immunodeficient (SCID) mice. The xenografted tumor cells expressing the stem cell surface markers could effectively propagate and histologically recapitulate the heterogeneity of the original tumor with the presence of both CSCs and more differentiated tumor cells. In contrast, the tumor cells lacking expression of the stem cell surface markers did not have tumorigenic potential. These early studies have since sparked enormous interest into CSC research. The first identification of prospective CSCs in solid cancers was made in breast cancer (Al-Hajj et al. 2003). Using a cocktail of cell surface markers that included Lineage⁻/CD44⁺/CD24^{-low}, they demonstrated that this breast cancer cell subpopulation was significantly enriched for tumorigenic potential, capacity of serial tumor propagation and the generation of a heterogeneous population of tumor cells. This initial demonstration of prospective CSCs in breast cancer, led to the later discovery of CSCs in an assortment of solid cancers including brain, colon, pancreas, melanoma, ovary, prostate and skin cancers (Table 1).

Tracking down Cancer Stem Cells

To study the biology of CSCs and understand the mechanisms underlying their functional phenotype with the aim of developing effective cancer treatments, an essential requirement is the ability to comprehensively and selectively isolate the CSC population from tumors. This has however proven to be technically challenging. The current CSC markers are either unable to selectively enrich for CSCs or only mark a small subset of the multiple CSC subpopulations within the individual tumors (Visvader and Lindeman 2012). Due to the difficulties associated with CSC markers and their specificity, CSCs are studied based on

their functional phenotypes such as self-renewal, multipotency and tumorigenic potential. Key functional assays to study the CSC phenotype include the *in vitro* tumorsphere assay and *in vivo* limiting dilution transplantation assay.

The sphere assay is a well established *in vitro* method for assessing the self-renewal capacity of a cell population in different model systems (Singec et al. 2006). Tumor cells are grown in low adherent conditions in the absence of serum, which selects for the growth of undifferentiated cells with high self-renewal capacity. Under these low-adherent and non-differentiating conditions, CSCs are hypothesized to be able to form three-dimensional tumorspheres containing stem-like cells and a heterogeneous population of progenitor cells with the ability to differentiate into multiple cell types. The self-renewal capacity of a cell population is assessed by the ability of the cells to form tumorspheres upon multiple passages and is proportional to the number of spheres formed. As multipotency is another key capacity of CSCs, this property is tested in the tumorsphere assay by exposure of the prospective CSCs to differentiating conditions by the addition of serum to the culture medium. Although the tumorsphere assay has been useful for studies of the self-renewal phenotype associated with CSCs, several limitations exist for this assay (Pastrana et al. 2011). A significant caveat of the tumorsphere assay is the tendency of cells to aggregate and the fusion of spheres even at low densities due to migration of non-adherent cells as well as movement of the culture plates (Coles-Takabe et al. 2008). Due to migration of cells and fusion of spheres, true clonality can in theory only be guaranteed by plating of a single cell per well. However, cell density also affects cell proliferation due to the requirement of cells for paracrine signals. In addition, the *in vitro* conditions of the tumorsphere assay do not completely recapitulate the complexity of the *in vivo* niche. While a cell line may show high self-renewal capacity and long-term passaging in the tumorsphere assay, the cells may not have the same self-renewal and tumor propagating capacity when exposed to *in vivo*

conditions. Another important caveat of the tumorsphere assay that may obscure the studies of CSCs is its inability to detect quiescent cells. The results obtained from the tumorsphere assay should thus be validated by *in vivo* experiments showing the tumor propagating potential of the cells in order to draw firm inferences about the self-renewal potential of CSCs (Pastrana et al. 2011).

The gold standard in methods to identify CSCs is the *in vivo* limiting dilution assay in which the tumor cell population of interest is serially transplanted at increasing numbers into animal models, to assess the tumorigenicity and tumor propagating capacity of the cells (Hu and Smyth 2009). To prove the presence of CSCs in the transplanted cell population, the prospective CSCs should demonstrate *in vivo* tumor propagating capacity upon serial passaging and the ability to reestablish the phenotypic heterogeneity present in the original tumor. In addition, the frequency of CSCs within the tumor cell population of interest can be deduced from the number of cells transplanted into the recipient mice that is required for tumor formation (Hu and Smyth 2009). CSC detection often utilizes a combination of approaches, such as isolation of different tumor populations using cell surface markers and FACS, followed by the validation of CSC phenotypes utilizing the tumorsphere or limiting dilution assay.

Although the limiting dilution assay serves as a valuable method for studying CSCs *in vivo*, several important caveats exist for this assay. The use of immune-compromised animal models does not accurately recapitulate the tumor environment and could largely impact the apparent frequency of tumorigenic CSCs (Quintana et al. 2008). Research by Quintana and colleagues using the limiting dilution assay demonstrated an increase in the frequency of tumor-propagating melanoma cells of several magnitudes when using a more immune-compromised animal model; 1 in 46,000 cells were tumorigenic when transplanted into immune-compromised NOD/SCID mice whereas this number increased to 1 in 9 when using

the highly immune-deficient NOD/SCID interleukin-2 receptor gamma chain null (IL2R γ null) mouse model and addition of matrigel. This denotes a nearly 5000 fold increase in tumor propagating capacity based on the immune status of the host. Moreover, the cross-species biological differences in the growth microenvironment and the possible inability of murine factors to engage human receptors could have an impact on the tumor forming ability of the cells, and thus alter the apparent frequency of tumorigenic CSCs. These limitations challenge the foundation of the xenotransplantation or limiting dilution assay by suggesting that it may simply be identifying subpopulations of cells that are capable of surviving in the immune-compromised mouse environment rather than identifying true CSCs.

Additional approaches have been used to identify and isolate prospective CSCs from different model systems. Though lineage tracing was originally utilized in the developmental field, it is emerging as a powerful tool to understand the evolution of tumors. Lineage tracing has led to the successful identification of the Sox9 expressing hair follicle bulge stem cells (Nowak et al. 2008), *Lgr5*⁺ epithelial stem cells in different tissues (Snippert et al. 2010), including the intestine (Barker et al. 2007), stomach (Barker et al. 2010) and hair follicle (Jaks et al. 2008). Combining tissue specific promoters with multi color reporters and live- cell imaging has allowed scientists to trace the fate of specific cells within a tumor, their contribution to tumor and metastatic growth as well resistance to drugs. Using the neural stem cell marker Nestin tagged with GFP, Chen et al identified glioma stem cells and further went on to demonstrate that the CSCs were resistant to chemotherapy (J. Chen et al. 2012). Another approach utilizes the lineage specific Cre-recombinase system with a reporter gene, such as the enzyme β -galactosidase or a fluorescent protein. Driessens and colleagues went on to demonstrate that benign papillomas follow a hierarchical model (Driessens et al. 2012). They identified two tumor populations in benign papilloma with a rarer fraction of the slower cycling, longer lived stem-like cells giving rise to the majority of the cells in the tumor. Hans Clevers work

has led the field with elegant multi-color lineage tracing experiments. The identification of the Lgr5⁺ tumor cells as the cell of origin for intestinal adenomas utilized the Cre-confetti reporter system and supports the CSC model (Schepers et al. 2012). Interestingly, it has been shown in a Wnt-dependent model of adenomas that Lgr5⁻ cells can give rise to Lgr5⁺ cells and form spheroids and tumors with the same efficiency as Lgr5⁺ cells. Intestinal carcinomas can thus be driven by both Lgr5⁺ and Lgr5⁻ cells (Schwitalla et al. 2013). Hence we must interpret the results from assays in the context of different cancers to understand which cancers follow the clonal evolution, CSC or a mixture of both models.

In vitro assays have been developed to identify CSCs based on molecular characteristics. These include the Aldefluor assay which measures expression of the enzyme aldehyde dehydrogenase (ALDH) (Ginestier et al. 2007; S. Deng et al. 2010). Tumor cells with high expression of the ALDH enzyme are able to detoxify the cytotoxic aldehyde intermediates produced upon exposure to chemotherapy agents and hence confer resistance to anticancer therapies. ALDH is found to be upregulated by a wide range of CSCs, however it is not usually sufficient for CSC identification and isolation (de Beca et al. 2013; Keysar and Jimeno 2010; Ricardo et al. 2011). Studies have shown that ALDH⁺ CSCs are more likely to form colonies in soft agar, form tumorspheres in serum-free media and exhibit chemoresistance when compared to the CD44⁺/CD24⁻ cells, as well as the inability of the CD44⁺/CD24⁻/ALDH1⁻ cells to form tumors at all (Ginestier et al. 2007; Tanei et al. 2009). Another popular method for the isolation of CSCs is the Hoechst stain exclusion assay which identifies CSCs based on their tendency to up regulate expression of ATP-binding cassette transporters (ABC transporters), which are active drug efflux pumps shown to be implicated in resistance to different anticancer therapies (Seigel and Campbell 2004). The identification of a “side population” (SP) with high expression of the drug efflux transporters and thus low intracellular concentration of the stain was used to prospectively sort for CSCs in breast and

other cancer types. But this assay must also be used with caution as it has been demonstrated that the SP cells isolated from glioblastoma multiforme brain tumors do not have self-renewal or tumor initiating properties (Broadley et al. 2011). On the other hand, label retention assays take advantage of the quiescent phenotype associated with a subset of CSCs, which due to their quiescent, non-dividing state, would retain more of a nucleotide label such as tritiated thymidine (Kuwahara et al. 2008). Another important technique used for CSC detection is immunohistochemistry (IHC). IHC has been commonly used to detect putative CSC markers like CD44, CD24 and ALDH in patient tissue sections and evaluate their correlation with distinct histological types (Lingala et al. 2010; Martin and Jiang 2014). Unfortunately, most of these assays do not specifically identify all CSC populations within a tumor as they are based on one particular CSC phenotype. Due to the presence of a number of CSC populations within a tumor, this often leads to the experimental set up failing to identify CSC subsets that do not share that particular phenotype.

A combination of enrichment techniques utilizing CSC markers to isolate tumor cells, followed by functional validation of the CSC phenotypes associated with the sorted populations is currently the best method to identify and validate the putative CSC fraction within a tumor.

Clinical importance of CSCs

Metastasis and tumor relapse are major causes underlying cancer-related deaths, and accumulating evidence point towards CSCs playing a critical role in this regard. A main cause of cancer relapse is attributed to the persistence of CSCs, which have been shown to possess an inherent resistance to standard anti-cancer therapies such as chemotherapy and radiation (Li et al. 2008; Phillips et al. 2006). Standard therapies thus target the tumor bulk but fail to eradicate the resistant CSCs, which gives a possible explanation for the relapse

observed in a major subset of patients undergoing standard treatments. Effective targeting of the CSC subpopulation is therefore essential for the complete eradication of a tumor and prevention of tumor relapse in patients.

However, accumulating evidence suggests that CSCs are not a fixed subset of a tumor but are able to convert between tumorigenic CSC states and more differentiated, non-CSC states (Gupta et al. 2011). This plasticity may be essential for tumor progression, as epithelial-to-mesenchymal transition (EMT) and its reverse cellular process, mesenchymal-to-epithelial transition (MET), are key steps in tumor invasiveness and metastasis (Elshamy and Duhe 2013; Sampieri and Fodde 2012; Shiozawa et al. 2013). Studies have demonstrated the ability of the tumor microenvironment to induce EMT in differentiated cancer cells to confer a CSC-like state with mesenchymal features greatly facilitating invasion and systemic dissemination from the primary tumor (Mani et al. 2008; Marjanovic et al. 2013). Oppositely, the disseminated tumor cells homing at distant organ sites may undergo MET and develop into metastatic lesions as a response to local stimulation from the microenvironment at the metastatic site. For example, TGF- β , which is recognized as an effective inducer of EMT, has been shown to efficiently promote the conversion of non-CSC basal breast cancer cells to confer a CSC state through the transcription factor ZEB1 (Chaffer et al. 2013).

In addition, heterogeneity within the CSC population of tumor cells may give rise to several distinct CSC subsets with diverse phenotypes or tumor propagating capacities. This may be regulated by cell-intrinsic factors or in response to distinct microenvironmental stimulation and largely impacts on the sensitivity of a tumor to therapy. Whereas one CSC population may show extensive self-renewal or proliferative capacity, another CSC subpopulation may be quiescent with the ability to drive tumor relapse upon activation years after anticancer therapy. Moreover, only a subset of CSCs may have the ability to undergo the complex metastatic process and colonize distinct microenvironmental niches. Diversity of CSC

populations is aptly demonstrated by studies in which the transcription factor Id1 marks glioblastoma stem cells (Anido et al. 2010). In these studies the Id1^{low} tumor cells were reported to have greater tumorigenic potential than the Id1^{high} subpopulation of glioblastoma cells, which however were shown to have the greatest self-renewal capacity of the CSC population. A deeper understanding of the factors controlling or inducing the CSC state as well as finding a common denominator of the distinct subsets of CSCs are thus of great importance for the development of effective cancer treatments.

Genomic approaches to deconvoluting tumor heterogeneity have also given us insights into the evolution of cancers. Analysis of the genomic, transcriptomic and epigenetic states has revealed extensive heterogeneity in Acute Myeloid Leukemia (AML) (Walter et al. 2012), chronic lymphocytic leukaemia (CLL) (Schuh et al. 2012), breast cancer (Navin et al. 2011; Nik-Zainal et al. 2012; Shah et al. 2012), renal cell carcinoma (Xu et al. 2012) and pancreatic cancers (Campbell et al. 2010; Waddell et al. 2015). Due to the rapid advances in the field, we can now not only look at whole tumor populations, but at single cell resolution. Interestingly, a recent paper from Lawson et al, demonstrated a hierarchical model for the establishment of metastasis. Using a single cell approach, they conclusively show that metastasis is established by cells with increased stem-like, mesenchymal and quiescence associated genes, which differentiate to form proliferative cells in advanced metastatic disease (Lawson et al. 2015). Studies aimed at identifying a gene signature have been hampered by our inability to isolate pure CSC populations. Several groups have used the current CSC markers available (see Table 1) to isolate CSCs from different tumor types and analyze the molecular pathways using array and RNA-sequencing technology. A breast CSC signature was identified using ALDH1 as a prospective CSC marker in 33 cell lines. A 413-gene expression profile was identified from the ALDH⁺ CSCs and an association with the CXCR1/IL-8RA pathway. An important step is to validate such signatures or genes using

CSC assays as detailed in earlier sections. Further functional assays confirmed that IL-8 was important for the self-renewal and metastatic phenotypes associated with CSCs (Charafe-Jauffret et al. 2009; Singh et al. 2013). Another approach has used the better characterized developmental counterpart of a cancer and extrapolated the signatures from pure stem cells to understand the genes driving tumorigenesis. Merlos-Suarez and colleagues identified an intestinal stem cell (ISC) signature using EphB2 and Lrg5 as markers of the ISC and found that this was also predictive of relapse in colorectal cancer (Merlos-Suarez et al. 2011). A similar strategy was adopted by Zhang and colleagues to demonstrate that the transcriptional profile of basal epithelial cells in the prostate was associated with aggressive prostate cancer (D. Zhang et al. 2016). CD44 has been used to isolate CSCs in breast cancer revealing a 31-gene signature which is also predictive of distant metastasis in patients (de Jong et al. 2015; Leth-Larsen et al. 2012; Linge et al. 2016). Continuous refinement of CSC markers with isolation of pure populations is the need of the hour for the generation of clinically relevant CSC gene signature sets.

Resistance to anticancer therapy

The mechanism of action of chemo- and radiation therapy has been intensely studied for decades. Chemotherapy covers cytotoxic chemical compounds that act by killing actively dividing cells in a non-specific manner by several distinct mechanisms such as stabilization of enzyme-DNA complexes, DNA fragmentation and inter- and intra-strand cross linking of the DNA, which interfere with cell division and results in the induction of apoptosis (Sarah and David 2008). Radiation therapy works by damaging the DNA of cancer cells by inducing single- or double-strand breaks (Lomax et al. 2013). This DNA damage is caused by either direct ionization of the molecular constituents of the DNA chain or indirectly by forming free radicals through ionization of water molecules that damage the DNA and results in

potentially cytotoxic or mutagenic lesions. Cellular stress responses to DNA damage results in cell cycle arrest and the activation of DNA repair mechanisms to protect genomic integrity. However, upon irreversible DNA damage, cell death by apoptosis is induced in the damaged cell.

Standard anticancer therapy thus aims to target the uncontrolled proliferative phenotype of cancer cells. However, increasing insight into intra-tumoral heterogeneity and cellular hierarchy indicates that standard anticancer treatments might miss the root cause of tumor progression and metastasis, namely CSCs as described previously. Due to the inherent persistence of therapy resistant CSCs, standard anticancer therapies such as chemotherapy and radiation affects the tumor bulk but fail to eliminate the CSC population. Cancer patients thus have a good initial response to therapy when targeting the tumor bulk, but a poor overall survival due to outgrowth of the remaining chemo-resistant CSCs (Carey et al. 2007) resulting in tumor relapse in patients. Therapy resistance is thus a major impediment to successful treatment, and effective targeting of the CSC subpopulation is essential for the complete eradication of a tumor.

The cellular mechanisms of therapy resistance in CSCs have been extensively studied and ascribed to several factors including anti-apoptotic properties and activation of pro-survival signaling pathways as well as increased expression of drug efflux transporters with broad specificity that lower the intracellular drug concentration through active efflux mechanisms. The resistance of CSCs to therapy is additionally thought to result from their capacity for active DNA-repair and the induction of a quiescent state characterizing a subset of the heterogeneous CSC population of tumor cells (Figure 1).

Quiescence

Resistance of CSCs to standard therapies is partly ascribed to the quiescence of a subset of

CSCs, which refers to a non-dividing state in which the cells maintain the capacity to reenter an active, proliferative cellular state. Standard treatments such as chemo- and radiation therapy predominantly act by targeting actively dividing cells. Thus, quiescence of CSCs poses an effective means of evading cancer treatments and conferring therapy resistance. Activation of CSCs from a quiescent state could then drive tumor relapse years after anticancer therapy. The acquisition of resistance to anticancer therapy in CSCs due to a quiescent state is supported by research in colorectal cancer by Touil et al (Touil et al. 2014). In this study, the fraction of cancer cells conferring resistance to 5-fluorouracil chemotherapy displayed a CSC phenotype based on markers, self-renewal and tumor propagating potential, and entered into a quiescent state upon exposure to the drug. Induction of quiescence in the chemo-resistant cancer cells upon exposure to 5-fluorouracil involved the activation of the c-Yes tyrosine kinase and its dissociation from the c-Yes/YAP complex, resulting in reduced nuclear levels of Yes-associated protein (YAP). This study suggests a mechanism by which the depletion of nuclear YAP, which is a transcriptional co-activator shown to be involved in stem cell self-renewal, arrests the cells in a non-dividing, quiescent state. Interestingly, in Alzheimer's disease, nuclear translocation of YAP has been reported to activate apoptosis related genes in response to accumulation of amyloid-beta-peptide in a p73 dependent mechanism (H. Zhang et al. 2011). Thus, the decrease in nuclear levels of YAP could be another way in which CSCs use a transcriptional co-activator to simultaneously impinge on the cell cycle and apoptotic pathways, and induce a state of quiescence. A recent study in glioblastomas further supports the hypothesis that CSCs utilize the induction of a quiescent state as a mechanism to acquire reduced sensitivity to standard therapies. By exposing tumorspheres containing both quiescent cells (as marked by increased label retention) and non-quiescent cells to chemotherapy, Campos et al reported higher frequencies of viable

quiescent cancer cells than their non-quiescent counterparts after chemotherapy treatment (Campos et al. 2014).

However, marked heterogeneity exists within the CSC subpopulation of tumor cells displaying distinct molecular and functional phenotypes with only a subset of CSCs in the state of quiescence. Research suggests that CSCs have the ability to convert between these quiescent, slow-cycling and more active states, thus adding a further level of complexity towards effective anticancer therapy.

Drug efflux transporters

An important factor contributing to therapy resistance in cancer patients is ascribed to the increased expression of drug efflux transporters in therapy resistant CSCs. These drug efflux transporters are mainly located in the cellular plasma membrane where they function normally to protect cells from harmful cytotoxic agents. Drug efflux transporters play an important role in protecting against cytotoxins and metabolic waste products especially in the blood brain barrier, liver, kidney and intestinal tissue (Gottesman et al. 2002).

In cancer, resistance of CSCs to therapy results in part from the up regulated expression of the transporters, as increased drug efflux across the plasma membrane lowers intracellular drug concentration (Gottesman et al. 2002). Due to their broad substrate specificity for morphologically and functionally distinct compounds, the increased expression of the drug efflux transporters may confer resistance to multiple anti-cancer drugs. Several drug efflux transporters have been linked to therapy resistance in CSCs. The ATP-binding cassette (ABC) family of transporter proteins, including multi-drug resistance protein 1 (MDR1, P-glycoprotein or ABCB1), MDR-associated protein 1 (MRP1 or ABCC1) and breast cancer resistance protein (BCRP or ABCG2) have been linked to therapy resistance (Z. Chen et al. 2016). These drug efflux transporters show extensive substrate specificity with the ability to

promote efflux of several distinct compounds including chemotherapeutics. Substrate binding to the active site of the drug efflux transporters causes an ATP-dependent conformational change with the resulting release and thus efflux of the substrate to the extracellular space. Targeting putative pathways that CSCs are dependent on for their survival upon exposure to drugs might be a therapeutic alternative worth exploring (Pardal et al. 2003). One such pathway that serves to protect the cells from external toxic agents or drugs is the over-expression of ABC transporter proteins. Therefore, targeting the ABC transporter proteins may be an alternative strategy to overcome therapy resistance. In combination with drugs such as Topotecan, Afatinib (a tyrosine kinase inhibitor) was found to suppress the efflux activity of only one member of the ABC subfamily (ABCG2) by inhibiting the ATPase activity and down regulating the expression of the transporter (X. K. Wang et al. 2014). This ultimately led to the accumulation of the drug in the breast cancer cells and reversal of the chemoresistant phenotype. Studies in adenoid cystic carcinoma have implicated the T-box transcription factor Brachyury in the mechanism by which CSCs acquire radiotherapy resistance (Kobayashi et al. 2014). Mechanistic studies demonstrated that knockdown of Brachyury lead to a decrease in expression of ATP- binding cassette transporter genes. Re expression of transcription factors such a Brachyury could be used as a strategy to resensitize the cells to the effects of radiotherapy.

Active DNA repair

As standard anticancer treatments including chemotherapy or radiation act by inducing DNA damage such as single- and double-strand breaks, their effectiveness largely rely upon the inadequate activity of DNA repair mechanisms in the cancer cells. Normally, DNA damage is recognized and repaired by a cellular DNA damage response (DDR) that plays an important role in maintaining genomic integrity and cellular function. Upon irreversible DNA damage,

cellular apoptosis or an irreversible state of senescence is induced in order to prevent abnormal growth and transformation of the damaged cell (Jeggo et al. 2016; Pearl et al. 2015). However, the DNA damage response that functions to protect genomic integrity and normal cellular function is additionally exploited by CSCs for protection against the DNA damaging effect of chemotherapy and radiation.

Damage to DNA causes a change in its spatial structure. Upon recognition of aberrant structures in the DNA by the DDR, cell cycle checkpoints are activated and cell cycle arrest is induced to ensure that the DNA damage is corrected before continuing cell division. The main sensors of DNA damage and regulators of checkpoint control are the ATM (Ataxia Telangiectasia mutated) and ATR (ATM and RAD3-related) protein kinases. Whereas ATM primarily recognizes double-strand breaks and disruptions in the DNA chain (Bakkenist and Kastan 2003), ATR responds to the presence of single-stranded DNA at stalled replication forks. Upon recognition of these structures, ATM and ATR phosphorylate downstream substrates in a signaling cascade ultimately leading to cell cycle arrest and recruitment of DNA repair effectors, or the induction of apoptosis upon irreversible DNA damage. Among the main substrates of ATM and ATR are CHK1 and CHK2. Phosphorylation of CHK1 and CHK2 leads to activation of a number of key proteins such as p53, which plays a key role in cell cycle arrest, DNA repair and initiation of apoptosis; and CDC25A which leads to cell cycle arrest by degradation of G1/S transition activators. Interestingly, several studies have observed an increased DDR response in the CSC population. A study by Bao et al. observed an enrichment of CD133⁺ glioma stem cells following exposure to ionizing radiation, thus indicating an inherent resistance of these cells to radiation compared to CD133⁻ glioma cells (Bao et al. 2006). This inherent resistance of the CD133⁺ glioma stem cells to radiation was conferred through an enhanced DDR, as increased activation of DDR factors ATM, CHK1 and CHK2 was observed in the CD133⁺ glioma stem cells following radiation. Moreover, the

observed resistance of the CD133⁺ glioma cells to radiation was reversed by inhibition of CHK1 and CHK2 (which are DDR factors), further supporting the key role of an enhanced DNA damage response and repair mechanisms in conferring resistance to therapy. This mechanism is further supported by studies in colon and pancreatic cancers. Gallmeier et al demonstrated that CD133⁺ colon CSCs were more resistant to Cisplatin than CD133⁻ CSCs through the increased phosphorylation of CHK1 (Gallmeier et al. 2011). This protective effect was reversed when the cells were treated with the CHK1 inhibiting agent SB218078. Similarly, treatment of chemoresistant CD24⁺/CD44⁺/ESA⁺ pancreatic CSCs to CHK1 inhibitors lead to resensitization to Gemcitabine (Venkatesha et al. 2012).

Cells have developed several repair mechanisms to account for the variety of damage inflicted on their DNA. These repair mechanisms include the nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), and the double-strand break (DSB) repair including homologous end joining (HE) and non-homologous end-joining (NHEJ). Several excision repair mechanisms exist to repair damage in a single DNA strand by removing the damaged DNA region and correctly synthesizing the new strand using the complementary strand as a template. These excision repair mechanisms includes BER, NER and MMR, which recognizes and corrects base changes in the DNA chain, nucleotide alterations normally caused by chemicals or radiation, and mismatch match errors arisen during replication, respectively. Mechanisms to repair double-strand breaks include NHEJ and HE. HE relies on the presence of a homologous sister chromatid and can thus only occur after replication, while NHEJ directly joins the two ends of the double strand break and therefore relies on the presence of complementary, compatible overhangs. However, due to the lack of a normal DNA strand, there is more likelihood of errors like deletions being introduced during repair of DSB. CSCs utilize a number of strategies to repair DNA damage and allow them to survive in spite of the toxic insults caused by chemotherapy. Venere and

colleagues (Venere et al. 2014) have demonstrated increased single stranded break (SSB) repair in GICs through the PARP1 pathway. Blocking of PARP1 reversed this protective effect and could be used in conjunction with other drugs to sensitize CSCs to the effects of therapy. Further evidence comes from the increased expression of DNA repair genes like PARP, ATM and BRCA1 through Progranulin in Glioblastoma human samples (Bandey et al. 2015) as well as up regulation of DSB repair genes in CD133⁺ CSCs in lung carcinoma (Desai et al. 2014). Increased DNA damage response and repair capacities in CSCs thus pose an effective mechanism by which CSCs evade the damaging effect of anticancer treatments. It must be emphasized that heterogeneity in the tumor population could lead to different strategies being employed by CSCs to evade the cytotoxic effects of therapy with enhanced DNA damage response just one weapon in their arsenal.

Defective apoptotic pathways and increased pro-survival signaling

Apoptosis functions in normal biology to remove damaged or abnormal cells. Apoptosis thus provides an important barrier against cancer and is induced by cellular stress responses to DNA damage or abnormal cellular processes. However, specific mutations enable some tumor cells to escape apoptosis and confer a more malignant phenotype. As such, the activation of pro-survival signaling pathways and inactivation of apoptotic signaling are critical means by which CSCs may confer drug resistance and evade apoptosis induced by cellular stress responses to DNA damage. The two main apoptotic signaling pathways are the intrinsic pathway, which acts through intracellular death signals in response to cellular stress, and the extrinsic pathway that acts through extracellular stimulation of pro-apoptotic cell surface receptors. The intrinsic and extrinsic signaling pathways ultimately converge and result in the same apoptotic process of cellular degradation (Hanahan and Weinberg 2000, 2011).

538 The intrinsic apoptotic pathway is initiated in response to intracellular stress signaling which
539 causes the release of cytochrome c and Smac/DIABLO pro-apoptotic proteins from the
540 mitochondria. Whereas Smac/DIABLO promote apoptosis by impeding the function of
541 inhibitors of apoptosis proteins (IAPs), cytochrome c binds and activates apoptotic protease
542 activating factor 1 (Apaf-1) and procaspase-9 to form the apoptosome. The apoptosome functions
543 to cleave and activate executioner caspases that trigger the apoptotic process of cell
544 degradation. The intrinsic pathway is regulated through a balance in expression of pro-
545 apoptotic proteins such as Bax, Bak, Bad, Bid, Bim, Bik, Noxa and Puma relative to the
546 expression of pro-survival proteins including Bcl-2, Bcl-X_L, and Mcl1 (Ouyang et al. 2012).
547 DNA damage induced by anticancer therapy such as chemotherapy or radiation results in the
548 accumulation of the tumor suppressor protein p53 (Schmitt et al. 2002; Z. Wang and Sun
549 2010). High cellular levels of p53 indirectly cause the induction of apoptosis through up
550 regulated levels of the pro-apoptotic Bax and Bak proteins.

551 CSCs evade the damaging effect of anticancer therapy by enhanced DNA damage response
552 (DDR) and repair mechanisms and additionally confer resistance to anticancer therapy
553 through increased pro-survival signaling and inactivation of pro-apoptotic proteins (Pearl et
554 al. 2015). Cellular levels of pro-survival proteins Bcl-2 and Bcl-X_L as well as several IAPs
555 were found to be significantly up regulated in chemo-resistant CD133⁺ glioma stem cells
556 compared to CD133⁻ glioma cells in a study by Liu et al., whereas the expression of pro-
557 apoptotic Bax was significantly decreased (G. Liu et al. 2006). In another study, the
558 inhibition of Bcl-2 anti-apoptotic activity resulted in activation of pro-apoptotic Bax and the
559 induction of apoptosis in glioblastoma cells (Tagscherer et al. 2008). Furthermore, the up
560 regulated expression of cell surface growth receptors such as HER2, FGF and EGF has been
561 shown to inhibit apoptosis in chemo-resistant CSCs through the down regulation of cellular

levels of the pro-apoptotic Bad protein, thus inhibiting the intrinsic signaling pathway and apoptosis (Economopoulou et al. 2012).

Increased pro-survival signaling and decreased expression of apoptotic genes would result in CSCs acquiring resistance as they could overcome or counteract the death signals caused by therapy-induced damage. The deregulated expression of the pro-survival and apoptotic pathways may be caused by genomic instability and accumulating mutations associated with tumor progression or as a consequence of the DNA damaging effect of standard anticancer therapy. Mutations leading to deregulated expression of anti-apoptotic proteins such as Bcl-2 and Bcl-xl, inactivation of pro-apoptotic factors such as p53, Bax and Bak, or an increase in pro survival signals such as Survivin and cFLIP could on their own or together cause apoptotic dysregulation and confer a survival advantage on CSCs (Vidal et al. 2014). For example, the anti-apoptotic cFLIP protein is found to be over expressed in colon, thyroid cancers and melanoma (Dutton et al. 2006; Griffith et al. 1998; Ryu et al. 2001; Todaro et al. 2006). Interestingly, the CD133⁺ CSC fraction of a leukemia cell line were found to over express cFLIP and protect cells from apoptosis. This could be reversed by depleting the cells of cFLIP in siRNA knock down experiments, thus restoring the extrinsic apoptotic pathway (Zobalova et al. 2008a; Zobalova et al. 2008b).

Caveats associated with CSC biology

Understanding the molecular circuitry of CSCs is critical for the design of new and improved therapies against this sub-population of tumor cells. Isolation of CSCs from a heterogeneous tumor mass to a high degree of purity has been a major stumbling block in the field (Visvader and Lindeman 2012). This is evident from xenotransplantation assays in which the number of CSCs identified in tumors is much lower than suggested by the markers, thus indicating lack

of specificity of the markers used to identify CSCs. As elaborated in Table 1, the classical CSC markers such as CD44⁺/CD24^{-/low} only identify a fraction of the CSCs in the breast. One of the major limitations in the CSC biology field are that the assays currently used to identify this subpopulation of cells based on critical phenotypes such as self-renewal. As discussed in detail in the previous section on tracking CSCs, the use of the tumorsphere or limiting dilution assays in isolation does not efficiently identify all the CSC populations that may exist in a tumor. While the *in vivo* recapitulation of an entire tumor from a single cell is considered the gold standard for assessing CSC activity, unfortunately the use of immune compromised model systems often means that crucial elements in tumor progression such as the immune system and microenvironment are compromised. The inefficiency of the current CSC markers in identifying a true CSC population combined with the lack of specific antibodies and the rarity of this population, have resulted in poor correlation in clinical studies (Keysar and Jimeno 2010). Although combinations of prospective CSC markers have improved the identification and isolation of CSCs in several cancer types, inter- and intra-tumoral heterogeneity complicates the work towards finding efficient and specific markers for the isolation and study of CSCs.

Discussion

Research over the past few decades has resulted in important breakthroughs in our understanding of cancer and lead to the development of better therapeutic options for patients. This has had a direct impact on better survival rates in many cancer types. But unfortunately, effective targeted therapy is still lacking for some cancer types like pancreatic cancer as well as metastatic cancer. These cancers still rely mainly on surgical intervention and non-specific chemo- or radio-therapy that is largely ineffectual.

The discovery of CSC populations in a number of cancers has confounded successful treatment due to the inherent therapy resistant phenotype associated with CSCs. One of the major hurdles in designing new therapies against CSCs is our inability to isolate CSCs to a high degree of purity, as the current “gold standards” in the field do not accurately identify the true CSC (Table 1). An alternative approach would be to use publically available data to identify genes critical for CSC phenotypes like self-renewal and chemo-resistance. Prospective CSC molecular markers can then be used to specifically and effectively isolate CSCs from a tumor. Utilizing genomic approaches, the molecular circuitry of the CSCs can be elucidated to build a CSC gene signature. This could further allow us to identify cell surface molecules that mark the true CSCs. Refinement in the methodologies used currently to identify CSCs based on phenotype is also essential. Combining assays that utilize *in vitro* methods like FACS and the tumorsphere assay to enrich for CSCs, followed by *in vivo* testing in animal models would allow for more accurate identification of this cell population. Unfortunately, often the rarity of CSC population makes validation in patient samples with associated clinical parameters like survival data implausible.

Since chemo- and radio- therapy mainly targets the rapidly dividing tumor population, it implicitly means that the slowly dividing or quiescent CSCs will be spared, which would ultimately re-grow to cause relapse in the patient (Hellman et al. 2008). CSCs utilize a number of mechanisms to circumvent the deleterious effects and ultimately cell death caused by chemo- and radio- therapy as shown in Figure 1.

Chemotherapy and targeted therapy have benefitted the management of many cancers. Unfortunately, many patients develop or acquire resistance to current modes of treatment. The contribution of CSCs and consequently intra-tumoral heterogeneity, to this phenomenon of acquired resistance leading to increased cancer mortality is now an area of active investigation (Vidal et al. 2014). Several studies support this hypothesis with work in the

leukemia field once again leading the way. For example, CD34⁺ (a marker of CSCs in AML) correlated with poor prognosis in AML patient cohorts after chemotherapy (van Rhenen et al. 2005). This is not surprising as AML CSCs have been experimentally shown to be resistant to the standard cytotoxic therapy cytarabine and the CD34⁺CD38⁻ cell population is enriched after treatment and indicative of relapse in patients (Gerber et al. 2012). Studies from carcinomas like breast, pancreatic and colorectal cancers have also lent credence to the hypothesis that CSCs contribute to acquired resistance. Breast CSCs identified by CD24⁺/CD44⁻ and ALDH1 have been found to be enriched in patient samples post treatment, while CD133⁺ cells from patient samples were more resistant to 5-fluorouracil and oxaliplatin than CD133⁻ cells (Todaro et al. 2007). The presence of therapy resistant CSCs in a tumor could provide an explanation for the phenomenon of acquired resistance. Adopting a two pronged strategy which debulks the tumor and targets the CSC population might be more effective in preventing relapse in patients.

One possible approach would be to target alternative pathways that are critical for CSC phenotypes like self-renewal. This would include the Notch, Shh, Bmi1 and Wnt signaling pathways (Borah et al. 2015). One important caveat of such an approach would be the side effects of targeting pathways common to both normal stem cells and CSCs. This could be overcome by specifically delivering the drugs to only the CSC population, thus preventing deleterious effects on normal cells.

The use of targeted antibodies against specific surface molecules or receptors which are unique to CSCs is another therapeutic option. Apoptosis is one of the important pathways that CSCs subvert in order to increase their resistance to chemotherapy. An interesting approach would involve the manipulation of expression of pro- and anti- apoptotic proteins like Bad and BCL-2, which could tilt the balance towards programmed cell death and thus elimination

of the CSC (Edinger and Thompson 2004). The potential side effects of such a therapeutic strategy on normal cells in tissues must be considered before implementation in the clinic.

Another emerging consensus is that CSCs can exhibit plasticity with the ability to convert between CSC and non-CSC states. It might therefore not be enough to only target one subset of CSCs in order to eradicate a tumor. To effectively ablate CSCs and prevent tumor relapse, a combination of CSC-specific therapies with current therapies targeting the tumor bulk will be required.

The tumor microenvironment (TME) is another important consideration in devising new therapies against CSCs. In a manner akin to the stem cell niche, it has been postulated that these are anatomically unique regions within the tumor, consisting of different cell types that contribute to the acquisition and maintenance of CSCs. The TME consists of a number of different cell types like Cancer Associated Fibroblasts (CAFs), Mesenchymal Stem Cells (MSCs) and inflammatory cells. The importance of the TME has been underscored in an elegant study by Quintana and colleagues (Quintana et al. 2010) where the tumor initiating frequency of melanoma cells varied significantly depending on the immune status of the recipient host. Studies have started to tease apart how the CSC niche could support the genesis of CSCs, aid in plasticity between non-CSC and CSC populations, in addition to contributing to metastasis and chemoresistance (Oskarsson et al. 2014; Ye et al. 2014). This process can be a two way street as demonstrated with breast CSCs which produce IL6, which in turn attracts and activates MSCs to produce the CSC-supportive cytokine CXCL7 (S. Liu et al. 2011). It naturally follows that the CSC niche could also play a role in the acquisition of resistance to conventional therapies. A classic example would be the hypoxic microenvironment within tumors which has been found to increase CSC pathways by the activation of TGF- β and Wnt signaling pathways (Anido et al. 2010; Scheel et al. 2011). Components of the tumor stroma could also act as a physical impediment for the access of

684 drugs to tumor cells. More recently, new data has supported the notion that the TME may
685 help to promote the conversion of non-CSCs to CSCs via induction of the EMT pathway in
686 tumor cells by tumor associated macrophages (TAMs) (Bruna et al. 2012; Cabarcas et al.
687 2011). One of the more intriguing concepts has been the establishment of the pre-metastatic
688 niche by the release of exosomes containing functional proteins, mRNA and miRNA
689 (Castellana et al. 2009; Jung et al. 2009; Muralidharan-Chari et al. 2010). Understanding the
690 mechanisms by which the tumor microenvironment forms a supportive CSC niche could be a
691 potential therapeutic avenue to explore.

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694 **Table 1. CSC markers in different organ systems.** The discovery of CSCs in multiple
 695 cancer types utilizing a combination of cell surface markers to determine the CSC frequency.
 696

Tissue type	Markers	Clinical importance	Reference
Leukemia	CD34 ⁺ /CD38 ⁻	It was reported that high expression of CD34 ⁺ /CD38 ⁻ stem cell markers are associated with a high minimal residual disease frequency in patients, particularly after the third course of chemotherapy. Thus CD34 ⁺ /CD38 ⁻ marker expression is directly correlated with poor prognosis.	van Rhenen et al. 2005
	CD33	CD33 is a well known marker for leukemia. Studies reported that more than 90% of the AML patient samples expressed the CSC marker CD33. Clinically it can be used as a suitable tumor-associated stem cell marker/antigen, predicting the target for	De Propriis et al. 2011

	<p>CD123</p> <p>BMI1</p> <p>c-kit</p> <p>CD44</p> <p>GLI-1</p>	<p>antibody-based AML therapies.</p> <p>Studies reported that CD123 is a marker of leukemia-initiating cells and a high percentage of CD123⁺/hMICL⁺ cells after chemotherapy represent a strong prognostic marker for relapse in patients.</p> <p>BMI1 is a marker for monitoring the tumor progression and invasion in AML patients, thus predicting a poor prognosis in patients with Chronic myeloid leukemia.</p>	<p>Testa et al. 2014</p> <p>Roug et al. 2014</p> <p>Saudy et al. 2014</p> <p>Wakita et al. 2011</p> <p>Zöller 2015</p> <p>B. Long et al. 2016</p>
Breast	<p>CD44⁺/ CD24^{-/low} or</p> <p>ALDH1</p>	<p>High percentage of CD44⁺/CD24^{-/low} and ALDH1⁺ markers were</p>	<p>Bane et al. 2013</p>

		identified in breast cancer patients. There is a significant correlation with expression of these markers and tumor grade as well as high-mitotic count in breast tumors, predicting poor prognosis and metastasis.	
	BMI-1	High expression of BMI-1 has a strong correlation with triple negative breast tumors and associated with poor survival	Guo et al. 2011
	CD44 ⁺	High percentage of CD44 ⁺ CSC are associated with invasive cancer related features such as advanced stage, grade, lymph node metastasis and poor prognosis .	Z. Han et al. 2015
	CD133	High expression of CD133 protein is associated with tumor grade, lymph node metastasis and significantly correlated with poor	Z. Han et al. 2015

	<p>CXCR4</p> <p>Beta1 integrin</p> <p>LGR5</p> <p>CD90/Thy1</p> <p>Hedgehog-Gli</p>	<p>prognosis of Infiltrating ductal carcinoma patients.</p> <p>Over expression of CXCR4 leads to tumor growth, invasion, angiogenesis, metastasis, relapse, advanced tumor-node-metastasis (TNM) stage and therapeutic resistance, predicting poor prognosis in cancer patients.</p>	<p>Chatterjee et al. 2014</p> <p>Nisticò et al. 2014</p> <p>L. Yang et al. 2015</p> <p>X. Wang et al. 2015</p> <p>Peiris-Pages et al. 2015</p>
Brain	CD133 ⁺ /Nestin ⁺	<p>Low percentage of CD133⁺/Nestin⁺, markers are associated with poor prognosis in glioma patients. It was reported that combined expression of Nestin⁺/CD133⁺ is an independent</p>	<p>M. Zhang et al. 2008</p>

	GD3 ^{high}	prognostic marker of gliomas. High expression of GD3 correlates with metastasis in brain tumors. GD3 synthase is involved in the characteristics of CSCs through the c-MET signaling pathway.	Yeh et al. 2016
	CD90 ^{thy}	Immunohistochemical analysis of high-grade gliomas from clinical samples revealed expression of CD90. CD90 plays a significant role in the differentiation of endothelial cells via the generation of tumor vasculatures.	He et al. 2012
Colorectal cancer	CD133 ⁺	High percentage of the CD133 ⁺ marker is associated with poor prognosis, recurrence, metastasis and chemotherapy resistance in patients.	Ren et al. 2013 B. B. Wang et al. 2016

	ALDH1	It has been shown that 40% patients with high expression of ALDH1 had distant metastases or local recurrence, while patients with low ALDH1 expression did not develop metastasis or local recurrence. Hence, ALDH1 expression correlates with poor prognosis.	Y. Deng et al. 2014
	LGR5	Studies shows that LGR5 mRNA and protein expression was notably over-expressed in more than 50% of the patients with primary colon cancer and LGR5 ⁺ patients have a poor prognosis rate compared to LGR5 ⁻ cancer.	Z. Liu et al. 2014
	Musashi	High expression of Musashi in the primary tumor is associated with increased metastasis rate, predicting poor prognosis in stage III and IV colorectal patients.	Espersen et al. 2015

	CD24 ⁺ /CD44 ⁺ CD166		D. A. N. Liu et al. 2014 Shafaei et al. 2013
Pancreatic cancer	CD24 ⁺ /CD44 ⁺ / EpCAM ⁺	Studies reported that CD44 ⁺ /CD24 ⁺ /EpCAM ⁺ stem cells are associated with high proliferative potential in clinical samples. High percentage of CD44 ⁺ and CD24 ⁺ is associated with tumour development, initiation, and metastasis in pancreatic cancer patients.	Ohara et al. 2013
	Nestin	Nestin, a CSC marker plays an important roles in pancreatic cancer cell migration, invasion and metastasis by selectively changing the expression of actin/cytoskeleton and cell adhesion molecules, predicting a therapeutic target in PDAC.	Matsuda et al. 2011
	ALDH1A1	ALDH1A1 expression is	Kahlert et al. 2011

	CD24 ABCG-2 ESA ⁺ c-Met CXCR4 Nodal-Activin	associated with tumour stage and low expression of ALDH1A1 significantly reduced survival in ALDH1A1 ^{low} patients.	Elkord 2012 F. Wang et al. 2010 Wei et al. 2011 Fotovati et al. 2011 Billadeau et al. 2006 Lonardo et al. 2011
Melanoma	ABCB5 ⁺ CD271	High expression of ABCB5 ⁺ cells are significantly associated with with increased risk of metastatsis and poor prognosis in melanoma patients especially in smokers. High expression of the CD271 cell marker is present in the tumour-initiating cell population, and	N. Lee et al. 2014 Boiko et al. 2010

	CD133/Nestin	associated with high metastatic burden and poor prognosis in melanoma patients. High expression of CD133/Nestin are associated with advanced disease stage and metastatic skin tumors. Thus predicting a potential therapeutic targets for malignant tumors of the skin.	Sabet et al. 2014
	ALDH1 ⁺ CD20 ⁺		Luo et al. 2013 Pinc et al. 2012
Ovarian cancer	CD44 ⁺ / CD117 ⁺	Overexpression of CD44 ⁺ /CD117 ⁺ is associated with tumor recurrence, metastasis and drug resistance in ovarian cancer patients.	Sacks and Barbolina 2015
	CD24 ⁺	In epithelial ovarian carcinoma patients, increased cytoplasmic	Kristiansen et al. 2002 Burgos-Ojeda et al. 2015;

	CD133 ⁺	<p>expression of CD24 is associated with shortened survival.</p> <p>High number of CD133⁺ cells in ovarian primary tumors is associated with self-renewal, multi-differentiation and tumor initiation capacities in patients</p>	<p>Gao et al. 2010</p> <p>H. Long et al. 2015</p>
Prostate cancer	ALDH1 ^{high} /CD44 ⁺	Prostate CSC markers ALDH1 ^{high} /CD44 ⁺ cells exhibit a higher proliferative and metastatic capacity in patients.	Yu et al. 2011
	ALCAM/CD166	High expression of ALCAM/CD166 in prostate cancer patients are associated bone metastases and promote survival and proliferation of prostate cancer cells in the bone microenvironment, predicting poor prognosis in patients.	Hansen et al. 2014
	ABCG2 CD105		<p>Guzel et al. 2014</p> <p>Fujita et al. 2009;</p>

[illegible]

	CD133 ⁺ /CD44 ⁺	Human liver cancer samples showed that CD133 ⁺ /CD44 ⁺ tumor cells was associated with portal vein metastasis and hematogenous metastasis in liver cancer patients .	S. Han et al. 2015
	CD90	CD90 expression in liver carcinoma is associated with increment in tumor growth and decreased survival rate.	Z. F. Yang et al. 2008
	CD133 ⁺	It was reported that CD133 ⁺ liver cancer cells promote angiogenesis, tumorigenesis and self-renewal through the IL-8 signaling cascade in liver cancer patients and predict poor prognosis.	Tang et al. 2012
Lung	ALDH1	Patients with ALDH1-positive lung cancer had poor prognosis whereas non-small cell lung cancer patients with high expression of ALDH1 have better prognosis and lower recurrence rates. Thus the effect of ALDH1	Huo et al. 2015

		expression depends on type of lung cancer	
	CD90 ⁺	High percentage of CD90 ⁺ cells are associated with high proliferation, self-renewal and tumorigenic capacity in lung cancer patients and hence could be a novel marker of lung CSCs.	Yan et al. 2013
	CD117	CD117 expression in lung cancer is associated with highly aggressive tumor behavior such as stage, size and grade.	Pelosi et al. 2004
	CD133	High percentage of CD133 cells are significantly associated with high proliferative potential, lymph node metastasis, thus predicting poor prognosis in lung cancer patients.	Tan et al. 2014
	ABCG2		Liang et al. 2015

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Figure legend

Figure 1. Acquisition of therapy resistance in CSCs occurs through a number of mechanisms. a) Schematic showing how chemo- and radio- therapy affects some of the known components of the intrinsic apoptotic pathway that leads to programmed cell death in tumor cells, b) Active DNA repair combines with defective apoptotic pathways and increased pro-survival signaling in chemoresistant CSCs, c) Multidrug resistance (MDR) is a major problem that prevents the successful eradication of cancer cells. A common mechanism is the over expression of ATP-binding cassette (ABC) efflux transporters in cancer cells such as P-glycoprotein (P-gp/ABCB1), multidrug resistance-associated protein1 (MRP1/ABCC1) and breast cancer resistance protein (BCRP/ABCG2) that limit the cell's exposure to anticancer drugs. d) Induction of quiescence is another means by which CSCs protect themselves from the deleterious effects of chemo- and radio- therapy. Exposure to 5-Fluorouracil activates c-Yes tyrosine kinase in chemoresistant CSCs. Phosphorylation of c-Yes leads to dissociation of the YAP/ c-Yes complex and translocation of nuclear Yap to the cytoplasm. Disruption of the YAP/ c-Yes complex prevents cell cycle progression and is a possible means by which CSCs induce quiescence. Note that circles and squares represent active and inactive forms of proteins respectively.